THE EVOLUTION AND FUNCTION OF SEA LAMPREY PHEROMONE COMPONENTS

By

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ABSTRACT

Sea lamprey (*Petromyzon marinus*) relies on a suite of complex chemical cues to mediate the reproductive stage of its complex life cycle. Pheromones are essential to ensure successful reproduction as anosmic individuals fail to locate spawning streams and mates. Three separate sets of pheromones have been characterized in lamprey: 1) A migratory pheromone released by stream resident larvae as metabolic byproducts that attracts adults into suitable spawning streams, 2) a sex pheromone comprised of bile acids released by sexually mature males that attracts females to a male nest and induces a suite of reproductive behaviors, and 3) a pheromone present in milt that is released during spawning events and hypothesized to signal sperm availability to females. In this dissertation, I test the overall hypothesis that different pheromones or their components function in their respective contextual factors (migration vs. reproduction, signaling location, and male spawning status) to influence female behavior and mate preferences. In Chapter 1, I provide a comprehensive review on our current understanding of chemical cues used by sea lamprey, what insights we have gained from management scale tests utilizing chemical cues, and the current unknowns and future research needs that should be addressed in order to implement chemosensory cues into the sea lamprey control program. In Chapter 2, data from field tests confirmed that sensory traps lead to reliable communication as females disassociated sexual and non-sexual signals using a pheromone antagonist during mating but not during reproductive migration. These results also suggest that the pheromone antagonist, PZS, may only be effective in controlling spawning populations and not for manipulating behavior during reproductive migration. Chapter 3 characterizes the functions of pheromones released via milt during spawning. Milt pheromones allowed females to discriminate among potential mates based on spawning status and milt pheromone concentration and attracted females to a nest while

retaining them there for times consistent with the entire multicomponent bile acid pheromone. In Chapter 4, behavioral tests confirmed that a male's nesting location impacted access to mates to a greater degree than the signal attribute of pheromone concentration, which is known to influence female preferences. These results highlight the dynamic nature of animal communication and the importance of integrating aspects such as a signaler's location, signal attributes, and transmission through the environment to understand how the interactions or lack thereof between them influence receiver responses. The studies conducted here broaden our understanding of sea lamprey pheromone evolution, the function of sea lamprey pheromone components, and sea lamprey reproductive ecology. This knowledge may be used when designing and implementing management tools that utilize pheromones or other chemosensory cues as supplemental controls. The implementation of chemosensory cues into control efforts is aided by a holistic understanding of the multiple chemosensory communication networks sea lamprey utilize which provides information on what aspects of chemical communication networks to target for manipulation and in what contexts these efforts may be most effective.

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INTRODUCTION TO DISSERTATION

All organisms communicate and provide information that influences behavioral or physiological responses in receivers (Bradbury and Vehrencamp, 1998). This information may be provided across a vast array of sensory modalities that could include acoustic, visual, olfactory, seismic, or electrical properties responsible for transmitting information (Bradbury and Vehrencamp, 1998; McGregor, 2005). Information may be conveyed using a single sensory modality or multiple modalities simultaneously (Bro-Jørgensen, 2010; Higham and Hebets, 2013). Signals are one specific way in which organisms communicate with each other and are defined as an act or structure that has specifically evolved to transmit information between the signaler and receiver where both parties benefit from the information provided (Bradbury and Vehrencamp, 1998; Smith and Harper, 2003). Signals can evolve in a variety of ways due to differing selection pressures such as the morphology and physiology of senders and receivers (Podos, 2001), social contexts such as intrasexual competition or the presence of predators or parasites (Rojas, 2017), and even properties of the environment (Giery and Layman, 2017). The ubiquitous nature of animal communication has led to extensive studies on signal properties, signal evolution, and receiver responses to signals (Johnstone, 1997; Laidre and Johnstone, 2013; Smith and Harper, 2003).

Despite extensive studies focused on animal signals, the majority of theoretical and empirical investigations have focused on visual and auditory sensory modalities, with studies focused on chemical communication being relatively less common (Symonds and Elgar, 2008). This disparity is partly due to our own sensory biases as we often cannot perceive chemical signals used by other organisms and also, in part, due to difficulties associated with isolating and testing chemical constituents of these signals. Pheromones are chemical signals that have evolved to elicit behavioral or physiological responses in conspecifics and may be comprised of

one or multiple chemical compounds (Wyatt, 2017). A vast majority of pheromone research has occurred in insect species (~80%, Symonds and Elgar, 2008), which provide useful models to investigate pheromones and chemical communication for pest management or invasive species control (Witzgall et al., 2010). Because pheromones and other chemical signals induce stereotypical behavioral responses, utilizing pheromones within control scenarios allows us to manipulate the behavior of pests or invasive species in predictable and reliable ways for our own benefit.

Sea lamprey, *Petromyzon marinus*, are a destructive invasive species within the Laurentian Great Lakes, and their dependency on chemical cues and pheromones during reproductive migration and mate search has led to extensive investigations focused on understanding sea lamprey chemosensory communication for control (Fissette et al., 2021; Li et al., 2007; Teeter, 1980; Twohey et al., 2003). Insect models that implement pheromones in control scenarios provide an overarching framework for developing and integrating tactics that can leverage knowledge of sea lamprey chemical ecology into the sea lamprey control program. Previous research on sea lamprey pheromone communication has been heavily focused on identifying chemical compounds that comprise the multicomponent migratory, mating, and milt pheromones along with describing their behavioral and/or physiological functions (Fissette et al., 2021). However, identifying the chemical constituents of pheromone blends and their function(s) in modifying behavior or physiology represents only aspect of sea lamprey chemical ecology. To better understand the role of pheromones within sea lamprey biology and learn how to better exploit the behaviors they induce, a broader and more holistic understanding of the ecological and evolutionary contexts of the pheromone communication system are needed. Understanding what contexts pheromones function in, why they function within these contexts, and how they

function across contexts provides additional information that pairs with the question of what behaviors these pheromones modulate. This additional information may be extremely valuable when developing specific, supplemental control tools (Siefkes et al., 2021) that likely need to vary based on the time of implementation, stream characteristics, and/or reproductive status of the sea lamprey being targeted. In order to broaden our understanding of the sea lamprey pheromone communication network, my dissertation research focused understanding different ecological and evolutionary contexts that shape sea lamprey pheromone communication and how these ultimately impact behavioral responses to pheromone components. I tested the overall hypothesis that different pheromones or their components function within specific contextual scenarios (migration vs. mate search, male spawning status, and male signaling location) to influence female behavioral responses and preferences.

In Chapter 1, titled "Progress towards integrating an understanding of chemical ecology into sea lamprey control" I synthesized and reviewed the research on sea lamprey chemical ecology in an effort to describe how this knowledge may be leveraged into the sea lamprey control program. I extensively cover our current understanding related to 1) the role pheromones and alarm cues play within sea lamprey reproductive ecology, 2) the chemical identities of sea lamprey pheromones and alarm cues, 3) how chemosensory information modulates sea lamprey behavior, 4) what insights we have gained from management scale tests incorporating pheromones, and 5) the current unknowns and future research needs that should be addressed before implementing chemosensory cues into sea lamprey control. This review compiles decades of research and presents a comprehensive overview of sea lamprey chemical ecology. Chapter 1 is published in the Journal of Great Lakes Research.

In Chapter 2, titled "Context-specific decoupling of a mimetic sex pheromone and its model in sea lamprey", I tested the hypothesis that female sea lamprey have decoupled the male sex pheromone and larval odor during reproductive migration. I specifically tested the prediction that migratory females do not use the compound petromyzonol sulfate (PZS) to discriminate between three-keto-petromyzonol sulfate (3kPZS) released by males and larvae. I provide evidence that females did not disassociate the sexual and non-sexual signals of 3kPZS and PZS during reproductive migration. PZS did not disrupt migratory responses to 3kPZS. Contrary to behaviors observed during mate search, migrating females were attracted to mixtures of 3kPZS and PZS at both the larval and male released ratios. Additionally, migratory females were attracted to both the migratory pheromone and male sex pheromone, but they did discriminate between them and preferred the migratory pheromone. The mechanism allowing discrimination was not differential ratios of 3kPZS:PZS present in male and larval odors but is likely facilitated by differences in chemical compositions between these two pheromones. These findings are one of the few studies to investigate how females shift their responses to mimetic or deceptive signals and provide additional evidence that females evolved to use PZS to avoid responding to larval released 3kPZS during spawning. This chapter highlights the importance of understanding the evolutionary contexts of pheromones because the selective pressures that drive pheromone evolution influence how and when pheromones influence receiver behaviors.

In Chapter 3, titled "Evidence that female sea lamprey use milt pheromones to discriminate among potential mates", I tested the hypothesis that pheromones present in sea lamprey milt allow females to discriminate among male mates. I specifically tested whether females discriminate males based on the presence/absence of milt pheromones and milt pheromone concentrations. I also determined whether milt pheromones influence female

behavior in the absence of bile acid pheromones. There are relatively few examples of pheromones associated with milt or seminal fluids and none have directly tested whether females discriminate among males based on this chemosensory information. Behavioral assays confirmed that compounds in milt are potent pheromones that attract females to the pheromone source and retain them there for extended periods of times in the absence of bile acid pheromones. Additionally, females used milt pheromones to discriminate among males base on the presence of milt and milt pheromone concentration. Increased retention times across all experiments suggests the milt pheromone helps maintain spawning groups on a nest. These results highlight how multiple signals influence female behavioral preferences within the social context of male reproductive status.

In Chapter 4, titled "Male nesting locations modulate female preferences for pheromone signals in sea lamprey", I tested the hypothesis that a male's nesting location impacts female preferences for pheromone signals. Male-female mating interactions are complex in nature and emphasis solely on secondary sexual traits may lead to simplistic interpretations of communication between senders and receivers. Male sea lamprey vary in the 3kPZS signals they produce and release (Buchinger et al., 2017; Fissette et al., 2020), and females prefer higher 3kPZS concentrations (Fissette et al., 2020; Johnson et al., 2009). However, there is variation in other aspects of signaling such as a male's nesting location and signal dispersal throughout the environment. I combined behavioral observation data showing variation in nest use and the number of females attracted to specific nesting sites with behavioral assays to assess how nesting location influenced female preferences for male pheromone signals. Behavioral assays at two separate experimental sites showed that nesting location had a greater impact on female preferences for pheromone signals than pheromone concentrations. I also provide indirect

evidence that pheromone plume dispersal influenced female behavior for nesting sites. These results highlight how spatial contexts are an important factor to consider when studying animal communication, and that focusing solely on signal attributes may provide an incomplete picture within male-female mating interactions.

My dissertation research provides several lines of evidence to support my overarching hypothesis that pheromones and their components function within specific contextual scenarios to influence female behavioral responses and preferences to male pheromones. The contexts of migration vs. mate search, male spawning activity, and male signaling location all had distinct effects on female responses to pheromones. Understanding these contexts not only gives us a better understanding of the sea lamprey pheromone communication network as a whole, but also provides useful information for integrating pheromones into control techniques. These results provide insights into which scenarios, contexts, and tactics pheromones may be most effectively implemented. For example, the pheromone antagonist, PZS, did not disrupt migratory female behavioral responses to the compound 3kPZS. Therefore, PZS is likely to be most effective in controlling spawning populations and a different pheromone antagonist may need to be utilized to control migratory individuals. Additionally, pheromone baited trapping should consider fine spatial scales when determining trapping locations because the location of a pheromone sources influences female preferences. Simply increasing pheromone concentrations within a trap will not necessarily equate to increased trap effectiveness or reliably compete with background odor sources. It is imperative to understand the evolution of pheromone signals and in what contexts pheromones function to influence behavior if we are to successfully integrate a knowledge of chemical ecology into control of this invasive species (Fissette et al., 2021).

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CHAPTER 1:

PROGRESS TOWARDS INTEGRATING AN UNDERSTANDING OF CHEMICAL ECOLOGY INTO SEA LAMPREY CONTROL

Fissette, S. D., Buchinger, T. J., Wagner, C. M., Johnson, N. S., Scott, A. M., & Li, W. (2021). Progress towards integrating an understanding of chemical ecology into sea lamprey control. *Journal of Great Lakes Research*, *47*, S660-S672.

ABSTRACT

The sea lamprey, *Petromyzon marinus*, is a destructive invader in the Laurentian Great Lakes that relies on several complex chemical cues to complete their life cycle. The central roles of chemical cues in sea lamprey reproduction provide opportunities to leverage knowledge of sea lamprey chemical ecology when developing alternative or supplemental strategies for sea lamprey control. A solid foundation has been laid regarding sea lamprey chemical ecology, with recent advances in our understanding of the migratory pheromone, male sex pheromone, and alarm cues broadening our fundamental understanding of the diversity, complexity, and evolution of chemical cues used by sea lamprey. Additionally, research applying semiochemicals in differing management scenarios has provided useful insights into the challenges of incorporating chemical cues into the sea lamprey control program. Here, we synthesize new findings related to fundamental research of chemosensory cues along with knowledge learned from management-based tests and explore options for integrating an understanding of chemical ecology into sea lamprey control in light of new knowledge. We also highlight current unknowns and future research needs that should be addressed prior to implementation of sea lamprey chemical ecology into the sea lamprey control program.

INTRODUCTION

The sea lamprey, *Petromyzon marinus*, is a destructive invasive species with a complex life history (Figure 1.1). Native to the Atlantic Ocean, sea lamprey invaded the Laurentian Great Lakes in the early 1900s and contributed to the collapse of ecologically, culturally, and economically important fisheries (Smith and Tibbles, 1980). Sea lamprey begin their complex life history by residing in streams as larvae for 3-17 years. Next, they metamorphose into parasitic juveniles that migrate downstream to lakes or the Atlantic Ocean and feed upon the blood of fish for 12-18 months. During this parasitic stage, a single sea lamprey may kill up to ~19 kg of fish (Swink, 2003) and elicit sub-lethal physiological responses in hosts that survive the initial attack (Goetz et al., 2016; Smith et al., 2016). Sea lamprey cease feeding in the late winter to early spring and migrate into spawning streams where they intermittently spawn over the course of 1-2 weeks before dying (Johnson et al., 2015a). The control program in the Laurentian Great Lakes target the larval and adult stages; lampricides kill larvae in streams, barriers block adults from spawning habitat, and electrofishing surveys assess larval density while traps assess adult population size (Marsden and Siefkes, 2019; Siefkes, 2017; Wilkie et al., 2019). Notably, sea lamprey rely on chemical cues throughout their life cycle (Buchinger et al., 2015) and therefore might be managed, in part, using tactics that leverage knowledge of chemical ecology (Sorensen and Johnson, 2016).

Adult sea lamprey use a suite of chemical cues to locate and navigate spawning streams and interact with mates (Figure 1.2). Pheromones, chemical signals that have evolved for communication between conspecifics (Karlson and Lüscher, 1959; Wyatt, 2014), have been the most extensively studied chemical cues in sea lamprey, though it is currently unclear whether the migratory pheromone is an evolved signal. During the spawning migration, adult sea lamprey

orient towards the odor of larvae (i.e., migratory pheromone) and away from the odor of dead conspecifics and potential predators (i.e., alarm cue) (Di Rocco et al., 2016a; Wagner and Bals, 2012). Later, sexually mature (spermiated) males release a multicomponent sex pheromone that stimulates sexual maturation in sexually immature males and females (Chung-Davidson et al., 2013a; Chung-Davidson et al., 2013b) and elicits mate search and spawning behavior in mature (ovulated) females (Johnson et al., 2012). Olfaction is critical for sea lamprey to reproduce; sea lamprey rendered unable to smell fail to locate larvae-laden streams (Vrieze et al., 2010; Vrieze et al., 2011) and mates (Johnson et al., 2006). Although larvae (Perrault et al., 2014; Wagner et al., 2016; Zielinski et al., 2005) and juveniles (Johnson et al., 2019; Kleerekoper and Mogensen, 1963) also respond to chemical cues, the chemical ecology of adults is much better understood and therefore closer to being exploited as part of the sea lamprey control program.

In this review, we summarize recent findings related to the chemical ecology of adult sea lamprey and outline questions that remain unanswered. Because chemical cues have critical roles at several points in the sea lamprey life cycle, tactics founded in chemical ecology are promising as a tier of an integrated control program and the focus of a theme of the Great Lakes Fishery Commission's sea lamprey research program. Research driven by the goal of developing control strategies has led to sea lamprey being a useful model for studying pheromones in a vertebrate and allowed interesting comparisons with teleost fishes (Li et al., 2018b; Stacey et al., 2015). Several previous reviews cover research towards the goal of integrating knowledge of chemical ecology into sea lamprey control (Buchinger et al., 2015; Li et al., 2003; Li et al., 2007; Sorensen and Hoye, 2007; Twohey et al., 2003), but an updated overview is needed to highlight new findings, reevaluate previous findings in light of new discoveries, and outline future research. Here, we synthesize recent advances in (1) understanding of the complexity and diversity of sea

lamprey chemical cues and their roles within sea lamprey ecology and (2) integration of chemical ecology into sea lamprey control. Lastly, we highlight unknowns and opportunities to guide future research into sea lamprey chemical ecology and its applications in sea lamprey control.

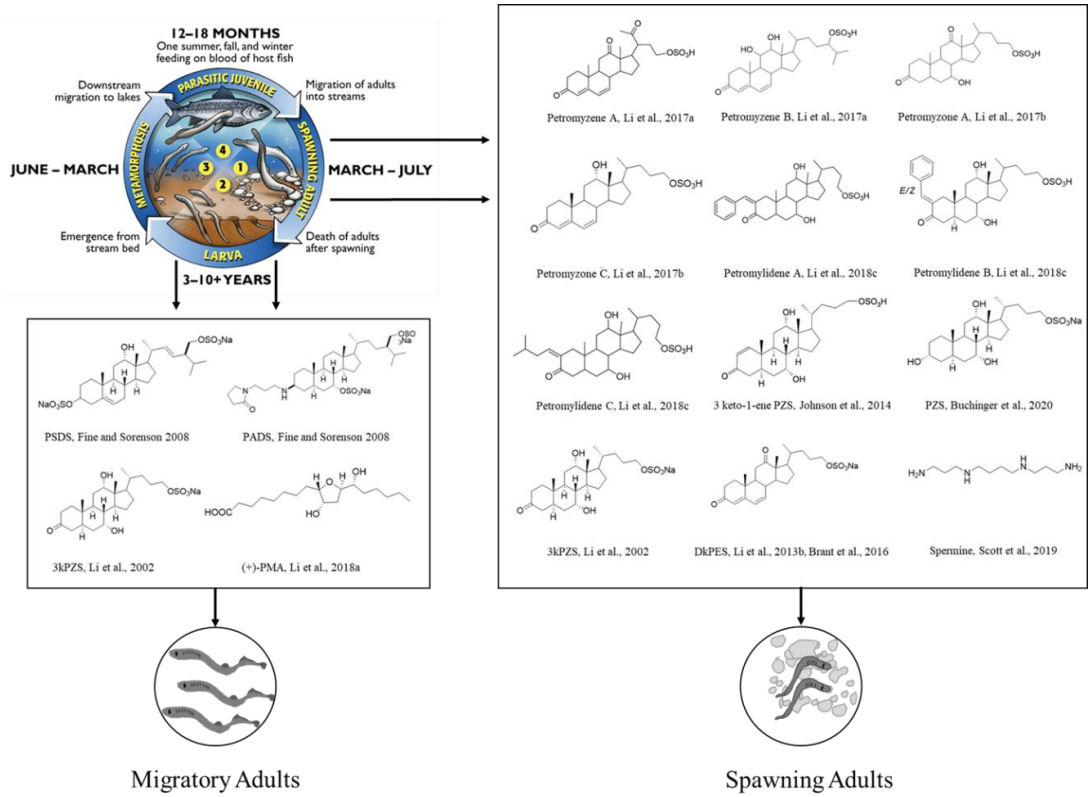

Figure 1.1. Sea lamprey life cycle and pheromone components released by various life stages. The sea lamprey life cycle (Image credit: Great Lakes Fishery Commission) and structures of behaviorally active pheromone components released by larvae that attract migratory adults and pheromones released by spermiating males that influence behavior of spawning adults. PSDS (petromyzosterol disulfate), PADS (petromyzonamine disulfate), (+)-PMA (petromyric acid A), 3kPZS (3-keto petromyzonol sulfate), PZS (petromyzonol sulfate), DkPES (3,12-diketo-4,6-petromyzonene-24-sulfate).

FUNDAMENTAL UNDERSTANDING OF SEA LAMPREY CHEMICAL ECOLOGY

Perception of chemical cues

The sea lamprey's well-developed olfactory system detects a diverse array of molecules with acute sensitivity and specificity (Buchinger et al., 2015; Li et al., 2018b). The anatomy of sea lamprey indicates a critical role of olfaction in their ecology; the olfactory bulb is proportionally large among vertebrates (Stoddart, 1990), the nasal capsule, which contains the olfactory organ, is larger than the brain (Kleerekoper and Erkel, 1960), and olfactory inputs to odorant receptor neurons are linked to locomotor outputs (Daghfous et al., 2018; Daghfous et al., 2016; Derjean et al., 2010). Interestingly, lampreys may also detect conspecific chemical cues using solitary chemosensory cells, which are especially abundant in migrating and spawning adults (Baatrup and Døving, 1985; Daghfous et al., 2019; Suntres et al., 2019). However, attraction to the migratory and mating pheromones requires an intact olfactory sense (Johnson et al., 2006; Vrieze and Sorensen, 2001), and receptors on the olfactory epithelium respond to conspecific odorants (Siefkes and Li, 2004). Indeed, the sea lamprey olfactory system detects a variety of compounds, including sulfated steroids (Li et al., 1995), fatty acids (Li et al., 2018a), amino acids (Li, 1994), and polyamines (Scott et al., 2019). The sea lamprey olfactory system is not only highly sensitive, detecting some compounds at concentrations of 10^{-14} molar or lower (Johnson et al., 2009; Scott et al., 2019), but also highly specific in discriminating between structurally similar compounds (Burns et al., 2011). For example, the olfactory system can discriminate the male sex pheromone 3-keto petromyzonol sulfate (3kPZS) from its likely precursor petromyzonol sulfate (PZS)(Brant et al., 2013), which differs by only two hydrogens (Siefkes and Li, 2004). Likewise, two larval-released fatty acids differ only in stereochemistry but elicit different olfactory and behavioral responses in migratory adults (Li et al., 2018a). A finely tuned olfactory system

allows sea lamprey to discriminate between multiple complex chemical cues that, in some cases, overlap in composition but convey different messages (Buchinger et al., 2020).

Migratory pheromone

Sea lamprey, as in many migratory fish (Bett and Hinch, 2016), navigate to spawning habitats following olfactory cues (Figure 1.2). Unlike anadromous Pacific salmon, sea lamprey do not home (Bergstedt and Seelye, 1995; Waldman et al., 2008) but rather enter spawning streams that contain populations of larvae (Moore and Schleen, 1980) guided by the larval migratory pheromone (Teeter, 1980). Adults conceivably benefit from using larval odors to select spawning streams because it indicates past spawning success and current larval productivity (Fine and Sorensen, 2010; Polkinghorne et al., 2001). As the larval odorants are generally conserved across species (Brant et al., 2016b; Buchinger et al., 2013; Fine et al., 2004; Robinson et al., 2009; Stewart and Baker, 2012; Yun et al., 2003a; Yun et al., 2011), the migratory pheromone likely involves adaptations in migratory adults but not specialized signaling by larvae. However, any benefits larvae may receive by attracting adults, such as being provided additional nutrients from decaying adults or adult pheromones influencing metamorphosis, have not been examined. More details on the chemical ecology of the migratory pheromone are available in several previous reviews (Buchinger et al., 2015; Moser et al., 2015; Sorensen and Vrieze, 2003) while more recent research on the migratory pheromone has focused primarily on the identity of its chemical constituents.

Intensive efforts to characterize the underlying chemistry of the migratory pheromone implicate multiple molecules with distinct behavioral effects. Initial chemical, electrophysiological, and behavioral experiments suggested two known bile acids — petromyzonol sulfate (PZS) and allocholic acid (ACA) — contribute to some, but not all, of the

activity of larval odor (Bjerselius et al., 2000; Li et al., 1995; Vrieze and Sorensen, 2001). Subsequent chemical fractionation of larval-conditioned water unveiled two novel steroid derivatives, petromyzonamine disulfate (PADS) and petromyzosterol disulfate (PSDS) (Figure 1.1), that biased migratory adult movement toward one of two laboratory channels (Fine and Sorensen, 2008; Sorensen et al., 2005). Furthermore, a field study using acoustic telemetry to track migrant behavior near a river mouth indicated PADS and PSDS increase the time migrating sea lamprey spend in river plumes (Meckley et al., 2014). However, PADS and PSDS did not lure migrants into the stream (Meckley et al., 2014) and, in two separate studies that also included PZS in the mixture, did not influence upstream movement or channel selection within a stream (Brant et al., 2016b; Meckley et al., 2012). Interestingly, 3kPZS, the major component of the male sex pheromone (Li et al., 2002), may also be a component of the larval migratory pheromone; 3kPZS is released into the water by larvae (Brant et al., 2016b; Buchinger et al., 2020b) and induces upstream movement of migratory males and females (Brant et al., 2016b; Brant et al., 2015). In fact, evidence that silver lamprey (*Ichthyomyzon unicuspis*) use 3kPZS as a component of their larval migratory pheromone, but not as a sex pheromone, indicates migration may be the original context in which 3kPZS functioned as a chemical cue (Buchinger et al., 2013). Intriguingly, one study reports evidence the effect of 3kPZS on upstream movement is synergized by a mixture of PADS, PSDS, and PZS (Brant et al., 2016b). In search of the molecules that bias migrants into a stream channel, a separate round of chemical fractionation identified multiple additional components of larval odor: petromyzonin (Li et al., 2013a), (+)- and (-)- petromyroxols (Li et al., 2015b), two *iso*-petromyroxols (Li et al., 2015a), and four fatty acids (-)- and (+)- petromyric acid A (PMA) and (-)- and (+)- petromyric acid B (PMB) (Li et al., 2018a). Of these, only (-)- and (+)- PMA have been tested in behavioral assays; (+)-PMA

(Figure 1.1) replicated larval odor in biasing migratory females into a stream channel (Li et al., 2018a). Taken together, these results suggest individual components, or sets of components, guide distinct behaviors potentially related to staging offshore prior to river entry (Meckley et al., 2014), orientation upstream (Brant et al., 2016b; Brant et al., 2015), and selection of tributaries within a river (Li et al., 2018a).

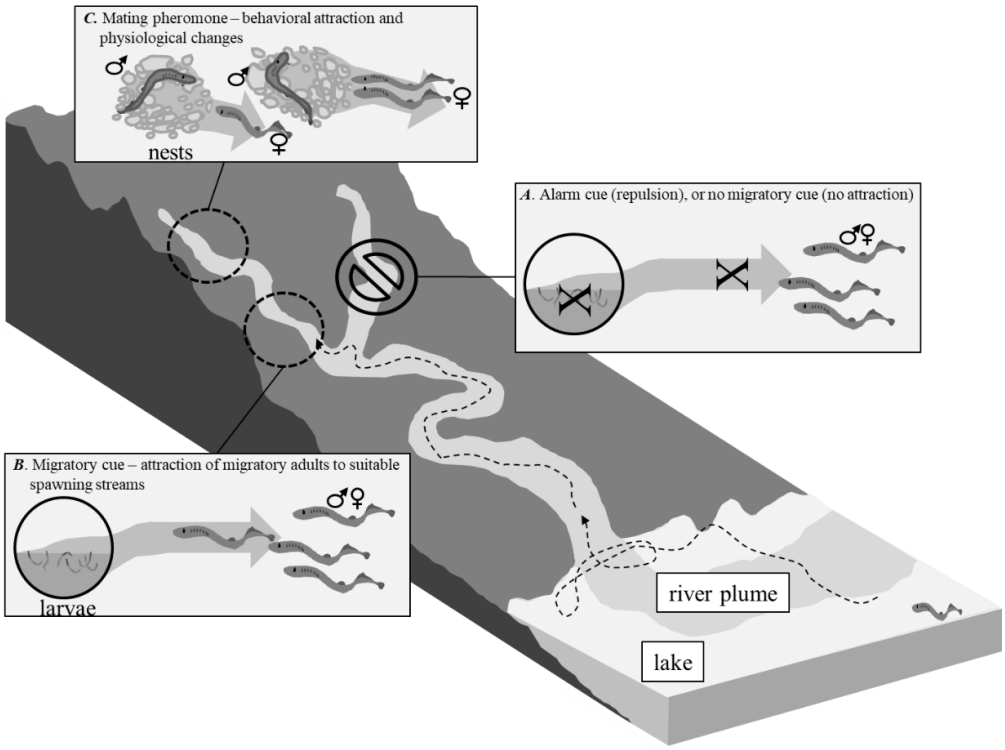

Figure 1.2. The function of alarm cues, migratory pheromones, and mating pheromones used during the sea lamprey reproductive migration. A) Rivers or tributaries with alarm cue or no larvae attract fewer migrating sea lamprey. B) Larval populations release migratory pheromone in rivers or tributaries with suitable habitat and attract migrating sea lamprey. C) Spermiated males release a suite of pheromone compounds that attract ovulated females and induce physiological changes in sexually mature and immature adults. Figure modified from Buchinger et al., (2015).

Predation-related semiochemicals

The ability to detect and appropriately respond to sensory information associated with predation risk is a primary determinant of animal fitness (Kats and Dill, 1998; Lima and Dill, 1990). In aquatic environments, this information is often chemical in form, and specifically, in sea lamprey, may be characterized as (a) alarm cues (compounds released by conspecifics during injury), (b) disturbance cues (compounds released by conspecifics after perceiving the threat of attack) that are inadvertently broadcast into the environment and operate as public information that benefit only receivers, and (c) predator odors, both from the animal and its feces (kairomones). Known anecdotally for at least 30 years (Imre et al., 2010), the tissue of Great Lakes sea lamprey contains a natural repellent, putatively an alarm cue (Wagner et al., 2011). Classification as an alarm cue is tentative as anecdotal evidence suggests sea lamprey and related species may release a disturbance cue when handled that elicits a similar avoidance response (Petersen, 2006). The sea lamprey alarm cue is contained in various tissues throughout the body, is released by injury or decay after death, and elicits an avoidance response in laboratory and field tests (Di Rocco et al., 2016a; Hume et al., 2015; Pietrzakowski et al., 2013; Wagner and Bals, 2012; Wagner et al., 2011).

There is evidence that larvae, newly transformed parasites, and adults respond to the alarm cue, and that the cues derived from juvenile and adult tissues elicit responses across life stages (Ayotte and Imre, 2016; Johnson et al., 2019; Perrault et al., 2014; Wagner et al., 2016). Sea lamprey also exhibit partial responses to alarm cues derived from heterospecific lampreys, presenting a typical pattern of reduced response with increased phylogenetic distance, implying a mixture of odorants compose the cue (Byford et al., 2016; Hume and Wagner, 2018; Wagner and Bals, 2012). Sea lamprey do not appear to respond to alarm substances derived from teleost

fishes or hagfishes (Hume and Wagner, 2018; Wagner and Bals, 2012) but see Imre et al., (2014) who observed an avoidance response to a heterospecific stimuli. Sexual maturation differentially affects the expression of the alarm response in males and females, with females exhibiting no response to the cue after ovulation, whereas males, after spermiation, continue to respond in laboratory conditions (Wagner and Bals, 2012). Additionally, exposure to alarm cue for 4 hours resulted in a loss of avoidance response in migratory stage sea lamprey (Imre et al., 2017). Investigations to identify the chemical constituents of the alarm cue are currently underway. The cue does not appear to involve lipid compounds (Dissanayake et al., 2016) but does include water-soluble nitrogenous compounds (Dissanayake et al., 2019).

The role of the alarm cue in guiding movement and habitat selection decisions has been most frequently studied in the context of the migration into rivers prior to spawning (Figure 1.2). During this movement from lake to stream, sea lamprey pass through an ecological transition zone partly defined by a different predator community. As these nocturnal migrants progress upstream, they enter increasingly shallow and narrow waterways where they often move in close proximity to river shorelines, which may ensure entry into tributaries emitting larval odor (Holbrook et al., 2015; Wagner et al., 2006; Wagner et al., 2009). However, such movement tendencies also increase the likelihood of contact with nocturnal shoreline predators, important consumers of diadromous fishes (Willson and Halupka, 1995). Prey detecting an alarm cue demonstrate antipredator behaviors such as flight, avoidance, reduced activity, or shelter seeking (Chivers and Smith, 1998; Wisenden and Chivers, 2006). Migrating sea lamprey exhibit context-specific responses to the alarm cue, all related to reducing exposure to the area where the odor is emitted. For example, when the alarm cue is restricted to one side of the channel, migrants move upstream on the opposite side of the channel (Bals, 2012; Di Rocco et al., 2016a; Hume et al.,

2015). However, when the cue is distributed throughout the channel, migrants will tend to increase their movement velocity, including entering rivers from the lake more rapidly, signifying a switch in anti-predator responses from spatial avoidance to temporal avoidance (Luhring et al., 2016). Interestingly, these migrants encounter the cue of their own volition as they move into the river plume from the lake. However, sea lamprey in daytime refuges in the river exhibit an initial delay in upstream movement when exposed to the cue (Luhring et al., 2016). This tendency is also observed in the larval response to alarm cue exposure where larval sea lamprey buried in river sediments reduce movement when exposed to the alarm cue (Perrault et al., 2014; Wagner et al., 2016). Overall, it appears the interplay between internal state (migrating vs. refuging/foraging, mature vs. immature), the spatial presentation of the cue (avoidable or unavoidable), and the temporal presentation of the cue (encountered when moving vs. stationary) mediates the selection of anti-predator tactics (active avoidance vs. hiding). In no reported case has the alarm cue induced escape behavior by migrating sea lamprey in the form of migratory abandonment (downstream movement and exit from the river). In fact, the observation that sea lamprey increased river entry with increasing alarm cue presence (Luhring et al., 2016) suggests the alarm cue also confirms the presence of conspecifics to the migrants (i.e., potential mates), a crucial piece of information for a solitary migrant that does not exhibit natal homing (Bergstedt and Seelye, 1995; Waldman et al., 2008) and therefore relies on simple aggregation to achieve reproductive success.

Although sea lamprey are anticipated to respond to odors directly associated with predators, little work has been reported to investigate responses to predator kairomones. Three laboratory studies report an aversive response to high concentrations of 2-phenylethylamine (PEA), a constituent of mammalian urine that is present in a presumed mammalian predator, the common

raccoon *Procyon lotor* (Di Rocco et al., 2016b; Imre et al., 2014). However, the odorant failed to elicit anti-predator responses in a subsequent field experiment (Di Rocco et al., 2016a). Work to identify additional odorants from predators that may augment response to the alarm cue, or create mixtures that may prove more resistant to behavioral habituation when used in management applications (Bals, 2012; Imre et al., 2016; Imre et al., 2017), is needed.

Male sex pheromones

Spermiated male sea lamprey release a mixture of diverse molecules that function as sex pheromones (Figure 1.1). Unlike many insect pheromones, sea lamprey sex pheromones appears to consist of multiple components that elicit distinct reactions rather than acting as a collective blend in which all components are needed to elicit a response (Buchinger et al., 2020b; Johnson et al., 2012; Johnson et al., 2009). However, the identities and functions of all pheromone components have not been characterized, limiting the investigation of possible synergistic effects in which mixtures elicit greater responses than individual components. Although our understanding of the mixture remains incomplete, recent studies have revealed unexpected complexity in the signaling molecules released by male sea lamprey and implicate a suite of additional components and functions.

A major component of the male pheromone mixture, 3kPZS, is released at high rates by spermiated males and elicits multiple physiological and behavioral responses in both males and females. Males synthesize 3kPZS in the liver, transport it via blood circulation to the gills, and release it into the water at high rates (~ 0.5 mg/h) (Brant et al., 2013; Siefkes et al., 2003). Once released into the water, 3kPZS modulates locomotor activity of ovulated females (Walaszczyk et al., 2016; Walaszczyk et al., 2013) and attracts ovulated females in laboratory and field assays (Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005). High inter-male variation in 3kPZS

release and female preference for the more concentrated of two adjacent pheromone plumes indicate 3kPZS likely guides female mate choice (Buchinger et al., 2017a; Fissette et al., 2020; Johnson et al., 2009). Indeed, biosynthesis of 3kPZS and possible precursors appears to be under sexual selection (Buchinger et al., 2019). Interestingly, 3kPZS signaling may also mediate interactions among males; recent studies have found evidence that intrasexual competition increases male 3kPZS release which may have consequences on female mate choice (Fissette et al., 2020) and that 3kPZS also attracts spermiated males in two-choice maze experiments (Buchinger et al., 2020b). Similarly, 3kPZS attracts migratory males and females (Brant et al., 2016b; Brant et al., 2015; Johnson et al., 2013) and stimulates sexually dimorphic changes in the hypothalamic-pituitary-gonadal axis by priming the neuroendocrine system via synthesis and release of gonadotropin releasing hormone (GnRH) (Chung-Davidson et al., 2013b). Although 3kPZS evokes a suite of responses in various contexts, multiple additional components contribute to the full activity of the male pheromone (Johnson et al., 2012; Johnson et al., 2009).

Several bile acid derivatives may act as additional components of the male sex pheromone that have independent effects as chemical signals. Ovulated females respond to 3kPZS and male-conditioned water similarly at long distances but do not remain near a nest or exhibit spawning behaviors in response to 3kPZS as they do to the complete male odor (Johnson et al., 2012; Johnson et al., 2009). The first identified minor component, 3 keto allocholic acid (3kACA), is a potent odorant released by males (Siefkes and Li, 2004; Yun et al., 2003b) and inhibits steroidogenesis in immature males (Chung-Davidson et al., 2013a) but has no observable behavioral effects on ovulated females (Johnson et al., 2012; Luehring et al., 2011). In contrast, a third component 3,12-diketo-4,6-petromyzonene-24-sulfate (DkPES) (Figure 1.1) attracts females when mixed with 3kPZS (Brant et al., 2016a; Li et al., 2013b). Furthermore, 3 keto-1-

ene PZS (Figure 1.1) appears as effective in attracting females as 3kPZS (Johnson et al., 2014), though whether it is an additional component of the blend or simply perceived as an analog of 3kPZS remains unclear. Additional molecules isolated from male odor include petromyzone A, petromyzene A and B, and petromylidenes A-C (Figure 1.1), which attracted ovulated females in two-choice maze tests (Li et al., 2017a; Li et al., 2018c; Li et al., 2017b), petromyzone C, which repulsed ovulated females in a two-choice maze test (Li et al., 2017b), and petromyzone B and petromyzestrosterol, which are potent olfactory stimuli but have negligible or unknown behavioral effects on ovulated females (Li et al., 2017b; Li et al., 2012). With these structures identified and initial behavioral assays conducted, future studies can test their contribution to the activity of male-conditioned water.

Seminal fluid produced by sea lamprey (*i.e.* milt) is a second source of male sex pheromones (Scott et al., 2019). In two-choice maze assays, milt attracted both migratory and spawning males and females. Subsequent experiments identified the polyamine spermine (Figure 1.1) as a component of male milt that is detected at low concentrations and attracts ovulated females but not spermiated males in a two-choice maze (Scott et al., 2019). Therefore, spermine likely acts as a component of the milt pheromone but additional components attract males and migratory females. Distinct components of milt acting as pheromones for spawning females versus males and migratory females is consistent with the different roles they may have for each sex and life stage; spawning females presumably use milt to locate males that are actively spawning and not spent whereas males and migratory females likely use milt to locate general areas supporting active spawning.

A recent study characterized PZS (Figure 1.1) as behavioral antagonist of 3kPZS for ovulated females (Buchinger et al., 2020b). Previously, electro-olfactogram (EOG) recordings indicated

PZS inhibits the olfactory response to 3kPZS (Fine and Sorensen, 2008; Raschka et al., 2018; Siefkes and Li, 2004). Interestingly, larvae and males both release PZS and 3kPZS but at opposite proportions; PZS is the more abundant molecule in larval odor whereas 3kPZS is more abundant in male odor (Buchinger et al., 2020b). Observations that larval odor contains 3kPZS (Brant et al., 2016b; Buchinger et al., 2020b) but does not attract ovulated females despite their strong preference for 3kPZS (Bjerselius et al., 2000; Brant et al., 2016b; Buchinger et al., 2020b) led to the hypothesis that ovulated females use PZS as an antagonist to avoid orienting towards larvae-released 3kPZS while tracking 3kPZS released by spermiated males (Figure 1.3). Indeed, PZS abated ovulated female preferences for 3kPZS in two-choice maze assays and field experiments (Buchinger et al., 2020b). These results add to the complexity of pheromone communication in sea lamprey and indicate the potential utility of using pheromone antagonists in control efforts.

Phylogenetic comparisons of pheromone production and behavioral responses to pheromones indicate lamprey pheromones are not entirely species-specific and implicate possible evolutionary mechanisms underlying complex signaling in sea lamprey. Larvae of several species release 3kPZS and at least two species respond to 3kPZS during migration (Brant et al., 2016b; Buchinger et al., 2013; Yun et al., 2011). However, sexual communication with 3kPZS appears limited to two species; of the 11 species sampled, only spermiated male sea lamprey and chestnut lamprey (*I. castaneus*) released 3kPZS at high rates (Buchinger et al., 2017b). Notably, all species tested detect 3kPZS with high sensitivity via their olfactory systems (Buchinger et al., 2017b; Robinson et al., 2009; Siefkes and Li, 2004; Yun et al., 2011) and synthesize 3kPZS and related bile acids (Buchinger et al., 2019). Chemical profiling and behavioral tests for cross-reaction indicate olfactory cues overlap among species and elicit asymmetrically species-specific

preferences; sea lamprey were attracted to olfactory cues from other Great Lakes species but other species generally preferred only conspecific odors (Buchinger et al., 2017c). A separate study found that chestnut lamprey, the only other species found to use 3kPZS as a sex pheromone, (Buchinger et al., 2017b) oriented toward male odorant from sea lamprey even when presented opposing male odorant from conspecifics (Buchinger et al., 2020a). These results indicate minor components are insufficient to maintain species specificity and raise the possibility sea lamprey reduce the reproductive success of native lampreys. Taken together, phylogenetic comparisons indicate that the various components of the male pheromone evolved through distinct evolutionary mechanisms, and led to the hypothesis that communication with 3kPZS evolved as a signaler adaptation and minor components may have evolved as receiver adaptations (Figure 1.3) (Buchinger et al., 2017c).

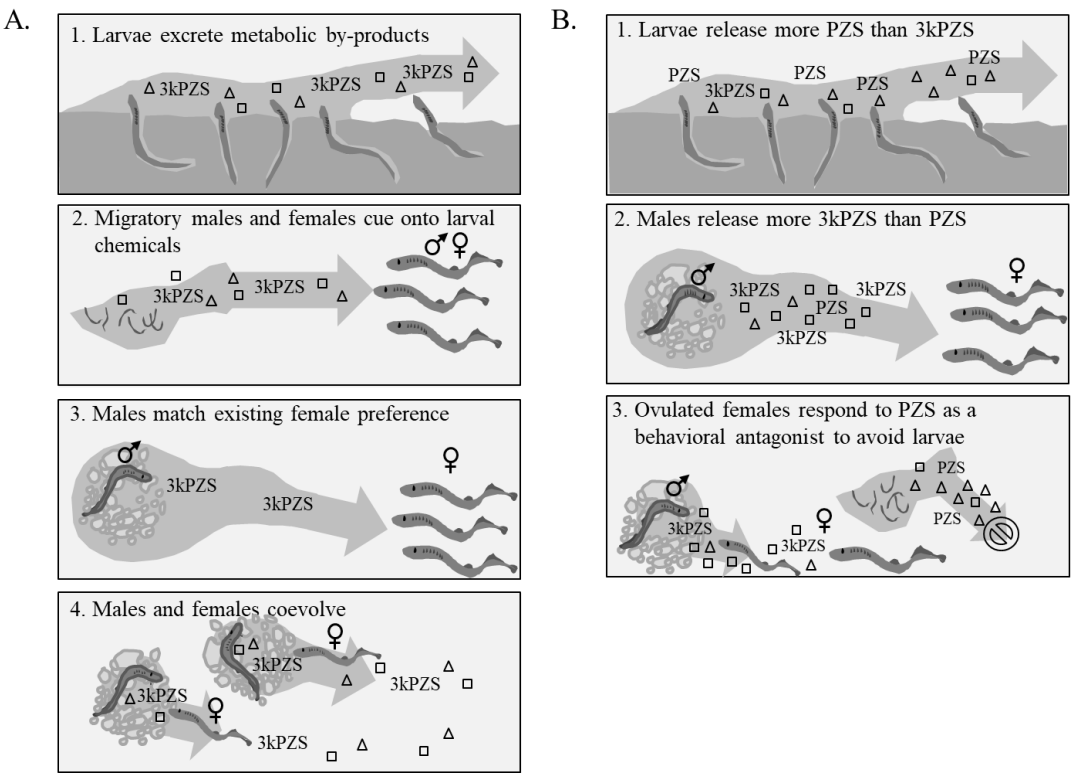

Figure 1.3. Evolution of sea lamprey pheromones. **A)** Schematic showing the hypothesized evolutionary steps of pheromone communication in sea lamprey. 1) Stream resident larvae release multiple chemical compounds, including 3kPZS, as metabolic byproducts. 2) Migratory adults cue into 3kPZS and other larval released compounds to locate suitable spawning habitat. 3) Males release 3kPZS that attracts females, exploiting the pre-existing preference for 3kPZS in the migratory context. 4) Female preference for increased 3kPZS release results in fitness benefits for males with elevated release and leads to the transition of 3kPZS as a broad scale migratory cue to a more proximate reproductive signal that coordinates reproduction. **B)** Schematic showing the evolution of PZS as a behavioral antagonist. 1) Stream resident larvae release more PZS than 3kPZS. 2) Spermiated males release 3kPZS at elevated rates, resulting in more 3kPZS than PZS released in pheromone plumes. 3) Female discrimination of pheromone plumes containing different 3kPZS:PZS ratios allows ovulated females to locate mates rather than larval habitat that may be present near spawning grounds. Figure modified from Buchinger et al., (2015).

INTEGRATING CHEMICAL ECOLOGY INTO SEA LAMPREY CONTROL

Exploiting the chemical ecology of sea lamprey remains a promising avenue for achieving the goals of the control program (Siefkes, 2017; Sorensen and Johnson, 2016). In general, an understanding of the chemical ecology of invasive species is useful for control programs because chemical cues are often taxon specific, reducing non-target effects, and chemically benign, being neither toxic nor persistent in the environment. In the case of the sea lamprey, these advantages are enhanced by the animal's strong reliance on chemical information when undertaking crucial habitat and reproductive decisions. Tactics that incorporate knowledge of chemical ecology include pheromone-based assessment, application of chemical cues to manipulate or disrupt sea lamprey behavior or physiology, and strategically implementing other control actions in ways that leverage the sea lamprey's reliance on chemical information (Li et al., 2007).

Chemical cues and pheromones are viewed as supplements to lampricides or barriers rather than alternatives (Siefkes, 2017; Siefkes et al. 2021). Development of technologies for sea lamprey control, including sterile male release (Johnson et al., 2021), pheromones (Friedricks et al., 2021), and genetic control (Thresher et al., 2019), requires significant research investments long before any benefits are realized and maintaining realistic expectations throughout these processes is critical to capitalize on the initial investments and produce useful management tools. Since the establishment of the sea lamprey management program, barriers and lampricides have been the closest options to "silver bullets" for controlling this invasive species. The goals, therefore, of integrated control with pheromones should be to supplement these highly effective technologies by minimizing the risk of lampricide resistance (Christie et al., 2019), extending the social license to maintain sea lamprey barriers (Hrodey et al., 2021) and apply pesticides to public waterways (Gaden et al., 2021), and targeting populations that are not effectively or

efficiently controlled using barriers or lampricides (Siefkes et al., 2021). Additionally, creating stream-specific combinations of tactics is likely necessary due to lampricides and supplemental controls working in a set range of environmental conditions that vary across stream and basin (Siefkes et al., 2021). As Twohey et al. (2003) stated in the proceedings of SLIS II (Sea Lamprey International Symposium II, Sault Ste. Marie, Michigan, USA, August 2000), "pheromone strategies that integrate and enhance existing management techniques are likely to be the most beneficial." Below, we briefly review previous and needed research on integrating an understanding of chemical ecology into sea lamprey control. By highlighting this research and the materials available for leveraging chemical cues against sea lamprey, we discuss the options available for managers to consider when developing specific control strategies. These are not recommendations for implementation which ultimately should be decided by management agencies.

Application of chemosensory cues to modify sea lamprey behaviors

The majority of research and discussion on the use of pheromones in sea lamprey control has focused on direct application of chemical cues to manipulate or disrupt sea lamprey behavior (Li et al., 2003; Li et al., 2007; Sorensen and Vrieze, 2003; Twohey et al., 2003). Below, we briefly review recent research on these approaches and highlight the contexts in which they may be useful.

Guide lamprey toward and into traps

Improving trapping success was prioritized for the development of management techniques that employ pheromones (Twohey et al., 2003) and has been investigated extensively during the 20 years since SLIS II. Sea lamprey traps are typically placed on or near the faces of dams to capture migrating individuals. In general, they suffer low and inconsistent performance due to

mismatches between trap placement and sea lamprey search behavior (Bravener and McLaughlin, 2013; McLean et al., 2015; Rous et al., 2017). Consequently, they do not achieve the removal rates necessary to achieve meaningful reductions in reproduction (Vélez-Espino et al., 2008; Young, 2005). Currently, traps are not used as a control measure (Miehls et al., 2020a; Miehls et al., 2020b), but several are operated as index sites to monitor adult sea lamprey abundance (Adams et al., 2021). Pheromone-baited trapping will need to be incorporated into the existing infrastructure of barrier-integrated traps or through the use of portable traps and could be labor intensive as daily operation is needed during the spawning migration (April-July).

In experimental environments with no competing cues, application of migratory pheromones (Wagner et al., 2006), sex pheromones (Johnson et al., 2005), and alarm cues (Bals, 2012; Di Rocco et al., 2016a) drastically alter movement paths, guiding lampreys toward or away from the odor source. When only considered in these simple circumstances, pheromones appeared to be highly promising tools to improve trap performance. However, follow-up research using sex pheromones and alarm cues in natural circumstances suggest greater refinement of the pheromone-trapping approaches are necessary to benefit the control program.

First, pheromone-baited traps must effectively compete with pheromone released by wild lamprey. Although high concentrations of 3kPZS lure some ovulated females away from natural male pheromone (as collected from sperimated males; (Johnson et al., 2009), mature females remain near natural male pheromone much longer than synthesized 3kPZS (Johnson et al., 2012; Johnson et al., 2009) and were less likely to approach 3kPZS-baited traps when background male odors were present (Luehring et al., 2011). However, given the ready availability of 3kPZS, an inexpensive deployment mechanism was developed (Wagner et al., 2018), and a number of management-scale field tests were initiated to test its efficacy in traps currently operated by the

control program. The goals of these efforts were to ascertain whether it would improve the performance of those traps by increasing captures of migrants or by attracting otherwise unavailable mature females. The results from these studies were highly variable, with capture increases ranging from 0 – 65% (Dawson et al., 2017; Hume et al., 2015; Johnson et al., 2020; Johnson et al., 2015b; Johnson et al., 2013; Wagner et al., 2018). Notably, increases in trap catch occurred when 3kPZS was applied to larger streams or streams containing few spawning sea lamprey (Johnson et al., 2015b). In another field test, a novel technique was developed that exploits a natural circumstance where 3kPZS would not be in direct competition with spermiated males (Wagner and Thomas, 2010). Sea lamprey not captured in barrier-integrated traps during the migration tend to collect in large numbers in the stretch of stream just below the barrier until the onset of maturation. At that time, they reverse direction and move downstream in search of mates. 3kPZS-emitting traps were placed downstream of the dam but upstream of the spawning habitat to 'intercept' these animals (termed reverse-intercept trapping) (Figure 1.4). The concept was supported in that trap captures increased with the observed downstream migration. However, as observed in the studies with existing traps, total capture success was low (10%). Although the low efficacy of pheromone-baited traps may be due to 3kPZS alone not replicating the full male odor (Johnson et al., 2009), subsequent studies indicated that even applying male-conditioned water yielded low catches, especially where many wild males were present (Johnson et al., 2015c). Although only sex pheromones have been used in management-scale tests, challenges such as competing with natural pheromone are also likely to occur when applying the migratory pheromone (Wagner et al., 2009). How, when, and where managers can implement pheromone-baited trapping that effectively competes with wild pheromone sources remains an important direction for future research. Based on the current information, pheromone-baited traps may be

most useful where sea lamprey occur at low densities, including, for example, areas with populations that are naturally small or have been reduced to low numbers or streams without barriers that aggregate sea lamprey in small areas. In these scenarios, each female removed is more likely to reduce recruitment and pheromone-baited traps may remove a larger proportion of the population more consistently.

Second, the pheromones applied with traps should be based upon the biology of sea lamprey most likely captured. So far, most studies on pheromone-baited traps applied sex pheromones (3kPZS or natural male pheromone) to barrier-integrated traps (Hume et al., 2015; Johnson et al., 2020; Johnson et al., 2015b; Johnson et al., 2013; Johnson et al., 2015c); however, barrier-integrated traps primarily catch migratory sea lamprey, which exhibit only weak behavioral responses to sex pheromones (Brant et al., 2016b; Brant et al., 2015). The sea lamprey alarm cue, a substance emitted from damaged tissue that elicits a strong repellency from migrants, has been field-tested in combination with 3kPZS in two push-pull trapping scenarios: barrier-integrated trapping (Hume et al., 2015) and free-standing traps in the river channel (Hume et al., 2020b). The alarm cue effectively guided migrating sea lamprey into the vicinity of the trap, reduced the time to encounter by 50% (Hume et al., 2015), and increased rates of trap encounter (Hume et al., 2020b) to levels that meet control targets (Haeseker et al., 2007). However, 'push', 'pull', and 'push-pull' configurations did not increase trap capture. This suggests a pheromone with stronger effects on migratory sea lamprey (i.e., the migratory pheromone) is needed to increase trap entry. Recent research has unveiled a suite of molecules that attract migratory sea lamprey in field tests but have not yet been tested in management-scale experiments (Brant et al., 2016b; Li et al., 2018a; Li et al., 2018b). Given migratory sea lamprey are the most likely to be caught and the migratory pheromone has profound effects on migratory sea lamprey behavior, these

molecules may be more useful for increasing trap catches. However, expectations should be set appropriately as management-scale tests require synthesis of pheromone molecules in large quantities, the blend is only partially characterized, and native lamprey above barriers likely introduce background pheromone (Wagner et al., 2009).

Taken together, previous research indicates pheromone-baited trapping may be a useful supplement to the current sea lamprey control measures in some circumstances, especially where adult sea lamprey density is low and the pheromone and trap combination matches the ecology of targeted life stage. However, the limited scope of most research on pheromone-baited traps (3kPZS-baited, barrier-integrated traps) precludes broad-stroke conclusions about pheromone-baited trapping prospects as a supplemental tool. Notably, authors of pheromone-baited trapping studies often noted the inefficacy of the fishing apparatus (trap) or the fishing practice (trap deployment), as evidenced by frequently improved encounter rates, but rarely improved capture rates (Hume et al., 2020b). The development of trapping devices that exploit the anguilliform swimming mode of sea lamprey (e.g., eel ladder trap entrances; (Hume et al., 2020a; Reinhardt and Hrodey, 2014) and its epibenthic swimming habit in rivers (e.g., bottom-oriented traps, S. Miehls pers. Comm., United States Geological Survey, July 24, 2020) may yield substantially different results of using pheromones to bait traps. More broadly, an important first step for researchers and managers is to consider which current or future issues might be addressed, wholly or in part, using pheromone-baited traps. As with the release of sterilized males (Johnson et al., 2021), the current approach to baiting traps with pheromone seems likely to be especially useful for controlling low-density populations, which will likely be an important challenge if the goal is to eradicate sea lamprey (Jones and Adams, 2021).

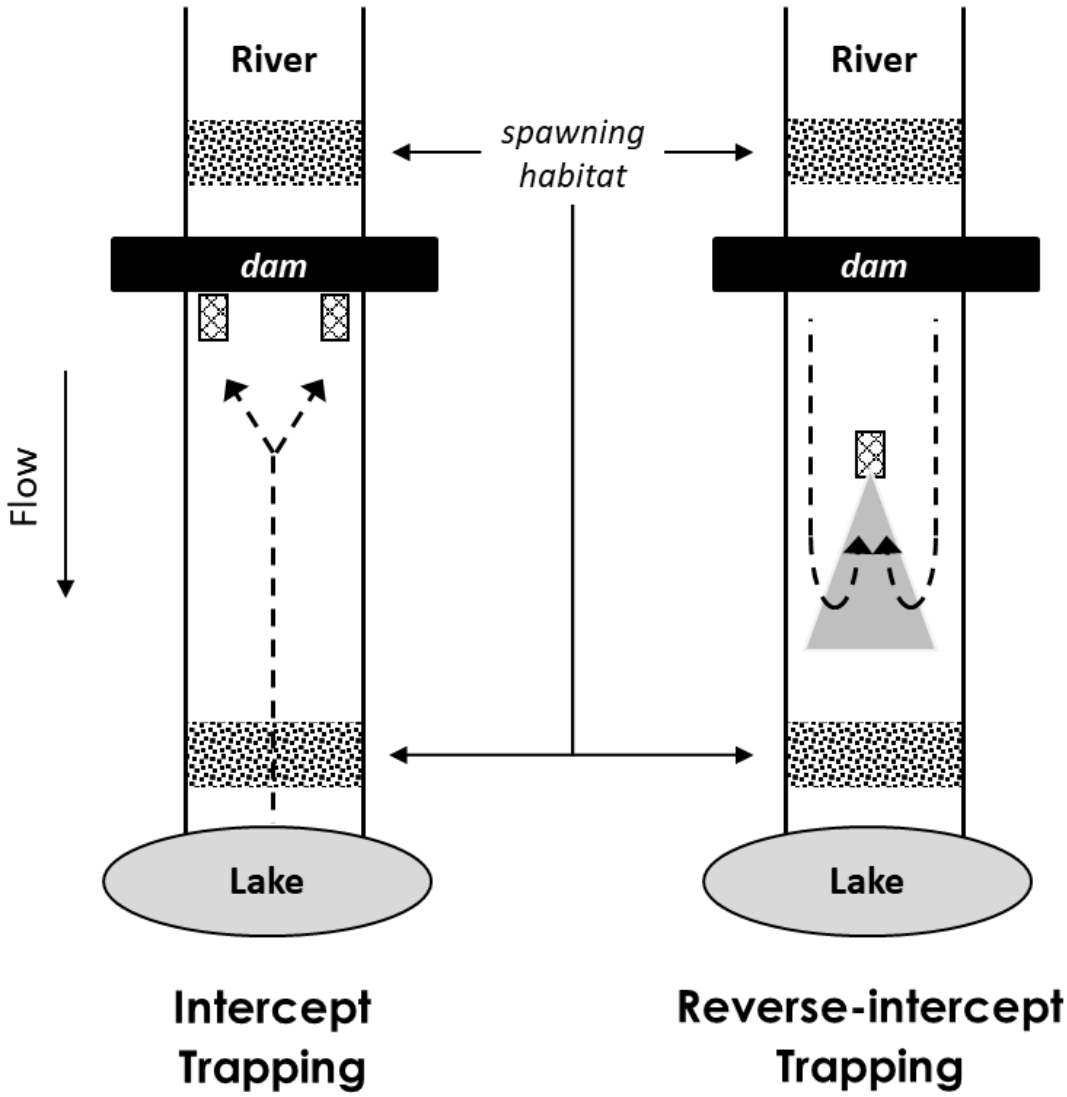

Figure 1.4. Potential trapping scenarios of adult sea lamprey using pheromones. Traditional trapping for sea lamprey assessment involves placing traps along a dam face to intercept migrating animals as they seek upstream spawning locations (dashed lines indicate the movement of migrating sea lamprey). Reverse-intercept trapping targets those animals that are not captured by the dam-integrated traps. When the blocked migrants 'reverse' their migration to seek alternate spawning habitat downstream, traps emitting a plume of attractant pheromone may intercept them. The pheromone emitted by the trap will not have to compete with spawning males, as the reverse-intercept trap is placed between the dam and the downstream spawning habitat.

Divert or draw adult sea lamprey into selected rivers or tributaries

Chemical cues could be useful in integrated sea lamprey control if they lure adult sea lamprey into streams with low natural survival (ecological trap) or that are effectively trapped or treated with lampricides. Indeed, larval odor appears to be a major driver of stream entry by migratory adults (Moore and Schleen, 1980; Vrieze et al., 2010; Vrieze et al., 2011). However, tests with the identified larval odorants PADS and PSDS failed to increase entry rates into the river but did increase the time sea lamprey spent in the river plume (Meckley et al., 2014), and a sex pheromone component, 3kPZS, failed to increase adult run size (Johnson et al., 2013). Alarm cue may also increase, rather than discourage, entry into streams; sea lamprey released near the mouth of Carp Lake River, MI were ~20% more likely to enter when alarm cue was applied to activate the entire discharge (Luhring et al., 2016). These effects may be bolstered by the addition of an attractant, similar to the idea behind push-pull strategies. Importantly, the success of diverting sea lamprey into selected streams depends on the presence of background odors; pheromones or alarm cues effectively bias (70-90% changes) sea lamprey into activated channels when no background odors are present but only moderately bias sea lamprey (10-20% changes) when tested against competing odors even when applied at concentrations 10 times higher than the natural source (Byford, 2016; Johnson et al., 2009; Wagner et al., 2006). As with trapping, diverting sea lamprey using pheromones may be useful in control but requires tests needing large amounts of natural and synthesized compounds, which are currently costly to synthesize for large-scale use, and is likely to be most effective in the absence of competing odors.

Disrupt mating or other life history events

Interfering with the chemical communication of sea lamprey may disrupt reproduction and reduce reproductive success. Indeed, anosmic sea lamprey fail to enter streams (Vrieze et al.,

2010; Vrieze et al., 2011), locate mates (Johnson et al., 2006), and spawn (Johnson, 2005). At least two techniques might be used to render sea lamprey effectively anosmic to particular pheromone components: first, attractive cues could be applied at rates high enough to cause sensory disruption, sensory adaptation, or camouflaging of natural pheromone plumes. For example, enshrouding natural male pheromone with 3kPZS reduced the number of females that located the male pheromone (Johnson et al., 2009). However, this approach required a very high concentration of 3kPZS (10^{-10} M) and only marginally reduced the number of females that locate the natural male pheromone (~ 75% still located natural pheromone). Another possible technique is application of antagonists that interfere with responses to pheromone components. Cross adaptation EOG recordings indicate specificity of sea lamprey olfactory receptors to certain compounds (Fine and Sorensen, 2008; Siefkes and Li, 2004) which may allow targeted application of antagonists that disrupt specific behavioral responses. Computer algorithm based screening of commercial compounds indicate many molecules may inhibit olfactory responses to 3kPZS (Raschka et al., 2018), with the larval odorant PZS confirmed empirically as being promising (Buchinger et al., 2020b). Indeed, maze and in-stream behavioral assays confirmed PZS abates attraction to 3kPZS when at the same or greater concentrations (Buchinger et al., 2020b). It is currently unknown whether PZS can block behavioral responses to the whole male odor, but given our current understanding of the pheromone system, it can be hypothesized that (1) because 3kPZS is a long distance component (Johnson et al., 2009), blocking the response of 3kPZS may inhibit long distance attraction and search behavior of females, effectively blocking the response to whole male odor despite not blocking it physiologically or in close proximity and (2) blocking responses to the natural pheromone blend will likely require higher concentrations of PZS than blocking responses to 3kPZS alone. Further research will determine how PZS

influences behavioral responses to the complete pheromone blend in various scenarios (Siefkes et al., 2021).

Current research continues to investigate how behavioral antagonists might be incorporated into sea lamprey control, including options such as (1) inhibiting the ability of female sea lamprey to find mates and reproduce by disrupting mate search behavior, (2) blocking detection of competing pheromone sources and luring sea lamprey into specifically targeted areas that are poor for reproductive success or are easier and more effective to treat with lampricides, or (3) blocking detection of background sources of 3kPZS and luring females into traps with a different attractive compound that is unaffected by the antagonist. Competition with background odors has impeded the effectiveness of chemosensory cues used in management scenarios, but the ability to block or disrupt background odors using antagonists may make strategies that rely on attracting sea lamprey to specified areas more effective (options 2 and 3). The effectiveness of behavioral antagonists also depends on a holistic understanding of the sea lamprey pheromone communication network that contains multiple communication channels. Using multiple antagonists, it may be possible to sequentially interfere with lamprey behaviors (i.e., river entry, upstream migration, and mate finding) that are mediated by multiple chemosensory cues in order to better redistribute or trap sea lamprey, or disrupt their life cycle.

Non-target effects of chemosensory cues

Non-target effects of chemosensory cues are likely to be less damaging than current control practices and more socially acceptable to stakeholders (Siefkes et al., 2021). First, chemosensory cues are applied at low concentrations and affect lamprey behavior or physiology but are not lethal. Effects on other fishes or animals are unknown but likely minimal as they have not evolved to communicate with these specific compounds. The evolution of distinct olfactory

receptors for different classes of bile acids between jawed (Cong et al., 2019) and jawless fishes (Zhang et al., 2020) provide a potential molecular mechanism that may limit the non-target effects of chemosensory cues on other fishes, but this needs further examination. Importantly, non-target effects on native lampreys (i.e., increased by-catch of native lamprey in trapping scenarios or impeded ability to find suitable spawning areas or mates when encountering antagonists) are possible given the migratory pheromone (Fine et al., 2004), sex pheromone (Buchinger et al., 2017b, 2017c, 2020), and alarm cue (Bals and Wagner 2012, Byford et al., 2016, Hume and Wagner 2018) appear at least partially conserved across species. Chemosensory cues are likely to be less harmful than current control practices of barriers and lampricides, but should nevertheless be considered given the substantial decline in native lamprey populations since sea lamprey control began (Neave et al., 2021).

Pheromone measurement as a tool for population assessment

Quantifying sea lamprey pheromones in river water could be useful for assessing the abundance and distribution of populations. Since SLIS II, many pheromone molecules have been identified (Li et al., 2018b), and their concentrations in stream water can be quantified or estimated using ultrahigh performance liquid chromatography-tandem mass spectrometry with low limits of detection: 3kPZS (<0.1ng/L), PZS (0.25ng/L), PADS (0.25ng/L), and PSDS (2.5ng/L) (Li et al., 2011; Stewart and Baker, 2012; Wang et al., 2013; Xi et al., 2011). While detection of pheromone compounds in river water has primarily involved quantification of PZS, PADS, PSDS, and 3kPZS (Stewart et al., 2011; Wang et al., 2013), the methods can be expanded to analyze other chemicals of interest based on assessment needs or objectives. Assessing larval and adult populations is critical for selecting streams for lampricide treatment (Jubar et al., 2021) and evaluating the performance of sea lamprey control (Jones, 2007). Notably, current

assessment methods, electrofishing surveys for larvae and trapping for adults, consume approximately 10% of the sea lamprey control budget (2020 budget; M. Siefkes pers. Comm., Great Lakes Fishery Commission, July 25, 2020). Supplementing current methods with pheromone quantification may yield an effective strategy for assessing sea lamprey populations, but additional research is needed to define the specific uses and appropriate methods for pheromone-based assessment.

Incorporating pheromone-based assessment requires further research on the high variation of pheromone concentrations in river water (Wang et al., 2013). Laboratory experiments indicate pheromone release varies in relation to feeding (Polkinghorne et al., 2001) and season (Brant et al., 2016b), for larvae and maturation status (Siefkes et al., 2003), and for body size (Buchinger et al., 2017a), and social context for males (Fissette et al., 2020). When measured in streams, pheromone concentrations have been highly variable and could be related to biological attributes of the population or abiotic factors such as stream discharge, water chemistry, and temperature (Wang et al., 2013; Xi et al., 2011). Whether the variation in pheromone concentrations measured in streams can be explained sufficiently to allow approximate assessment of population demographics remains unknown. One promising method to more accurately measure average pheromone concentrations is the use of passive sampling, which involves deploying pheromone samplers in streams for extended durations of time (e.g., 1 week) rather than collecting samples at a single time point (Stewart and Baker, 2012). Optimizing methods using passive samplers may require characterization of river hydrodynamics (Stewart and Baker, 2012), design of sampling arrays that provide accurate estimates of the stream-wide population as well as distribution of the population, and validation of samplers for target compounds. Addressing the

high variation in riverine pheromone concentrations requires studies that optimize the sampling methods as well as explain the biological variation in pheromone release.

The design of pheromone-based assessment tools must also incorporate information about release of pheromone molecules across life stages and species. Larval and male sea lamprey produce and release many of the same compounds, including 3kPZS (Buchinger et al., 2019; Buchinger et al., 2015; Li et al., 2018b). Likewise, larval compounds appear to be largely conserved across lamprey species (Fine et al., 2004) and male sex pheromones are partially shared among species (Buchinger et al., 2017b; Buchinger et al., 2017c). Given the overlap in distributions of lampreys across life stage and species (Applegate, 1950; Morman, 1979), the source of pheromones measured in river water requires direct consideration. Nevertheless, measuring pheromones has potential to provide specific information for population assessment. For example, discriminating 3kPZS release by larvae vs spermiated males may be possible based on the concentration (or, for example, spikes in concentration during the spawning season) as spermiated males release 3kPZS at many fold higher rates than larvae (Brant et al., 2013; Brant et al., 2016b) or based on the ratio of 3kPZS to other components, which differs between larvae and males (Buchinger et al., 2020b). Similarly, high concentrations of 3kPZS are likely attributable to spawning sea lamprey in most rivers as only chestnut lamprey, which have a range limited to the east coast of Lake Michigan, have been found to use 3kPZS as a sex pheromone (Buchinger et al., 2017b). Sampling above and below barriers may also help account for pheromones released by native lamprey populations located above barriers (Neave et al., 2021). Further characterization of known and unknown compounds is likely to unveil compounds and/or ratios that are stage- and/or species specific (Buchinger et al., 2017c). Lastly, chemical information may also be paired with detection of environmental DNA (eDNA) to enhance the

species specificity of pheromone quantification (Docker and Hume, 2019; Gingera et al., 2016). While eDNA can provide accurate species detection between native lampreys and sea lamprey, it cannot distinguish the sex or stage of lampreys within a stream and is not as efficient at detecting larvae, especially at low densities (Gingera et al., 2016; Schloesser et al., 2018). Combining pheromone and eDNA assessment may provide a more accurate assessment of lamprey populations within streams, especially with specifically designed, seasonal testing and in instances where native populations persist above barriers, resulting in populations below barriers due to larval drift.

Taken together, the existing data indicate much potential in measuring pheromones for assessment and raise important directions for future research. As a first step, researchers and managers should consider which current objectives of assessment are likely achievable using pheromone quantification. Given that release of pheromone compounds often correlates with body mass (Scott and Ellis, 2007), future research might indicate pheromone concentrations produce useful estimates of lamprey biomass (Polkinghorne et al., 2001); however, barring major advances in our ability to explain variation in pheromone release, pheromone quantification is unlikely to be useful for quantitative estimates of population size or the body size of larvae, which inform the selection of streams for lampricide treatment (Jubar et al., 2021). Nevertheless, measuring pheromones could achieve current or new objectives less focused on quantitative estimates of lamprey populations. Passive samplers could be deployed to streams scheduled for qualitative surveys that define the distribution of larvae in a stream or evaluate the need for quantitative assessment (Jones, 2007). Likewise, samplers placed at river mouths may allow assessment of relative attractiveness to migrants that may better direct larval assessment in following year(s). Alternatively, repeated pheromone quantification could provide useful indices

that, for example, assist with evaluating the success of lampricide treatments. Lastly, measuring pheromones might achieve objectives that are not currently feasible given the available techniques and resources; for example, sampling 3kPZS during the spawning season could be used to assess the size and duration of spawning, which is currently not assessed other than through intensive nest surveys done for specific research studies. Indeed, pheromone quantification, although unlikely useful for all assessment objectives, is a potential addition to current assessment methods.

MOVING FORWARD

The two decades since SLIS II have brought about many advances in our fundamental understanding of sea lamprey chemical ecology and the challenges to incorporate it into the control program. Of the many ideas put forth in the SLIS II proceedings and subsequent theme papers (Li et al., 2003; Li et al., 2007; Sorensen and Vrieze, 2003; Twohey et al., 2003), only baiting barrier-integrated traps with 3kPZS has been evaluated at a management scale (Johnson et al., 2020; Johnson et al., 2015b; Johnson et al., 2013). Most of the options of how to incorporate an understanding of chemical ecology into sea lamprey management remain viable, albeit in need of study. Managers and researchers must identify the specific needs of the sea lamprey control program that might be addressed using an understanding of chemical ecology, especially through integration of current or other emerging control methods (e.g., sterile male release, electrical barriers, or improved trap designs). Additionally, the current high costs of synthesizing these unique compounds needs to be considered when developing management strategies. Pheromone-based assessment and direct application of semiochemicals or their antagonists hold promise to provide additional tools for sea lamprey control. Further research is needed to optimize the methods, identify the contexts in which each tool is likely to be most useful, and develop economical means to synthesize a diverse suite of chemical compounds.

Fundamental research on the complexity of sea lamprey semiochemicals and related physiology and behaviors is critical to define when, where, and how they can be applied for control. For example, studies on the molecular mechanisms of pheromone detection may yield potent antagonists for pheromones or pheromone receptors. Elucidating evolutionary mechanisms underlying sea lamprey pheromones and chemical cues will allow us to better understand and exploit their behavioral functions. Likewise, characterizing the interacting roles

of multiple components of a cue (e.g., 3kPZS and DkPES) and multiple cues (e.g., alarm cue and migratory pheromone) will open opportunities to develop mixture formulas that compete more potently or effectively with background semiochemicals. Lastly, understanding the internal, social, and environmental circumstances that drive variation in responses to semiochemicals remains a fundamental need in the study of sea lamprey behavior. This need is paramount as the success or failure of management tools is defined both by the magnitude of the desired effect and the context within which the tools perform.

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CHAPTER 2:

CONTEXT-SPECIFIC DECOUPLING OF A MIMETIC SEX PHEROMONE AND ITS MODEL IN SEA LAMPREY

ABSTRACT

The sensory trap model of signal evolution suggests that males manipulate females into mating using traits that mimic cues used in a non-sexual context. Despite much empirical support for sensory traps, little is known about how females evolve in response to these deceptive signals. Female sea lamprey (*Petromyzon marinus*) evolved to dissociate a male sex pheromone from the larval odor it mimics and orient only towards males when searching for mates. Larvae and males release the attractant 3-keto petromyzonol sulfate (3kPZS), but spawning females avoid larval odor using the pheromone antagonist petromyzonol sulfate (PZS), which larvae, but not males, release at higher rates than 3kPZS. We tested the hypothesis that females have also decoupled their responses to the male pheromone and larval odor during migration, when they navigate into spawning streams using larval odor and before they begin mate search. In-stream behavioral assays revealed that, unlike spawning females, migratory females did not discriminate between mixtures of 3kPZS and PZS applied at ratios typical of larval versus male odorants. Our results indicate females dissociated the sexual and non-sexual signals during but not outside of mating and show sensory traps can lead to reliable sexual communication without females shifting their responses in the original context.

INTRODUCTION

Many sexual signals exploit pre-existing responses that originated outside of the mating context (Christy, 1995; Endler and Basolo, 1998; Ryan, 1990; Ryan and Cummings, 2013). For example, male lyrebirds (*Menura novaehollandiae*) mimic alarm calls to prevent females from leaving courtship arenas (Dalziell et al., 2021) and male characin (*Corynopoma riisei*) have opercular ornaments that mimic ants and elicit foraging responses from females (Kolm et al., 2012). An understanding of mimetic signals such as these, known as sensory traps (Christy, 1995; West-Eberhard, 1984), helped to expand the discussion around signal evolution beyond classic models that suggest preferences for mating signals arise according to the benefits they provide or indicate (Andersson, 1994; Hosken and House, 2011; Møller and Jennions, 2001). Despite much evidence for sensory traps, if and how receivers evolve in response to these deceptive signals remains largely unknown and subject to debate (Arnqvist, 2006; Basolo, 1996; Dawkins and Guilford, 1996; Holland and Rice, 1998; Rodríguez, 2009; Ryan and Cummings, 2013).

Some evidence indicates that sensory traps can lead to reliable sexual communication if receivers evolve to dissociate the mating signal from the non-sexual cue it mimics (Stuart-Fox, 2005). In splitfin fishes (Goodeniae), males signal to females using a yellow tail band that mimics damselfly larva and invokes foraging responses in females (Garcia and Ramirez, 2005). The deceptive signal imposes costs on females as they become less efficient at foraging when male tail bands distract them from actual prey (Garcia and Lemus, 2012). These costs drove females to evolve a higher response threshold to the stimulus (a quivering yellow shape), which was then matched by exaggeration of the male tail band. The ensuing co-evolution of signal elaboration and resistance led females to decouple the male yellow band and damselfly larvae

and stop responding to the stimulus as a feeding cue. However, as expression of the yellow band is costly, females seem to benefit from using it to assess male quality (Garcia and Ramirez, 2005). These studies support the theory that exploitation of female preferences is costly to females and is met by resistance (Arnqvist, 2006; Holland and Rice, 1998) but show that this resistance can lead to reliable sexual communication (Stuart-Fox, 2005).

Male sea lamprey (*Petromyzon marinus*) signal to females using a sex pheromone that mimics the odor of conspecific larvae (Buchinger et al., 2013). During the pre-spawning migration into streams, lamprey follow chemical cues released by larvae that reside in nursery habitats near spawning grounds (Fissette et al., 2021; Teeter, 1980). The larval odor consists of fecal metabolites including the bile acid 3-keto petromyzonol sulfate (3kPZS), which has been found to attract migratory males and females (Brant et al., 2016b; Brant et al., 2015; Johnson et al., 2013). After several weeks in a stream, males become sexually mature (spermiated; Applegate, 1950; Johnson et al., 2015), build nests on gravel bars, and signal to sexually mature (ovulated) females using a multi-component pheromone (Fissette et al., 2021). As with larval odor, the male pheromone consists, in part, of 3kPZS; males release 3kPZS at high rates (~0.25 mg/hr) via specialized gill cells (Li et al., 2002; Siefkes et al., 2003) and females are attracted to 3kPZS over large distances (Johnson et al., 2009; Siefkes et al., 2005). Evidence that other lamprey species use 3kPZS as a component of larval odor but not a male pheromone indicates the non-sexual migratory response to 3kPZS likely originated before male signaling with 3kPZS (Buchinger et al., 2017; Buchinger et al., 2013). Taken together, these studies support the hypothesis that the male pheromone is a partial mimic of the non-sexual larval cue.

Despite the deceptive origin of 3kPZS signaling, female sea lamprey evolved to use the male pheromone for reliable sexual communication (Buchinger and Scott et al., 2020). Sea lamprey

die after a single spawning season, and females have only a few days to a week to find males and spawn (Applegate, 1950; Johnson et al., 2015). Therefore, 3kPZS signaling conceivably benefits females by reducing the costs of mate search. However, females searching for mates encounter 3kPZS from both males and larvae, as larvae reside in habitats immediately downstream of and sometimes interspersed with spawning grounds. Consistent with expected costs of confusing larval odor (the model) and the male pheromone (the mimic), ovulated females discriminate against larval odor and orient only toward the male pheromone during spawning (Buchinger and Scott et al., 2020). A mechanism underlying this discrimination is the pheromone antagonist petromyzonol sulfate (PZS), which abates female preference for 3kPZS when mixed at equal or greater concentrations than 3kPZS and which larvae, but not males, release at higher rates than 3kPZS (Figure 2.1A; Buchinger and Scott et al., 2020). Discriminating of larval odor and male pheromone by ovulated females enables reliable sexual communication, but it remains unknown if female responses have shifted in the nonsexual context as observed in splitfins (Garcia and Lemus, 2012).

Here, we tested the hypothesis that females have also decoupled the male pheromone and larval odor in the non-sexual migratory context. Specifically, we used in-stream behavioral assays to determine responses of migratory females to 1) mixtures of synthesized 3kPZS and PZS at ratios typical of larval (1:10) and male (100:1) odorants and 2) the complete, natural odors of larvae and males. Our results indicate females did not shift their responses to dissociate 3kPZS released by males versus larvae during migration as they did during spawning and suggest a previously undocumented evolutionary trajectory of sensory traps.

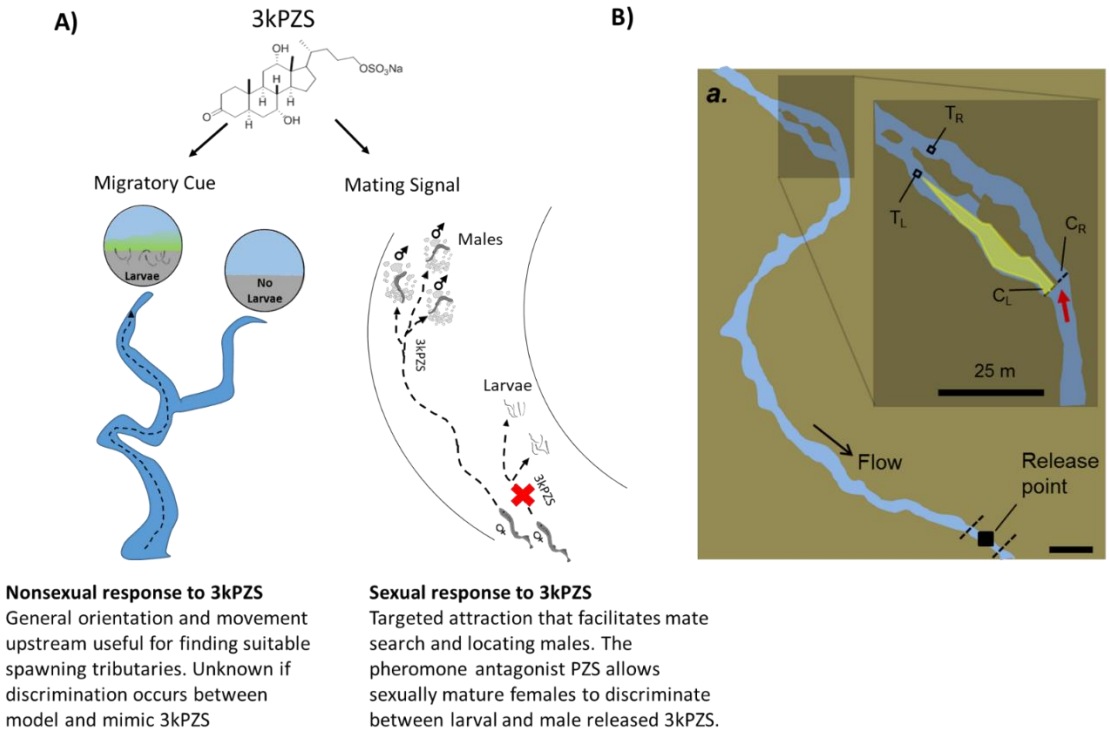

Figure 2.1. Context dependent responses to 3kPZS and how they were tested within the non-sexual context of reproductive migration. **A)** The behavioral responses to 3-keto petromyzonol sulfate (3kPZS) are different within the contexts of migration and sexual reproduction. During reproductive migration, migratory sea lamprey display general orientation and upstream movement to larval released 3kPZS which helps guide them to suitable spawning streams and tributaries within them (Brant et al., 2016, Johnson et al., 2013). During sexual reproduction, 3kPZS facilitates mate search, induces targeted attraction to a male's nest, and helps ensure successful reproduction (Johnson et al., 2009), and PZS acts as a pheromone antagonist to allow ovulated females to discriminate between male and larval odor. **B)** The ~250 m experimental bioassay used to test whether migratory sea lamprey discriminate between the larval cue and its mimic in the non-sexual context. An island at the upstream end splits the river into two sub-channels and mimics a natural scenario of tributary selection. Individual lamprey were implanted with passive integrated transponder (PIT) tags, and their behavior was monitored using several PIT antennas throughout the experimental site. Antennas were used at three locations: 1) two antennas at the upstream end where odors were applied in the left or right channel (T_L and T_R), 2) two antennas at the channel choice point (C_L and C_R), and 3) two antennas at the downstream end where experimental subjects were released, one above and one below the release point.

METHODS

Experimental Animals

Sea lamprey were trapped in tributaries of Lake Huron from May-June by the United States Fish and Wildlife Service. Lamprey were then transported to United States Geological Survey, Hammond Bay Biological Station (HBBS), Millersburg, Michigan and held in 1000 L aerated tanks that were continually fed with Lake Huron water at ambient temperatures. All experimental procedures followed protocols approved by the Michigan State University Institutional Animal Care and Use Committee (PROTO202100029). Two days prior to experiments, a group of sexually immature females (N = ~250) was taken to the Ocqueoc River and held in steel mesh cages to allow 1) acclimation to river conditions and 2) the natural sexual maturation process to remain ongoing in warmer river temperatures that are not available in holding conditions at HBBS (holding tanks contain much colder Lake Huron water). Every 2-3 days, additional migratory stage females (75-150) were transported to holding cages in order replace the females used in daily experimental trials (N = 45-54) and/or any individuals that died. The total number of females held in cages ranged from 205-307 for the duration of the experiment. No sexually mature females were used during this experiment, and they were differentiated from migratory stage individuals by applying gentle pressure to the abdomen and checking for oocyte expression (Siefkes et al., 2003).

Experimental Procedures – Passive Integrated Transponder Tagging

For each trial, 15-18 females were fitted with a 23 mm half-duplex passive integrated transponder (PIT) tag (Oregon RFID, Portland OR). In each female, a PIT tag was inserted via a small incision in the abdomen anterior to the first dorsal fin. After inserting the tag, the incision was closed using VetBond tissue adhesive (3M, St Paul MN). When tagging was complete,

experimental subjects were held in 200 L aerated tanks until being taken to the Ocqueoc River to acclimate for ~20 hours prior to the following day's experimental trials.

Experimental Procedures – Behavioral Assays

In-stream assays were used to evaluate behavioral responses of migratory stage females to natural and synthesized odors released by larvae and sexually mature males, and because migrating sea lamprey are primarily nocturnal (Applegate 1950), behavioral experiments were conducted at night. Assays were conducted in a ~250 m stretch of the Upper Ocqueoc River (Figure 2.1B) from May 29, 2021 to June 20, 2021. This stretch of river contains an island at the upper reaches that splits the main channel into two sub-channels. This mimics a natural scenario faced during sea lamprey migration, where tributaries within river systems meet and create a decision point for lamprey, where they must select the proper tributaries to maximize opportunities for successful reproduction (Buchinger et al., 2015; Fissette et al., 2021). The Ocqueoc River is a historic spawning site (Applegate, 1950) but currently, sea lamprey are blocked from the upper stretches by a barrier. This ensures no background pheromone odor from naturally spawning populations are present during experimental trials.

Female behavior was monitored using a PIT array with four separate PIT antennas to determine distribution within the experimental assay during experimental trials (Figure 2.1B). Two antennas, one within each channel and spanning the entire stream channel width, were placed ~3-5 m upstream from the sub-channel confluence and were used to determine which sub-channel an individual entered. Two additional antennas (1 m^2) were placed in each sub-channel at the upstream end of the island ~ 5 m below where the main river channel splits. Treatment odors were applied within the 1 m^2 upstream PIT antennas. PIT antennas monitored the proportion of females entering each sub-channel. Females used during experiments were held in

release cages ~200 m downstream from the odor application point. Three experimental trials were conducted each night ranging from ~21:40 to 01:15, with the first trial beginning 15 minutes after sunset. Each experimental trial was conducted for one hour. After 15 minutes of odor application, females were released from cages, and their behavior was monitored for 45 minutes. Odors were applied using a peristaltic pump (Masterflex L/S EW-07554-90, Cole Parmer Vernon Hills IL). Unique PIT tag numbers were used for each individual and allow behavior to be analyzed only for the trial in which they were released.

To determine if migratory stage females discriminated between the larval and male odorants, we compared behavioral responses across seven different treatments that include synthetic (Bridge Organics Inc., Vicksburg, MI, USA) and natural pheromone mixtures and were alternately applied to each sub-channel: 1) MeOH: water (1:1) – negative control, 2) larval extract (LE, migratory cue) – positive control, 3) spermiated male washings (SMW, male sex pheromone), 4) 3kPZS: PZS (1: 10), larva ratio, 5) 3kPZS: PZS (100: 1), male ratio, 6) 3kPZS: PZS (1: 10) vs. 3kPZS: PZS (100: 1), and LE vs. SMW. Details on odorant collection are described below. The negative control allowed assessment of channel bias within the assay. The positive control (LE) replicated the whole migratory pheromone released by larvae and confirms the behavioral assay functions to capture the intended behaviors. Testing larval and male released 3kPZS:PZS ratios is a direct test of whether females have decoupled the male pheromone and larval odor during migration. Individual ratios of 3kPZS: PZS showed whether migratory females are attracted to these different ratios on their own, and the application of these ratios simultaneously (3kPZS: PZS 100:1) versus (3kPZS: PZS 1:10), with one odor in each sub-channel, showed whether migratory females, like ovulated females, have a direct preference for either ratio. SMW applied alone showed if migratory stage females are attracted to the male sex

pheromone, and the application of LE and SMW simultaneously (LE versus SMW) showed if migratory females discriminate and prefer the natural larval or male pheromone.

To help avoid confounding factors of channel bias and time of night, we applied each treatment to the left versus right sub-channels during approximately the same number of trials and distributed treatments across all experimental times of night (early, middle, late trials). All treatments were applied to reach in-stream concentrations of 1.00E-13 M 3kPZS which has been shown to induce behavioral responses in females (Johnson et al., 2009). Sub-channel discharges were taken every 2-3 days or after every rain event to calculate the amount of each odorant needed to reach desired concentrations. The 3kPZS: PZS ratios for synthetic odors (3kPZS: PZS, larva = 1: 10, male = 100: 1) were chosen based on previous work (Buchinger and Scott et al., 2020). The ratios of 3kPZS: PZS in our natural odorant treatments (LE and SMW) were not identical to the synthetic ratios but showed the same overall trends in compound concentrations, with more PZS than 3kPZS released by larvae and more 3kPZS than PZS released by sexually mature males (3kPZS:PZS, LE = 1:3.74, SMW = 40.3:1).

Experimental Procedures – Natural Pheromone Collection

LE was collected by holding high densities of larval sea lamprey in large tanks at HBBS, and collection methods followed previously described methods (Li et al., 2018). LE from 6 separate years were pooled together, mixed thoroughly, and then aliquoted into 1L bottles. SMW was collected by holding 14 sexually mature males in 50 L of continually aerated Lake Huron water for 7 hours. Males were then removed, the water thoroughly mixed, and aliquoted into 1 L bottles.

Experimental Procedures – Chemical Quantification

Quantification of chemical components present in LE and SMW was conducted using liquid chromatography tandem mass spectrometry (LC-MS/MS; Fissette et al., 2020) with slight modification. For LE samples, 1 ml aliquots were centrifuged at 12,500 *g* for 20 minutes at 4º C (accuSpin Micro 17R, Fisher Scientific, Hampton, NH, USA). The supernatant was collected and filtered through 0.22 µm PVDF membrane filter (Millex ® - GV, Merck Millipore Ltd., Ireland). Prior to the LC-MS/MS analysis, the filtrate was diluted with an equal volume of methanol (1:1, v/v). For SMW samples, 10 ml aliquots were freeze dried. The residue was dissolved in 1 ml methanol and centrifuged at 12,500 *g* for 20 minutes at 4º C. The supernatant was freeze dried and reconstituted in 100 µl of 50% methanol in water for LC-MS/MS analysis. Samples were analyzed on a ACQUITY H-Class UPLC™ coupled to a Xevo TQ-S triple quadrupole mass spectrometer (both Waters Corporation, Milford, MA, USA). A BEH C18 column (2.1×100 mm, 1.7 µm particle size, 130 Å; Waters Corporation) was used with 10 mM trimethylamine (TEA) in water as mobile phase A, and methanol as mobile phase B. The injection volume was 10 µl, and the HPLC flow rate was 0.25 ml/min. Mass spectra were acquired using multiple reaction monitoring (MRM) mode with electrospray ionization in negative ion mode. MassLynx 4.2 software was used for data acquisition and data were processed using TargetLynx XS (Waters Corporation).

Statistical Analyses

All statistical analyses were conducted in R v3.5.1 (R Core Team, 2018). The proportions of females entering each sub-channel were analyzed using a mixed-effects logistic regression model with a binomial distribution. A separate model was run for each treatment, and all models evaluated the effect of odor on which sub-channel a female entered and tested for channel bias.

All analyses used the lme4 (Bates et al., 2015) and car (Fox and Weisberg, 2019) packages (α = 0.05). For all treatments, only females that swam upstream from the release point and entered a sub-channel were included in statistical analyses: (MeOH: water (1:1), 29/128 (23%); LE, 58/129 (45%); SMW 49/133 (37%); 4) 3kPZS: PZS (1: 10), 32/125 (26%); 3kPZS: PZS (100: 1), 43/122 (35%); 3kPZS: PZS (1: 10) vs. 3kPZS: PZS (100: 1), 31/140 (22%); LE vs. SMW, 51/111 (46%). For the negative control, a "treatment" side was randomly assigned for the first trial and alternated across subsequent trials.

RESULTS

Migratory females did not discriminate between 3kPZS:PZS mixtures regardless of the mixture ratio

When odors were tested individually, migratory females were attracted to 3kPZS: PZS mixtures at the ratio released by males (1:10) (χ^2 (1) = 6.77, P < 0.01, Figure 2.2A) and the ratio released by larvae (100:1), (χ^2 (1) = 21.9, P < 0.001, Figure 2.2A), and showed no preference when exposed to both ratios of 3kPZS:PZS mixtures simultaneously (χ^2 (1) = 0.09, P = 0.76, Figure 2.2A).

Migratory females were attracted to both the larval and male odor individually but discriminate between them when exposed simultaneously

Migratory females showed no preference for either sub-channel during negative control trials (χ^2 (1) = 0.63, P = 0.43, Figure 2.2A). Migratory females were attracted to both the larval cue (LE) (χ^2 (1) = 28.1, P < 0.001, Figure 2A) and the male sex pheromone (SMW) (χ^2 (1) = 17.0, P < 0.001, Figure 2.2B) but when individuals were exposed to both simultaneously, with a different odor in each sub-channel, migratory females preferred the sub-channel baited with LE over the sub-channel baited with SMW, (χ^2 (1) = 6.51, P < 0.02, Figure 2.2B).

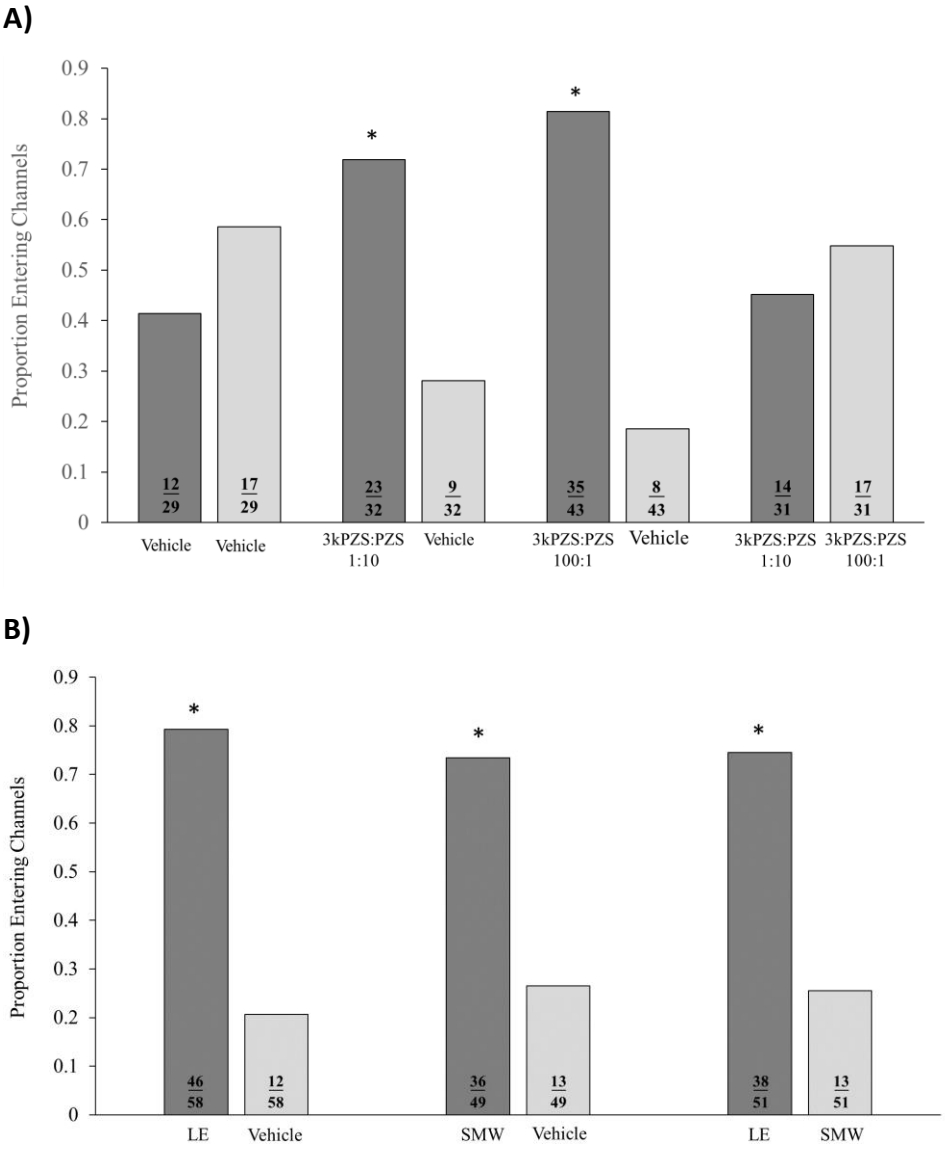

Figure 2.2. Behavioral responses of migratory sea lamprey to mixtures of natural and synthetic odorants. The proportion of migratory individuals entering each sub-channel during behavioral experiments. **A)** Natural odor mixtures tested were the migratory pheromone released by sea lamprey larvae (LE) and the mating pheromone released by males (SMW). **B)** The synthetic pheromone mixtures tested were a mixture of 3-keto petromyzonol sulfate (3kPZS) and petromoyzonol sulfate (PZS). These were tested at the larval released ratio (3kPZS:PZS, 1:10) and male released ratio (3kPZS:PZS 100:1) as previously reported (Buchinger and Scott et al., 2020). All treatments were standardized by 3kPZS application to reach in-stream 3kPZS concentrations of 1.00E-13M. Separate mixed-effects logistic regression models with a binomial distribution were used to analyze each treatment in order to assess how different natural or synthetic pheromone mixtures influence attraction via channel selection. Asterisks indicate $p < 0.05$, and the numbers within each bar represent the proportion of migratory females entering the specific sub-channel over the duration of the experiment.

DISCUSSION

A fundamental question regarding sexual signals that evolve via sensory traps is how communication systems subsequently evolve due to costs of being deceived in either the sexual or nonsexual contexts (Rodríguez, 2009). Do individuals evolve to resist deception by discriminating between the mimic and its model and use the signal only in the mating context, only in the original context, or in both contexts (Arnqvist, 2006; Rodríguez, 2009)? Little empirical data exists to explain these outcomes and studies investigating how communication systems are subsequently shaped in each context following the evolution of sexual signals via sensory traps appear to be limited to Goodeniae fish species (Garcia and Lemus, 2012; Garcia and Ramirez, 2005) and sea lamprey (Buchinger and Scott et al. 2020). This limitation precludes a full understanding of how sensory traps shape the evolution of communication systems. We tested the hypothesis that female sea lamprey decoupled larval odor and the male pheromone that mimics it in the nonsexual migratory context. In-stream behavioral assays revealed that, unlike spawning females, migratory females do not discriminate between larva- and male-typical ratios of the attractant 3kPZS and its antagonist PZS. These results suggest that females have not evolved to dissociate 3kPZS released by males versus larvae during migration as they have during spawning and illustrate how a sensory trap can lead to reliable communication without female resistance.

We postulate that deceptive signaling by male sea lamprey led to little if any costs to females during migration. Confusing larvae and male odor during migration is unlikely costly because the migratory response to 3kPZS is non-targeted and attracts females to a general area (Brant et al., 2016b; Brant et al., 2015). While the larval cue is needed to find suitable spawning areas (Fissette et al., 2021), this may also be aided by following male released 3kPZS. The larval and

male pheromones provide some overlapping information to migratory females and responding to male sex pheromone in the absence of a larval cue could still aggregate individuals into spawning tributaries and/or spawning areas to facilitate reproduction. Unlike the migratory context, expected costs of confusing larval and male released 3kPZS during mate search and spawning are high due to females having limited time (Applegate, 1950; Johnson et al., 2015) and energy reserves (William and Beamish, 1979) to locate mates and fertilize large egg stores before dying. 3kPZS likely reduces mate search costs by facilitating quick localization of males, and the evolution of PZS as an antagonist to discriminate among pheromone sources ensures attraction to only the male signal during reproduction. The differences in expected costs across non-reproductive and reproductive contexts in sea lamprey likely play a role in females not disassociating 3kPZS released by males or larvae during migration; a result counter to observations in splitfin fishes, where females faced direct costs when foraging and evolved reliable communication to yellow tail bands via resistance.

Our results provide further evidence that the male pheromone is only a partial mimic of larval odor. Several lines of evidence suggest the male sex pheromone is a mimic of the larval cue: 3kPZS is released by both larvae and males, exaggerated rates of 3kPZS release by male sea lamprey likely evolved via a receiver bias, and 3kPZS elicits non-sexual migratory responses in lamprey species that do not utilize 3kPZS as a sex pheromone (Buchinger et al., 2017; Buchinger et al., 2013). Migratory females were attracted to both the model and its mimic when tested alone but did discriminate between them when simultaneously exposed to both odors. While the mechanism allowing discrimination between the larval cue and male sex pheromone cannot be fully described here, our results indicate the pheromone component PZS does not act as an antagonist during migration as it does for sexually mature females (Buchinger and Scott et al.,

2020). This discrimination may be explained by the deceptive male sex pheromone not being a perfect mimic to begin with and given the role of pheromones within sea lamprey reproductive ecology, an imperfect mimic seems most plausible. Both the migratory cue and sex pheromone are blends comprised of multiple components, and although there is some overlap in chemical composition (Buchinger et al., 2015; Fissette et al., 2021), they each contain distinct pheromone components that guide different behavioral responses within each context (Fissette et al., 2021; Johnson et al., 2012; Li et al., 2018; Sorensen et al., 2005). Specific compounds released by larvae guide behaviors suited to migration such as staging offshore prior to upstream migration (Meckley et al., 2014), general orientation upstream (Brant et al., 2016b), and tributary selection within rivers (Li et al., 2018). In contrast, components of the male sex pheromone have evolved to specifically facilitate mate search, resulting in targeted upstream movement, attraction to the odor source, and subsequent retention at the source that all facilitate reproductive behaviors (Brant et al., 2016a; Johnson et al., 2012; Johnson et al., 2009; Siefkes et al., 2005). The physiological mechanism for discrimination may occur at the level of odorant receptors and subsequent signal transduction pathways as 3kPZS and PZS are discriminated as separate odors by the olfactory epithelium (Siefkes and Li, 2004), and behavioral results add further support because 3kPZS is needed in a pheromone mixture containing PZS to induce behavioral responses during migration (Brant et al., 2016b).

The evolution of receiver responses in the presence of deceptive signals remains a major question regarding signal evolution via sensory traps. Our results highlight how a previously undocumented evolutionary trajectory of a sensory trap leads to reliable communication across contexts. In sea lamprey, female resistance or dissociation of the mimic and its model does not occur as seen in species of splitfin fishes. Rather, females showed no shift in responses to 3kPZS

released by larvae or males during the non-sexual context of migration, and by using different mechanisms across contexts, migratory and spawning lamprey respond to larval and male released 3kPZS with context appropriate behavioral responses to ensure reliable communication in each. The lack of dissociation displayed by migrating females has implications for invasive species control, where pheromones and pheromone antagonists are being investigated as control tools for sea lamprey in the Laurentian Great Lakes. The inability of PZS to disrupt migratory behavior suggests its effectiveness in manipulating sea lamprey behavior is likely limited to spawning populations.

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CHAPTER 3:

EVIDENCE THAT FEMALE SEA LAMPREY USE MILT PHEROMONES TO DISCRIMINATE AMONG POTENTIAL MATES

ABSTRACT

Female ability to select mates who are able to fertilize eggs directly impacts female fitness, but male ornaments, signals, or signaling strategies that attract females may not directly correlate to male fertility. In externally fertilizing species, chemical signals may offer females the opportunity of assessing male fertilization ability or capacity because ejaculates are released directly into the environment and are available to be detected. Pheromones associated with sperm release have only been described in a few species, and the evidence for female discrimination among males using these signals remains limited. Sea lamprey (*Petromyzon marinus*) rely on pheromone communication for reproduction and utilize multiple pheromones from different sources to attract females; a potent bile acid sex pheromone released across gill epithelia and a polyamine in milt released during spawning. Unlike males in other fish species who control pheromone release via urinary pulses, male sea lamprey have limited capacity to modulate bile acid pheromone release, and female attraction to this potent pheromone occurs regardless of male status (actively spawning on a nest vs. freely swimming or hiding). This presents a challenge to females where effort may be wasted on locating mates who are not immediately prepared to spawn. We tested the hypothesis that milt pheromones aid females in discriminating among male mates based on spawning status. In-stream behavioral assays revealed that females discriminate between males not only using the presence of milt pheromones but also the concentration, and that pheromone components in milt function independently to attract females to and retain them on a male's nest. Our results show that milt release signals sperm availability to females and that males and females likely benefit from the behaviors induced by a milt pheromone.

INTRODUCTION

The information signals and cues provide about mate quality and how that influences mate choice and fitness maximization is a core tenet of sexual selection theory (Andersson, 1994). Female assessment of potential male mates is arguably the most critical step within the mate choice process, and therefore, choosing mates who are ready and able to fertilize available eggs benefits females and could be a target of selection (Sheldon, 1994; Wedell et al., 2002). Female fitness may be hindered via sperm limitation (Wedell et al., 2002) caused by differing strategies of male sperm allocation (MacDiarmid and Butler IV, 1999; Pizzari et al., 2003; Rubolini et al., 2006), variable mating opportunities (Pitnick, 1993; Puurtinen and Fromhage, 2017), or sperm depletion due to multiple matings (Svensson et al., 1998; Warner et al., 1995). These may drive the evolution of female preferences for males with better fertilization capacity (Harris and Moore, 2005; Markow et al., 1978; Ruther et al., 2009; Sato and Goshima, 2007). Much research has focused on elaborate male advertising strategies or ornaments that signal male quality (Andersson, 1982; Andersson and Simmons, 2006; Johansson and Jones, 2007), which may (Evans et al., 2003; Helfenstein et al., 2010; Matthews et al., 1997; Rowe et al., 2011) or may not (Birkhead et al., 1997; Parker et al., 2006; Pilastro et al., 2008) be correlated or serve as proxies for sperm quality or fertilization ability.

Olfaction may allow females to directly assess sperm availability, sperm quality, or male fertility, especially in externally fertilizing animals where gametes and associated fluids are released into the environment. Pheromones present in milt, seminal plasma, or associated with sperm availability have been found in a few species and may influence female ovulation (Van den Hurk and Resink, 1992), induce spawning behaviors (Carolsfeld et al., 1997), increase oviposition and egg release (Smith et al., 2018), or attract females (Lambert and Resink, 1991;

Scott et al., 2019), even at distance in a species with dissociated sperm transfer (Zizzari et al., 2017). However, these studies are relatively few and have not directly tested whether females discriminate among males based on cues associated with sperm. Sea lamprey, *Petromyzon marinus* rely on chemical signals from multiple sources to communicate with conspecifics and offer a useful model to study how multiple signals may convey differing information that allows discrimination among male mates based on sperm availability.

Female sea lamprey face a challenge when locating male mates as they must discriminate between males who have resources available to not only fertilize eggs (sperm availability) but also ensure embryo survival (e.g., nest construction, Manion and Hansen 1989). In contrast to other fish species which release sex pheromones via the urine in pulses (Appelt and Sorensen, 1999; Barata et al., 2007; Rosenthal et al., 2011), all male sea lamprey, regardless of their status (e.g., on a nest/on spawning grounds vs. hiding in refugia) begin continually producing and releasing a potent, multicomponent bile acid sex pheromone across gill epithelia (Siefkes et al., 2003) at the onset of spermiation. The function of the bile acid pheromone is well described; it facilitates mate search and reproduction by attracting females to a male's location and induces spawning behaviors (Buchinger et al., 2015; Fissette et al., 2021a; Johnson et al., 2012). A major component of the gill released sex pheromone, 3-keto petromyzonol sulfate (3kPZS; Li et al., 2002), attracts females over long distances and at a range of in-stream concentrations (Johnson et al., 2009). Female attraction to this pheromone occurs regardless of male location, but given the constraints of a narrow reproductive window (Applegate, 1950; Dhamelincourt et al., 2021) and limited energy stores due to a lack of feeding (William and Beamish, 1979), locating males who are on a nest, actively spawning, and have sperm available or gravel patches with spawning activity is likely advantageous. Our current understanding of the bile acid pheromone suggests it

is ill-suited in allowing females to discriminate among males based on spawning activity or sperm availability. However, males also release pheromones via their milt when spawning, including an odorous polyamine released via the seminal plasma (Scott et al., 2019), and these milt pheromones are hypothesized to signal male sperm availability to females (Scott et al., 2019).

We hypothesized that pheromones present in sea lamprey milt allow females to discriminate between spawning and non-spawning males or locate areas of spawning activity (or both). To test this hypothesis and evaluate female discrimination among males, we recorded behavioral responses of sexually mature females to 1) the odor of a spawning male (bile acid pheromone + seminal plasma) against the odor of a sexually mature but non-spawning male (bile acid pheromone alone), 2) the odor of spawning males where one male is releasing 2x the amount of milt compared to the other male, and 3) seminal plasma applied individually in the absence of any gill released pheromones at two separate distances. We provide evidence that pheromones present in sea lamprey milt enable females to discriminate among males based on the presence and concentration of milt pheromones. These results indicate milt pheromones are a signal directly associated with the release of gametes, signal sperm availability to females, and that males and females likely benefit from the spawning behaviors they induce.

METHODS

Animal Collection

Sea lamprey were collected via traps from tributaries of Lakes Michigan and Huron by the US Fish and Wildlife Service and Fisheries and Oceans Canada and then transported to the US Geological Survey Hammond Bay Biological Station (HBBS), Millersburg, Michigan (MI). At HBBS, sea lamprey were held in 200-1000 liter tanks that were constantly supplied with aerated, ambient temp Lake Huron water. To collect sexually mature males and females for experiments, sea lamprey were held in cages (0.25-1m^3, ~25 individuals per cage) in the Ocqueoc River, Millersburg MI. To confirm sexual maturity, lamprey were checked daily for gamete expression with gentle abdominal pressure (Siefkes et al., 2003). Sexually mature individuals were returned to HBBS and held in 200 L tanks until experimental use. All experimental procedures followed approved protocols and methods by the Michigan State University Institutional Animal Care and Use Committee (AUF numbers 02/18-025-00 and PROTO202100029).

Seminal Plasma Collection

Bulk collections of milt and subsequently seminal plasma occurred during June and July 2019 and followed methods outlined in Scott et al., (2019). To collect milt for application during behavioral experiments, gentle abdominal pressure was applied to sexually mature males to express milt into 50 mL centrifuge tubes. During collection, milt was held on ice to prevent degradation. Following collection, milt was centrifuged (10 minutes, 1,020 g at 4℃) to separate seminal plasma from sperm. The seminal plasma was then collected and held at -80℃. The volume of milt each male produced and how many individual males were sampled was not directly recorded for these bulk collections, but estimations can be made based on related sampling events. On July 8^{th,} 2019, 231 mL of milt was collected from 137 males, resulting in an

average of 1.69 mL milt per male. During bulk collections in 2019, ~1000 mL of milt was collected which equates to ~592 males sampled. The percentage of milt comprised of seminal plasma was also estimated via a separate sampling experiment. Milt was collected from 10 males, and the volume collected from each individual was recorded prior to centrifuging. The volume of seminal plasma was then recorded after centrifuging and resulted in an average composition of 96.1%. For use in experimental trials, seminal plasma collected during bulk collections in 2019 was thawed, pooled together, thoroughly mixed, and then redistributed into 8.65 mL aliquots in 15 mL centrifuge tubes and stored at -80℃ until use. This batch of seminal plasma was used for all behavioral experiments.

3kPZS Quantification in Seminal Plasma

Liquid chromatography tandem mass spectrometry was used to determine whether behavioral effects of seminal plasma on females was driven by the gill-released pheromone 3kPZS (which is present in blood/plasma; Brant et al., 2013) or additional components. A 1 mL aliquot of seminal plasma was centrifuged at 12,500 *g* for 20 min at 4 ºC. The supernatant was collected and filtered through 0.22 µm PVDF membrane filter (Millex ® - GV, Merck Millipore Ltd., Ireland). The filtrate was then diluted with an equal volume of methanol (1:1, v/v) and used for LC-MS/MS analysis. 3kPZS concentrations were quantified based on previously established methods (Fissette et al., 2020). Briefly, the samples were analyzed on a ACQUITY H-Class UPLC™ coupled to a Xevo TQ-S triple quadrupole mass spectrometer (both Waters Corporation, Milford, MA, USA). A BEH C18 column (2.1×100 mm, 1.7 µm particle size, 130 Å; Waters Corporation) was used with 10 mM trimethylamine (TEA) in water as mobile phase A, and methanol as mobile phase B. The injection volume was 10 µl, and the HPLC flow rate was 0.25 ml/min. Mass spectra were acquired using multiple reaction monitoring (MRM) mode with electrospray

ionization in negative ion mode. MassLynx 4.2 software was used for data acquisition and data were processed using TargetLynx XS (Waters Corporation).

Milt Application During Experimental Trials

Milt was applied during behavioral trials to simulate a male consistently spawning over the course of one hour. To calculate this volume, we combined behavioral observations that counted the number of spawning events during a given time with estimated milt release during a single spawning event. To calculate the number of spawning events, spawning nests (n=25) were observed (June 5 – July 3, 2018) in local tributaries for a 30 min period, and the number of spawning events was recorded. Spawning events are easily identifiable as males attach to the female's head with their oral disc, coil around the female while moving their tail to the ventral side of the female's tail to align urogenital papillae, and then rapidly undulate simultaneously to release gametes while stirring up sediment within the nest (Johnson et al., 2015). The average number of spawning events during the observational period was 7.2 (Supplemental Table 1) and extrapolated to 1 hr of spawning (duration of behavioral trials) the average is 14.4. We have no precise methodology to measure the amount of milt released per spawning event. Given that during milt collection males, on average, had 1.69 mL of milt available we estimated milt release to be 25 µl. Spawning events last ~5 s (Johnson et al., 2015) so 1 hr of constant spawning equates to 720 total spawning events, resulting in a total of 18 mL of milt released (720 x 25 µL). Because milt is 96.1% seminal plasma, 17.3 mL of seminal plasma would be released over the course of an hour during constant spawning. This volume of seminal plasma was then applied during behavioral trials to simulate consistent male milt release over the course of an hour.

Spermiated Male Washing Collection and Application

Two separate batches of spermiated male washings (SMW) were collected for use in behavioral experiments. SMW contains the complete bile acid pheromone mixture released by males that induces robust mate search and spawning behavior in females (Johnson et al., 2012; Siefkes et al., 2005). The first batch was collected on June 24, 2020 and used for all behavioral experiments during 2020. Individual spermiated males (n=13) were held separately in 5 L of ambient temperature Lake Huron water that was constantly aerated (temp. start = 14.1 ℃, temp. end = 16.5 ℃) for 5 hrs. After 5 hrs, males were removed from the holding water, all holding water was pooled, mixed thoroughly, aliquoted into 1 L bottles, and then stored at -20 ℃ until use in behavioral experiments. 3kPZS concentrations in pooled samples were analyzed using LC-MS/MS methods previously described.

The second batch of SMW was collected on June 5, 2021 using slightly different methods and was used for all behavioral experiments during 2021 and 2022. Spermiating males (n=14) were held together in 50 L of ambient temperature Lake Huron water (temp. start = 12.0 ℃, temp. end = 19.0 ℃) for 7 hrs. After 7 hrs., males were removed, the water thoroughly mixed, aliquoted into 1L bottles, and then stored at -20 ℃ until use in behavioral experiments. 3kPZS concentrations in pooled samples were analyzed using LC-MS/MS methods previously described.

To be consistent with seminal plasma application during behavioral trials, SMW was applied to simulate male release of bile acid pheromones over the course of one hour rather than being applied to reach a specified in-stream concentration of 3kPZS based on stream discharge. When using the 2020 batch (*Experiments 2 and 3),* 1 L was applied over the course of an hour (65 L / 13 males / 5 hrs = 1 L per male per hr) which equated to 0.63 mg hr^{-1} 3kPZS. When using the

2021 batch, 510 mL was applied over the course of an hour (70 L /14 males / 7 hrs = 510 mL per male per hour) which equated to 0.85 mg hr^{-1} 3kPZS.

General Experimental Procedures – Behavioral Assays

In-stream behavioral assays were conducted to evaluate behavioral responses of ovulated females to pheromone treatments. All behavioral experiments were conducted in the upper Ocqueoc River, a historic sea lamprey spawning site (Applegate 1950). Currently, an electric barrier downstream restricts sea lamprey migration to this stream reach and ensures no background pheromones from naturally spawning populations are present during behavioral experiments.

Behavioral responses of ovulated females were quantified using a passive integrated transponder (PIT) system (Oregon RFID, Portland, OR, USA). The PIT array varied by experiment with the number of PIT antennas ranging from two to four. Each behavioral experiment included two 1 m^2 adjacent PIT antennas set ~1m apart that simulate spawning nests where pheromone treatments were applied. Two additional cross stream antennas located 20 m and 25 m downstream of the odor application point were used to monitor lamprey movement upstream in some experiments. Each experimental subject was fitted with a 23 mm half-duplex PIT tag (Oregon RFID) sheathed in plastic tubing and attached dorsally via a suture. Unique tag numbers ensure individual lamprey are analyzed only for their respective trial.

Groups of experimental subjects (n=10-20 per trial based on lamprey availability) were held in 0.5 m^2 release cages either 50 m or 100 m downstream of the odor application point, and they were acclimated to river conditions for ~2 hours prior to experimental start time. Due to the general nocturnal behavior of sea lamprey (Applegate 1950), behavioral experiments started 30 min after sunset. Each experimental trial was conducted over the course of an hour with a 15-

minute pumping period prior to lamprey release and a 45-minute behavioral observation period after release. Behavioral parameters measured via PIT array varied by experiment but may include pheromone localization (the proportion of females that located the treatment source), first odor choice (the first PIT nest a female entered), and retention time (duration of time each female spent within each PIT nest where odors were applied). To calculate retention times, the time between PIT detection was used, and if a female was not detected for 15 s they were considered to have left the odor source. Treatment odors were applied using a peristaltic pump (Masterflex L/S, item EW-07554-90, Cole Parmer, Vernon Hills, IL, USA). Odorants were mixed with river water to make a total volume of 10 L and applied at 167 ml min^{-1} in each of two adjacent 1 m^2 PIT antennas.

Experiment 1: Does milt act as an independent pheromone?

To determine if pheromones in milt act independently from gill released pheromones and assess their potential at influencing female behavior at differing spatial scales, in-stream assays compared behavioral responses of females across four treatments and two distances (50 and 100 m) using the following metrics: pheromone localization and retention time at the odor source. The four treatments included a negative control (vehicle, MeOH:Water, 1:1), 3kPZS control (Bridge Organics Inc., Vicksburg, MI, USA) (0.0047 mg/hr, concentration quantified in 17.3 mL of seminal plasma), seminal plasma (17.3 mL), and SMW (510 mL, 0.85 mg/hr 3kPZS). These treatments were tested with females released 50 m (June 28 – July 19, 2021) and 100 m (June 9 – June 26, 2022) downstream of the treatment application point. The 3kPZS control treatment reached in-stream concentrations ranging from 2.33×10^{-15} – 9.95×10^{-15} M (50 m) and 1.34×10^{-15} – 3.19×10^{-15} M (100 m). SMW application equated to 0.85 mg hr^{-1} 3kPZS and in-stream concentration of 3kPZS ranging from 4.53×10^{-13} – 1.81×10^{-12} M (50 m) and 3.94 x

10^{-13} – 5.01 x 10^{-13} M (100 m). A PIT array incorporating four PIT antennas as described in *General Experimental Procedures – Behavioral Assays* was utilized to monitor behavioral responses of females. Treatments were applied at the center of 1 m^2 PIT nest antenna, with a treatment applied in one nest and a vehicle control (MeOH:Water, 1:1) applied in the other. The PIT nest in which treatments were applied was alternated throughout the duration of the experiment to demonstrate females' ability to locate the treatment source at separate locations and to avoid the possibility of detections due to behavioral swimming preferences for certain stream and flow attributes. The total number of females released varied by treatment: negative control (50 m, n=89; 100 m, n=98), 3kPZS (50 m, n=79; 100 m, n=85), seminal plasma (50 m, n=104; 100 m, n=111), and SMW (50 m, n=75, 100 m, n=67). Any females that died during the acclimation period were discarded from all behavioral metrics and statistical analyses: 3kPZS (50 m, n=3; 100 m, n=1), seminal plasma (50 m, n=7; 100 m, n=2), and SMW (50 m, n=3). These mortality rates are expected given females in reproductive condition who die quickly after becoming sexually mature and spawning (Applegate 1950).

Experiment 2: Do females discriminate between males based on sperm release?

To determine if milt pheromones allow females to discriminate spawning from non-spawning males, in-stream assays compared female first odor choice and odor source retention between SMW + seminal plasma and SMW applied simultaneously in adjacent PIT nests. Only 2 PIT nest antennas were used during this experiment; cross stream PIT antennas were not utilized. In each PIT nest, 1 L (0.63 mg hr^{-1}) of SMW was applied, and in one PIT nest 17.3 mL of seminal plasma was added to the SMW. The PIT nest containing seminal plasma was alternated throughout the duration of the experiment. Behavioral trials (n=6) were conducted from June 28 – July 2, 2020, and only females that swam upstream and entered a treatment source were used

for statistical analyses (n=44). Females that died during acclimation (n=8), did not leave the release cage (n=6), or did not enter a treatment nest (n=22) were not used for statistical analyses. SMW application for each PIT nest equated to 0.63 mg hr^{-1} 3kPZS and in-stream concentrations of 3kPZS ranging from 2.59 x 10^{-13} – 2.67 x 10^{-12} M.

Experiment 3: Do females use milt pheromone concentration to discriminate among males?

To determine if females discriminate between males based on milt pheromone concentrations, in-stream assays compared female first odor source and odor source retention to two different mixtures of SMW + seminal plasma. Only 2 PIT nest antennas were used during this experiment and cross stream PIT antennas were not utilized. In each adjacent PIT nest, 1 L (0.63 mg hr^{-1}) of SMW was applied and mixed with one of two concentrations of seminal plasma, 17.3 mL or 8.65 mL. This concentration difference was chosen based on female responses to the bile acid pheromones, where a 100% increase in pheromone concentration has distinct effects on female behavior (Johnson et al., 2009, Fissette et al., 2020). Which PIT nest received each concentration was alternated throughout the experiment. Behavioral trials (n=12) were conducted from July 3-13, 2020 and only females that swam upstream and entered a treatment source were used for statistical analyses (n=61). Females that died during acclimation (n=39), did not leave the release cage (n=31), or did not enter a treatment nest (n=120) were not used for statistical analyses. SMW application for each PIT nest equated to 0.63 mg hr^{-1} 3kPZS and in-stream concentrations of 3kPZS ranging from 4.75 x 10^{-13} – 6.53 x 10^{-13} M.

Statistical Analyses

All statistical analyses were performed using R v4.2.2 (https://www.r-project.org/). For behavioral experiments, generalized linear mixed effects models with a binomial distribution were used to evaluate females' first odor choice (*Experiments 2 and 3)* and pheromone

localization at distances of 50 and 100 m (*Experiment 1).* For each instance evaluating first odor choice, two separate models were built, one containing only odor treatment as a covariate and one including both odor treatment and nest bias. For each model, random effects of stream discharge and trial ID were included. Likelihood ratio tests determined the best fitting model between the two options. For evaluating pheromone localization, models included odor treatment as a covariate and random effects of stream discharge and trial ID. For Experiments 2 and 3, a mixed effects linear model was used to evaluate the effect of treatment on odor source retention. These models included odor treatment as a covariate and included a random effect of Fish ID for a paired analysis of retention times across the simultaneously applied pheromone treatments. All analyses incorporating mixed models used the glmer and lmer functions in R package lme4 (Bates et al., 2015) and Anova function with type III sums of squares (α=0.05) (car package, Fox and Weisberg, 2019). Odor source retention times for Experiment 1 were analyzed using non-parametric Kruskal Wallis tests (α=0.05) and Dunn's test (α=0.05) with Bonferroni correction for post hoc analyses due to normality assumptions being violated (50 m distance) and low sample sizes (100 m distance) for the vehicle control (n=1) and seminal plasma 3kPZS (n=5) treatments. Additionally, the vehicle control treatment at 100m was removed from post-hoc analyses due to only having one observation.

RESULTS

Milt pheromones attract and retain females on a nest from distances of 50 and 100 m

During behavioral experiments, pheromone treatments differentially affected females' ability to localize the odor source at 50 m (χ^2 (3) = 64.3, P < 0.001, Figure 3.1A) and 100 m (χ^2 (3) = 34.2, P < 0.001, Figure 3.1B). Treatment effects were consistent across both distances with SMW increasing localization over all other treatments ($p < 0.05$, Tukey's HSD, Figure 3.1) and seminal plasma increasing localization over the 3kPZS and the vehicle controls ($p < 0.05$, Tukey's HSD, Figure 3.1). The 3kPZS control did increase localization over the vehicle control at 50 m but this result was not significant ($p = 0.053$, Tukey's HSD, Figure 3.1A), and no difference was observed at 100 m ($p = 0.28$, Figure 3.1B).

Pheromone treatments also influenced female retention time at the odor source across distances of 50 m (χ^2 (3) = 12.6, P < 0.001, Figure 3.2A) and 100 m (χ^2 (3) = 12.6, P = 0.006, Figure 3.2B). At each distance, females spent more time at seminal plasma and SMW when compared to the vehicle control and seminal plasma (50 m, ~700%; 100 m, ~2400%) ($p < 0.05$, Dunn's tests with bonferroni correction, Figure 3.2), but were not different from each other ($p > 0.05$, Dunn's test with bonferroni correction, Figure 3.2). The vehicle control and seminal plasma were not different from each other at 50 m ($p > 0.05$, Dunn's test with Bonferroni correction, Figure 3.2A) and were not compared at 100 m due to only having one observation for the vehicle control (Figure 3.2B).

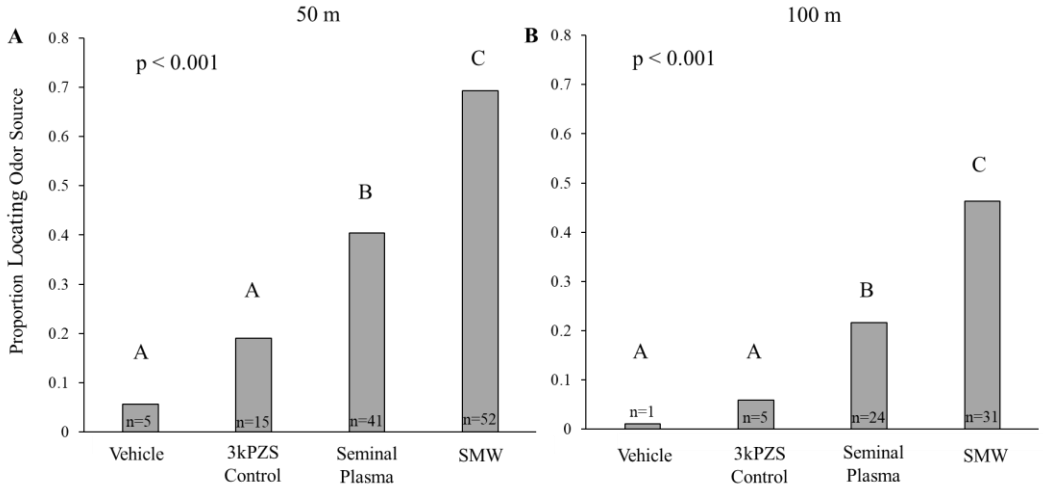

Figure 3.1. Sexually mature females localize milt pheromones applied within a 1 m^2 passive integrated transponder (PIT) nest. Proportion of all females released that moved upstream from release cages and entered the 1m^2 PIT nest baited with a pheromone treatment at 50 m (**A**) or 100 m (**B**). Treatment odors included a vehicle control (MeOH:Water, 1:1, absence of pheromone), a 3kPZS control treatment applied at the concentration quantified in seminal plasma used during behavioral experiments, seminal plasma, and spermiated male washings (SMW, contains entire gill released pheromone). Seminal plasma and SMW were applied at the rate of 1 male hr^{-1}. The n within each bar indicates the total number of females of those released that entered the 1 m^2 PIT baited with a pheromone treatment. Female attraction to pheromone treatments differed across treatments at 50 m ($P < 0.001$, mixed-effects logistic regression with binomial distribution) and 100 m ($P < 0.001$, mixed-effects logistic regression with binomial distribution). Letters above each bar indicate differences in pheromone localization across treatments (Tukey's HSD post hoc tests, $\alpha<0.05$) which were consistent at both distances tested. No differences were observed between vehicle control and the 3kPZS treatment, whereas seminal plasma increased pheromone localization when compared to vehicle control and the 3kPZS treatment. A greater proportion of females were attracted to SMW than any other treatment.

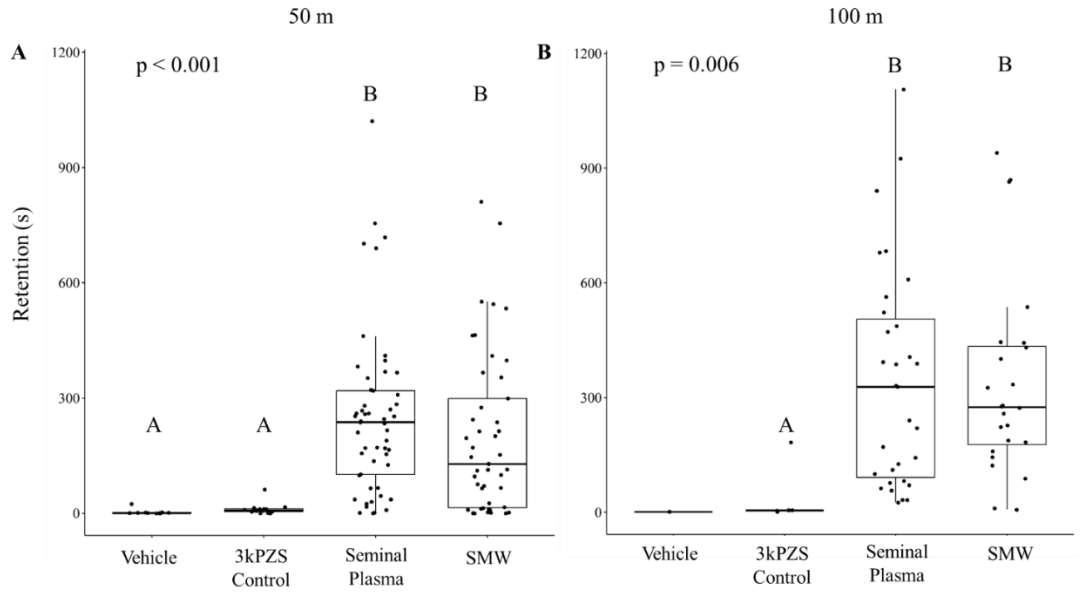

Figure 3.2. Milt pheromones retain females at the PIT nest for times consistent with the entire male multicomponent bile acid pheromone. Boxplots show female retention time (s) within a 1 m^2 passive integrated transponder (PIT) nests where pheromone treatments were applied at distances of 50 or 100 m. Each point represents an individual observation, and the boxplots display the median, interquartile range, the maximum (Q3 + 1.5*IQR), the minimum (Q1 – 1.5*IQR), and any outliers. Treatment odors included a vehicle control (MeOH:Water, 1:1, absence of pheromone), a 3kPZS control treatment applied at the concentration quantified in seminal plasma used during behavioral experiments, seminal plasma, and spermiated male washings (SMW, contains entire gill released pheromone). Treatments differentially affected retention times at both 50 m ($P < 0.001$, Kruskal Wallis Test) and 100 m ($P < 0.001$, Kruskal Wallis Test). Letters above each bar indicate differences in retention across treatments (Dunn's tests, $\alpha < 0.05$), and at 100 m, the vehicle control was removed from post-hoc tests due to only having one observation. Females spent similar amounts of time at seminal plasma and SMW treatments, and they spent more time at these treatments when compared to the vehicle and 3kPZS control treatments where retention times were no different from each other.

Females discriminate between spawning and non-spawning males

A likelihood ratio test indicated the best fitting model included odor treatment and side bias as covariates with random effects of stream discharge and trial number (χ^2 (1) = 12.5, $p < 0.001$). Behavioral experiments confirmed that females prefer male odor that contains seminal plasma. The addition of seminal plasma to SMW increased female first odor choice (χ^2 (1) = 9.65, p = 0.002, Figure 3.3A), and a strong side bias was also apparent (χ^2 (1) = 7.96, p = 0.004) and accounted for within our model. Females tended to spend more time on nests baited with SMW and seminal plasma, but this trend was not significant (χ^2 (1) = 2.88, p = 0.09, Figure 3.3B)

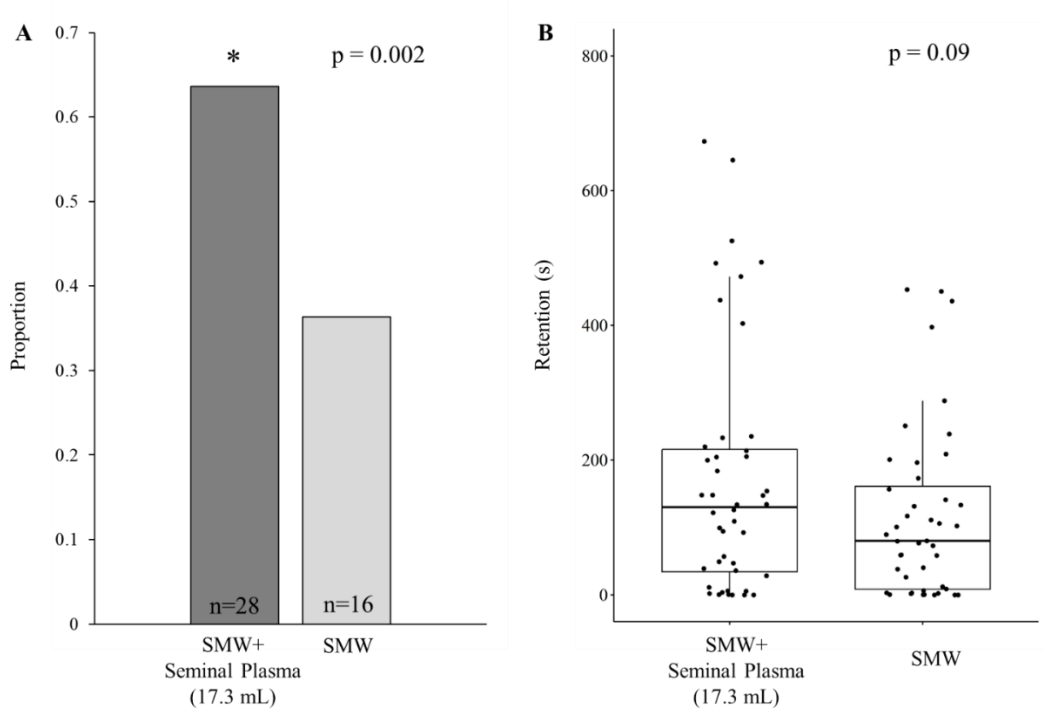

Figure 3.3. Sexually mature female sea lamprey were more likely to enter and stayed longer at passive integrated transponder (PIT) nests that contained the odor of a male releasing milt. A) Proportion of females choosing to enter a pheromone treatment first during behavioral experiments. Treatment odors simulated a non-spawning male by applying pooled water from sexually mature males (SMW, contains entire gill released pheromone) and a spawning male releasing milt (SMW + seminal plasma). The n within each bar indicates the total number of females choosing that odor first over all behavioral trials. Females preferred the odor of spawning males (P=0.002, mixed-effects logistic regression with binomial distribution). **B)** Boxplots of female retention time (s) within 1 m^2 PIT nests baited with the odor of a non-spawning or spawning male. Each point represents an individual observation, and the boxplots display the median, interquartile range, the maximum (Q3 + 1.5*IQR), the minimum (Q1 – 1.5*IQR), and any outliers. Females tended to stay longer at the odor of a spawning male, but this result was not significant (P=0.09, mixed effects linear model).

Females discriminate milt pheromone concentrations

A likelihood ratio test indicated the best fitting model included odor treatment and nest bias as covariates with random effects of stream discharge and trial number (χ^2 (1) = 5.35, p = 0.02). Behavioral experiments confirmed that females prefer higher concentrations of milt pheromones. A 100% increase in seminal plasma increased female first odor choice (χ^2 (1) = 3.96, p = 0.047, Figure 3.4A) and the time spent at the odor source (χ^2 (1) = 11.90, p < 0.001, Figure 3.4B). A nest bias was also apparent (χ^2 (1) = 5.07, p = 0.02).

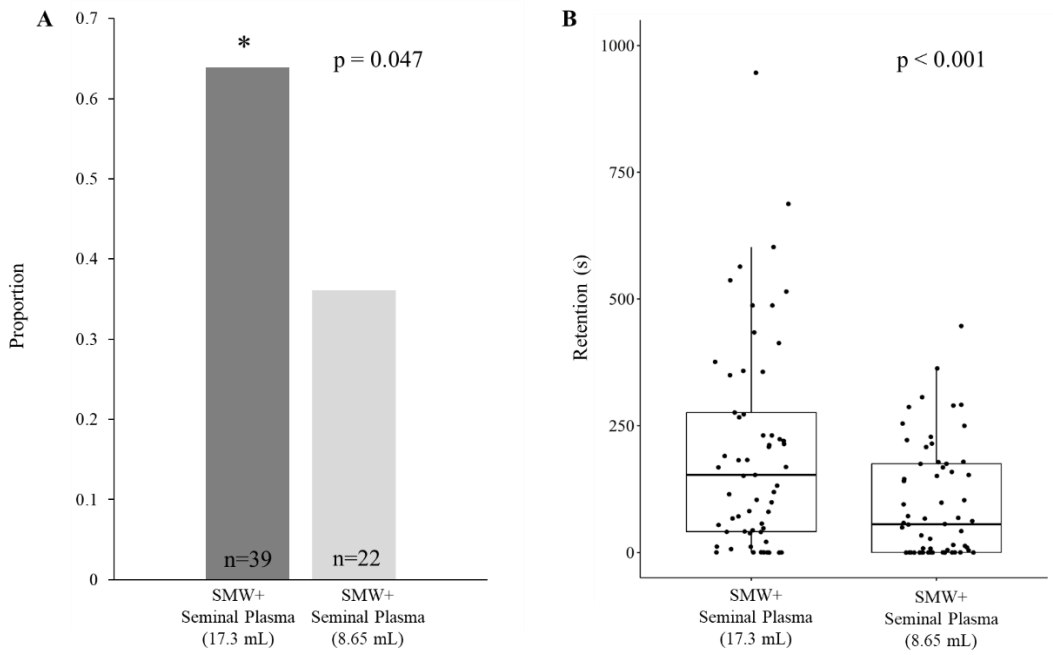

Figure 3.4. Sexually mature female sea lamprey were more likely to enter and stayed longer at passive integrated transponder (PIT) nests that contained the odor of a male releasing more milt. A) Proportion of females choosing to enter a pheromone treatment first during behavioral experiments. Treatment odors simulated two spawning males, one releasing 2x milt (SMW+seminal plasma – 17.3 mL) than the other male (SMW+seminal plasma – 8.65 mL). The n within each bar indicates the total number of females choosing that odor first over all behavioral trials. Females preferred the odor of a male releasing more milt (P=0.047, mixed-effects logistic regression with binomial distribution). **B)** Boxplots of female retention time (s) within 1 m^2 PIT nests baited with the odor of a male releasing 2x the milt of another male. Each point represents an individual observation, and the boxplots display the median, interquartile range, the maximum (Q3 + 1.5*IQR), the minimum (Q1 – 1.5*IQR), and any outliers. Females stayed longer at nests baited with males releasing more milt (P<0.001, mixed effects linear model).

DISCUSSION

In support of our hypothesis that pheromones present in sea lamprey milt allow females to discriminate actively spawning males, areas of spawning activity, or both, we found that 1) females preferred the odor of spawning males to non-spawning males, 2) females discriminated among males based on milt pheromone concentrations, and 3) milt pheromones independently influence female sea lamprey reproductive behaviors such as attraction, localization, and retention at distances of 50 and 100 m. Olfactory cues or pheromones present in the seminal vesicles or male seminal materials can influence female physiology (Van den Hurk and Resink, 1992) or reproductive behaviors (Carolsfeld et al., 1997; Lambert and Resink, 1991; Scott et al., 2019; Smith et al., 2018; Zizzari et al., 2017), but evidence for whether the information provided by these influence female mate preferences is limited. Our results provide evidence that female sea lamprey evaluate males based on the presence milt pheromones and gain additional information about mate status or quality during mate choice via multiple pheromone signals. Males may gain additional benefits by attracting females via gamete release. The use of separate pheromone signals for differentiating male mates may be especially important in sea lamprey because males continuously release the bile acid pheromone once sexually mature (Li et al., 2002; Siefkes et al., 2003) and do not have the same level of control over pheromone release as species with urine based pheromones (Appelt and Sorensen, 1999; Barata et al., 2007; Rosenthal et al., 2011). As a result, females may waste their limited time and energy reserves (William and Beamish, 1979) during mate search locating males that are unequipped to fertilize eggs or to ensure embryo survival (e.g., hiding males or males without a nest, Manion and Hansen 1980).

Sea lamprey spawn via external fertilization (Johnson et al., 2015) and are reliant on chemical cues or signals for reproductive migration (Vrieze et al., 2011) and mate search

(Johnson et al., 2006), therefore the evolution of chemical information signaled via the release of sperm is most likely to influence female reproductive behaviors surrounding mate search and reproduction. Females not only discriminated between actively spawning vs. non-spawning males, but also among males based on the volume of milt released. These suggest milt pheromones serve a primary function in female evaluation and discrimination of males during mate search. Females of the collembolan species, *Orchesella cincta*, discriminate male spermatophores based on density (Zizzari et al., 2009) or male-male competition (Zizzari et al., 2013) and chemical cues associated with sperm release appear to be used in discriminating between males with dissimilar MHC genotypes in rose bitterling, *Rhodeus occellatus* (Smith et al., 2018). We showed pheromones released via seminal fluid are used by females to discriminate male spawning status, and that females also discriminated among males based on milt pheromone concentrations. Discrimination between varying milt concentrations is consistent with behavior observed to gill released pheromones where females orient to 3kPZS or SMW that is 2x the concentration than an adjacent pheromone source (Fissette et al., 2020; Johnson et al., 2009). Discriminating differing milt volumes suggests male variation in milt pheromone concentrations, differences in spawning activity (increased spawning events = higher volumes of milt release), or the volume of milt a male releases can influence female behaviors. It is probable that variation among milt signals exists and may be acted on by sexual selection because; 1) male variation in production and release of bile acid pheromone components (Buchinger et al., 2017; Buchinger et al., 2019) directly influence female preferences (Fissette et al., 2020; Johnson et al., 2009) so it is plausible the same variation could occur in milt pheromones and influence behavioral responses and 2) males exhibit variation in sperm concentrations and quality (Ciereszko et al., 2002), and genetic compatibility influences hatching success (Rodríguez-

Muñoz and Tregenza, 2009), so its plausible milt pheromone composition or concentrations related to specific components may be correlated with these metrics and signal fertilization ability, a cue used by females in other species (Harris and Moore, 2005; Ruther et al., 2009; Wedell et al., 2002). Because the same batch of seminal plasma was across all behavioral experiments, our results can only provide a mechanistic explanation for overall pheromone concentration differences, but cannot provide insights as to differences in pheromone composition or the concentration of individual pheromone components. The additional information females gain from the presence of milt pheromones is not just limited to the presence of milt pheromones, but also the volume and/or concentration.

Milt pheromones also appear to serve additional functions in attracting females to a male's nesting location and retaining females on a nest for extended periods of time to ensure fertilization of large egg stores. While female behavioral responses of localization and retention to the milt pheromone overlap with those of the gill released pheromone (Johnson et al., 2012; Johnson et al., 2009; Li et al., 2002; Siefkes et al., 2005), the preference for the odor of spawning males to non-spawning males provides strong evidence that females glean additional information from this signal during mate search. Retention time increases across all experiments provide additional evidence that milt pheromones help maintain spawning groups (Scott et al., 2019), which are most often comprised of one male and multiple females on a nest (Johnson et al., 2015), or an alternative explanation is that increases in retention are an extension of nest entry results and females just have preferences for actively spawning males. Increased time spent around potential mates could conceivably lead to mating opportunities and result in fitness benefits as spawning events typically occur every 4-5 minutes (Johnson et al., 2015; Manion and Hanson, 1980). Despite 3kPZS's presence in the seminal plasma used during behavioral trials

and its potent function as a pheromone in attracting females from long distances at multiple concentrations (Johnson et al., 2009), these do not appear to confound or explain the observed female behaviors for several reasons; 1) seminal plasma increased female retention consistently across all behavioral experiments and 3kPZS only has modest influence on female retention (Johnson et al., 2012), 2) seminal plasma applied alone retained females at rates consistent with the SMW treatment which contained 182x the 3kPZS, 3) seminal plasma applied alone increased female localization of the odor source compared to the 3kPZS control treatment, and 4) seminal plasma, when mixed with SMW, only increased 3kPZS concentrations 0.75% when increases of ~100% were necessary to induce similar female preferences to adjacent odor sources (Fissette et al., 2020; Johnson et al., 2009). These all suggest milt pheromones provide additional information to females that ultimately influence localization, retention, and mate preferences.

Males of many species have deceptive components of their signals that may exploit females' preexisting sensory biases (Endler and Basolo, 1998; Ryan and Cummings, 2013) or inaccurately reflect some status or quality indicator to receivers (Backwell et al., 2000; Bee et al., 2000). Male sea lamprey have deceptive components of their gill released pheromones; elevated 3kPZS release evolved via a sensory trap that exploits female preference for 3kPZS during reproductive migration (Buchinger et al., 2020; Buchinger et al., 2013), increased pheromone signaling by small males influences female behavior (Buchinger et al., 2017), and males increase pheromone release when detecting competition (Fissette et al., 2020). Multiple or alternative signals may allow females to glean reliable information in order to make preferential decisions (Bro-Jørgensen, 2010; Candolin, 2003; Higham and Hebets, 2013). In sea lamprey, the information milt pheromones provide may be more difficult to fake compared to what information gill pheromones signal. Releasing milt, and therefore signaling sperm availability is a binary

scenario, a male is either spawning and releasing sperm or not whereas gill pheromones are released continually during sexual maturity and males have some ability to modulate their 3kPZS release (Fissette et al., 2021b; Fissette et al., 2020). Females may use milt pheromones as a more reliable indicator of male fertilization ability or male nest resources to reduce errors during mate search.

Based on the behavioral results here, we conclude that chemical compounds in sea lamprey milt, despite inducing overlapping behavioral responses to the gill released pheromone of localization and retention, comprise a functionally distinct pheromone from the bile acid mating pheromone. Chemical cues in sea lamprey milt provide additional information to females and allow them to discriminate between spawning and non-spawning males and perhaps even among males, important distinctions in a species with a single reproductive event and a limited, seasonal spawning window. These results advance the understanding of attractive chemical cues in male ejaculates, display how multiple signals within the same modality can be used during mate choice, and provide evidence that females may discriminate among males using milt pheromone concentrations.

SUPPLEMENTAL INFORMATION

Table S3.1. Cumulative number of spawning events during behavioral observations of 25 sea lamprey nests

Nest Number	Spawning Events
1	0
2	0
3	7
4	7
5	1
6	6
7	8
8	5
9	10
10	6
11	12
12	14
13	7
14	6
15	7
16	5
17	7
18	0
19	5
20	6
21	29
22	9
23	14
24	4
25	5
AVG (30 min)	7.2
AVG (60 min)	14.4

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CHAPTER 4:

MALE NEST LOCATIONS MODULATE FEMALE PREFERENCES FOR PHEROMONE SIGNALS IN SEA LAMPREY

ABSTRACT

Animal communication is dynamic; signal attributes, signaler and receiver positioning, and the environment can create potential interactions that influence how information is conveyed and/or received. These aspects of communication networks are often studied independently, and many times, we lack an understanding of how these attributes interact within dynamic scenarios to influence receiver responses, mate choice, and potential fitness impacts. Using sea lamprey, (*Petromyzon marinus*), we tested the hypothesis that male signaling location impacts mate availability by influencing female preferences for pheromone signals. Male sea lamprey aggregate on spawning grounds where individuals build nests and use sex pheromones to attract females. The dynamic nature of signaling can be tested within this context because nests vary in spatial distribution, males vary in production and release of pheromone signals, females show preference for pheromone signals with higher concentrations, and pheromone transmission is mediated by the environment. Using in-stream behavioral assays at two experimental sites, we showed variability in the existence and strength of interactions between nesting location and pheromone concentrations, which had distinct effects on female behavior. Female preferences were mediated to a larger degree by nesting location rather than pheromone concentrations, and a male's nesting location may directly impact mate availability. These results highlight the importance of integrating signaling behavior, signal attributes, and the environment to better understand the mechanisms underlying receiver responses within communication networks.

INTRODUCTION

A plethora of sexual signals, displays, and behaviors exist to attract potential mates and influence mating preferences (Andersson, 1994). These are shaped via biotic and abiotic factors such as the direct or indirect benefits communicated to mates (Kokko et al., 2003; Møller and Jennions, 2001), the exploitation of preexisting sensory biases (Christy, 1995; Ryan, 1990), the heterogeneous nature of signaling environments (Boughman, 2002; Endler, 1992), intrasexual competition (Bee and Bowling, 2002; Candolin, 1999; Fissette et al., 2020), or predation (Endler, 1980; Stoddard, 1999; Zuk and Kolluru, 1998). A major focus of research surrounding signal evolution is that of signal attributes and the information they convey, which may have implications for mate assessment and choice that drive the elaboration of male traits (Andersson and Iwasa, 1996; Andersson, 1994; Kirkpatrick, 1996). However, signal attributes which relate to male quality and influence female behavior do not occur within a vacuum and represent only one aspect of communication networks (Endler and Basolo, 1998; Hutton et al., 2015). Behavioral decisions related to the location from which an individual signals may also influence receiver responses in dynamic ways (Cummings and Endler, 2018; Endler, 1992).

The possibility for dynamic interactions between location and environmental variables can further impact signal efficacy and mate preferences due to slight adjustments or misalignments in animal positioning having consequences for the information received or conveyed (Echeverri et al., 2021). Signal detectability and consequently its efficacy in communicating desired information may be affected by environmental factors such as ambient light (Endler and Thery, 1996), temperature (Conrad et al., 2017), humidity (Webster and Cardé, 1982), substrate (Elias et al., 2004), anthropogenic noise (Hanna et al., 2011), flow dynamics (e.g. air or water currents) responsible for chemical dispersal (Riffell et al., 2008), and the overall transmission medium

(e.g. water vs. air) (Endler and Basolo, 1998). A signaler's location and its interaction with multiple environmental variables add even further nuance into communication networks as display sites and the environment are not always static. Despite the knowledge that signaling location impacts signal attributes such as body coloration (Marchetti, 1993; Seehausen et al., 2008), acoustic parameters (Goutte et al., 2016; Halfwerk et al., 2017; Nemeth et al., 2001; Parris, 2002), and chemical dispersion (Fares et al., 1980; Murlis et al., 2000), the processes surrounding signaling location and orientation and their impacts on behavioral preferences of receivers remain less emphasized and may rely on broad assumptions about signal attributes or spatial positioning (Echeverri et al., 2021).

Sea lamprey, *Petromyzon marinus*, provide a useful model to study the interactive effects of signaling location and signal attributes on female preferences. Once sexually mature, male sea lamprey build nests in suitable gravel substrate and continuously release a mixture of bile acid pheromones via their gill epithelia that attracts mates (Applegate, 1950; Li et al., 2002; Siefkes et al., 2003; Siefkes et al., 2005). Pheromone communication is essential for reproduction (Fissette et al., 2021a) as it facilitates long distance mate search (Johnson et al., 2009) along with nesting and reproductive behaviors (Johnson et al., 2012a). Spawning occurs in lek like aggregations where nests vary in spatial orientation, there is typically one male per nest which is defended from intruders, and females are polyandrous and have the opportunity to discriminate among mates (Applegate, 1950; Johnson et al., 2015; Manion and Hanson, 1980). Males vary in the pheromone signals they produce and release (Buchinger et al., 2017; Buchinger et al., 2019; Fissette et al., 2021b), and females prefer pheromone signals with higher concentrations of a main pheromone component, 3-keto petromyzonol sulfate, 3kPZS (Buchinger et al., 2017; Fissette et al., 2020; Johnson et al., 2009). Due to the interaction between the heterogeneous

nature of stream habitats and currents, spatial variation of nests, and male variation in pheromone signals, the distribution, transmission, and perception of chemical information carried via male pheromone plumes likely varies based on the nest location (Moore and Crimaldi, 2004; Moore et al., 2000; Webster and Weissburg, 2009). These interactions may have direct implications for female mate choice, where males who have preferred signals may not be perceived that way due to the influence of environmental factors on signal transmission.

We hypothesized that nest location impacts male fitness by influencing female preferences for pheromone signals. To test this hypothesis, we 1) we surveyed the spatial distribution and use of sea lamprey nests in a wild stream to assess variation across these metrics and 2) conducted behavioral experiments that manipulated pheromone concentrations between paired nesting sites in two separate stream reaches to assess how signal quality impacted female preferences for nest locations and retention time within each nest. We provide evidence that nest location impacts female behavioral responses and mate preferences, which vary based on interactions or the lack thereof between nest location and pheromone concentration. These results highlight how a male's signaling location may directly impact female mate choice despite the quality of a male's signal and show how environmental impacts on signal transmission can influence female behavior.

METHODS

Animal Collection

During their reproductive migration, sea lamprey were trapped from tributaries of Lakes Michigan and Huron by the US Fish and Wildlife Service and Fisheries and Oceans Canada. Lamprey were then transported to the US Geological Survey Hammond Bay Biological Station (HBBS), Millersburg, Michigan (MI) and held in 200-1000 L tanks that were constantly supplied with aerated, ambient temp Lake Huron water. To collect sexually mature adults for pheromone collection and experiments, male and female sea lamprey were held in cages (0.25-1 m^3, ~25 individuals per cage) in the Ocqueoc River, Millersburg MI. To confirm sexual maturity, lamprey were checked daily for gamete expression by using gentle abdominal pressure (Siefkes et al., 2003). Sexually mature individuals were transported back to HBBS and held in 200 L tanks until experimental use. All experimental procedures followed protocols and methods approved by the Michigan State University Institutional Animal Care and Use Committee (PROTO202100029).

Spermiated Male Washing Collection for Behavioral Experiments

Spermiated male washings (SMW) were collected on July 1, 2022 and contain all the bile acid pheromone components released by males via gill epithelia (Johnson et al., 2012a; Siefkes et al., 2003). Twelve spermiating males were held in 60 L of ambient temperature Lake Huron water (temp. start = 14.8℃, temp. end = 20.0℃) for 10 hrs. After 10 hrs., males were removed, the water thoroughly mixed, aliquoted into 1 L bottles, and then stored at -20℃ until being used as pheromone treatments during behavioral experiments.

Nest Surveys of a Wild Sea Lamprey Spawning Population

To assess the spatial distribution and frequency of nest use by males and females, visual surveys were conducted in a ~270 m section of the Ocqueoc River located directly below a barrier that blocks upstream migration. Surveys occurred on 12 nights from June 3, 2022 – June 15, 2022 and were conducted from ~21:30-23:30 each night. Two separate surveys were conducted each night along the ~270 m transect, and during each transect, data was collected on nesting locations and the number of males and females present on each nest. Each nesting location was given a unique ID using a brightly colored and numbered rock to allow paired observations for each nest location across the spawning season. At the end of the second survey each night, all newly built or re-built nests were destroyed by filling in the crescent shaped nest depression with gravel and destroying the downstream piles of larger rocks (Applegate, 1950; Johnson et al., 2015). During surveys, only newly built nests or nests that had been re-built in a previously used location had data collected from them. Defined nest characteristics such as large anchor rocks at the upstream end, a noticeable depression where larger rocks are removed and only smaller gravel remains, and a rim of larger rocks piled at the downstream end of the nest (Applegate 1950; Johnson et al., 2015) make it easy to distinguish between newly built and rebuilt nests from destroyed nests, ensuring accurate data collection. Additionally, males and females are sexually dimorphic and have distinct secondary sexual characteristics: males have a thick, dorsal rope made of fat and females have large swollen bellies and a distinct, ventral ridge located near their cloacal aperture (Johnson et al., 2015).

Behavioral Experiments

Behavioral experiments were conducted at two separate sites within the 270 m observation area to determine how interactions between a male's nesting location and pheromone

concentration influence female preferences during mate choice. Experimental locations were chosen in order to directly compare a nest that was frequently built and attracted females during the spawning season to a nest that was less frequently built and attracted fewer females. These nests were also roughly parallel with each other within the stream channel to avoid any confounding spatial component on female behaviors related to nests being too far up or downstream from each other. Nesting locations were evaluated on three metrics: the number of days a nest was used (rebuilt and empty or actively being used), the cumulative number of females on the nest, the cumulative number of males on the nest. Nests were ranked based on these criteria and then chosen as sites for behavioral experiments.

At experimental site 1 (upstream site), the observation data for two separate nests (nests 36 & 37) were combined into one frequently used location as they were each used repeatedly and always built within 0.5 m of each other. These nests were each built consistently (#36, 12 days; #37, 8 days), had large total numbers of males and females (#36, 15 females and 16 males; #37, 21 females and 9 males), and had males (#36, 12 days; #37, 9 days) and females (#36, 9 days; #37, 9 days) present a majority of survey days. The infrequently used nest was used more sporadically (n = 5), had fewer total females (n=10) and males (n=5), and fewer total days with males (n=5) and females (n=5) present. These nests were nearly parallel within the stream and only 2.25 m apart as measured from the center of each nest location.

At experimental site 2 (downstream site), the frequently built nest was constructed on 11 days, had a large total of females (n=27) and males (n=11), and had females and males present most sampling days (n=11 for females; n=10 for males). In contrast, the infrequently built nest was only constructed on one day and had one male and one female present. At this site, nests

were 3.9 m apart and the infrequently built nest was located ~1 m upstream of the frequently built nest.

In-stream behavioral assays were conducted at the upstream site from July 13-22, 2022 and at the downstream site from July 1-12, 2022. Female behaviors were quantified using a passive integrated transponder (PIT) system (Oregon RFID, Portland, OR, USA). The PIT array utilized two separate 1 m^2 antennas placed directly over the nesting locations outlined above and served as the application points for pheromone treatments. Each female was fitted with a 23 mm half-duplex PIT tag (Oregon RFID) which was sheathed in plastic tubing and attached dorsally via a suture. Unique PIT tag numbers ensure females are analyzed only for their respective trial. Experimental subjects (n=12-20 depending on animal availability) were held in 0.5^2 m release cages either 75 m (upstream site) or 80 m (downstream site) downstream of PIT nests. Females were acclimated for ~ 1 hour prior to the start of behavioral experiments, and behavioral experiments were started ~30 min after sunset. Behavioral experiments were an hour in length, with a 15 min pheromone pumping period prior to release and a 45 min behavioral observation period after release. Two separate behavioral parameters were measured via the PIT system: the first PIT nest a female entered, and the total time spent within each $1m^2$ antenna (retention time). Retention times were calculated using PIT detection times, and if a female was not detected for 15 s, they were considered to have left the PIT nest.

To assess the influences of nest location, pheromone concentration, and any interactions between them, three separate pheromone treatments were utilized during behavioral experiments: 1) a control (500 mL SMW applied in each nest), 2) higher pheromone concentration in the frequently built nest (500 mL SMW in frequently built location, 250 mL SMW in the infrequently built location), and 3) lower pheromone concentration in the frequently built nest

(250 mL SMW in frequently built location, 500 mL SMW in the infrequently built location). The volumes of SMW application were chosen using 2 parameters. 500 mL of SMW represents the total pheromone released by one male over the course of an hour (60 L / 12 males / 10 hrs = 500 mL per male per hr), and females show behavioral preferences for adjacent pheromone sources that vary by 2x the concentration (i.e. 500 mL vs. 250 mL, Fissette et al., 2020, Johnson et al., 2009). Treatment odors were applied using a peristaltic pump (Masterflex L/S, item EW-07554-90, Cole Parmer, Vernon Hills, IL, USA). Odorants were mixed with river water to make a total volume of 10 L and applied at 167 ml min^{-1} in each of two adjacent 1 m^2 PIT antennas.

Stream Mapping and Measuring Pheromone Distribution at Experimental Sites

Each experimental site was mapped in order to create visual displays of pheromone dispersal from the nesting locations to release cages. To map each experimental site, a straight line transect was created from the upstream to the downstream end using posts and string. Transects perpendicular to this line were then marked every 2 m for the first 20 m and then every 5 m for the remaining distance to release cages. The distance to each bank was measured along these transects and drawn onto grid paper to create a map with an outlined stream channel. Any large log jams or notable impediments were also marked onto the map. At each perpendicular transect, the river was divided into ten equal sections, and the center of each section was marked with a zip tie to ensure sampling location was consistent across all data.

At each experimental site, fluorescent dye was used to measure pheromone plume dispersal within the river channel. To measure relative pheromone distributions and concentrations, 300 mL of red fluorescent dye (Cole Parmer, item EW-00298-06, Vernon Hills, IL, USA) was mixed with 10 L of river water, pumped into the nest location, and then recorded at each sampling point using a DataBank Datalogger with a Cyclops-7 rhodamine dye sensor (Turner Designs, San Jose,

CA, USA). At each transect, 12 fluorescent dye measurements (left bank, right bank, 10 sections) were taken. This process was conducted while pumping at each nesting location utilized during behavioral experiments. These measurements were then used to create pheromone plume distribution maps for each nest location.

Creating Maps of Pheromone Distribution

The images for dye concentrations were generated in Python 3.11 (https://www.python.org/) using the matplotlib 3.7 (https://matplotlib.org/) library. Sampling points were provided along horizontal transects of the river. These transects were 2 m apart for the first 20 m, then 5 m apart thereafter. Each transect has sampling points at each edge of the river and in the center of 10 equally sized bins for the given transect. B-splines (https://www.datatechnotes.com/2021/11/b-spline-fitting-example-in-python.html) were used to generate values along any x-axis between the river edges, normalized with an input value between 0 and 1 as a linear relationship between the left and right edge of the transect. To interpolate a given value z at coordinates (x_0, y_0), the first step is to determine the relative distance from the left shoreline. This was accomplished by interpolating the river edges as b-splines and using the estimated edges calculate a normalized x-value we will call x_1. The next step is to create a new vertical b-spline using the x_1 value as an input for every horizontal transect. The desired value for z is then found using the vertical b-spline. Mesh points to generate the images were sampled in a hexagonal grid with a radius of $\sqrt{3}/8$ meters. Linear color scaling was used to color the maps. Trees, nests, and release cages were added by estimating positions from hand-drawn maps.

Statistical Analyses

All statistical analyses were performed using R v4.2.2 (https://www.r-project.org/). For behavioral experiments, exact binomial tests with expected probability of 0.5 were used to

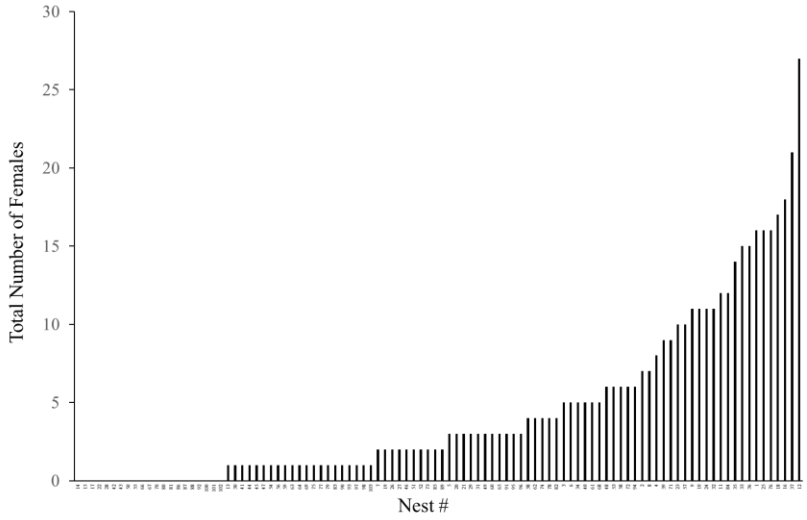
compare female choice of the frequently built nest to the infrequently built nest within each pheromone treatment. Generalized linear mixed effects models with a binomial distribution and post hoc Tukey's tests were used to evaluate female nest choice across treatments and included a random effect of trial time (1^{st} or 2^{nd}). Mixed effects linear models were used to evaluate the effect of treatment on odor source retention, and each model included a random effect of Fish ID for paired analyses of retention times for simultaneously applied pheromone treatments. All analyses incorporating mixed models used the glmer and lmer functions in R package lme4 (Bates et al., 2015) and Anova function with type III sums of squares (α=0.05) (car package, Fox and Weisberg, 2019).

RESULTS

Variation in Cumulative Number of Females at Nesting Locations and Nest Building Frequency

During behavioral observations, a total of 102 nesting locations observed. Large variation was observed in the cumulative number of females on each nesting location (range 0-27, 4.48 ± 5.46, mean ± SD, Figure 4.1A), cumulative number of males on each nesting location (range, 0-18, 3.85 ± 3.49), and nest building frequency (range, 1-11, 4.29 ± 2.80 Figure 4.1B). Females were not evenly distributed amongst nests, with 20% of the observed nesting locations accruing 61% of the total females.

A)



B)

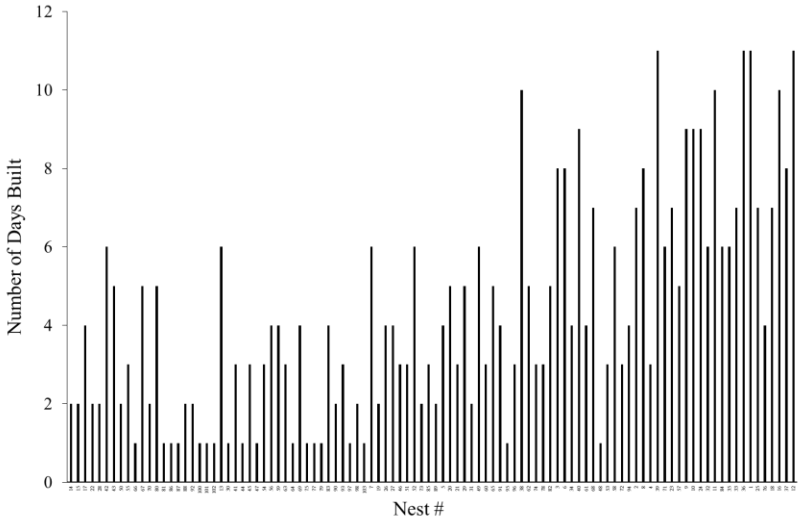


Figure 4.1. Variation in the cumulative number of females observed on a nest (A) and the frequency of times a nest was built (B) during behavioral observations. For twelve days, behavioral observations were collected at night along a ~270 m stretch of the Ocqueoc River, Millersburg MI. Two transects of this section were conducted each night, and these observations were used to quantify the number of females on a nest and the frequency nests were built for each nesting location. Each nesting location was marked with a brightly colored and number rock to allow repeated measures of observations across days. A total of 102 nesting locations and 457 females were observed during the course of this experiment. The total number of females and the number of times a nest was built (occupied or unoccupied) were quantified for each nesting location. During our observations, we had no way to determine whether females were unique across sites or days. Each bar represents the total for an individual nesting location and nesting locations are presented in the same order for each bar chart. The total number of females on a nest was heavily skewed, with 20% of the nests accounting for 61% of the total number of females.

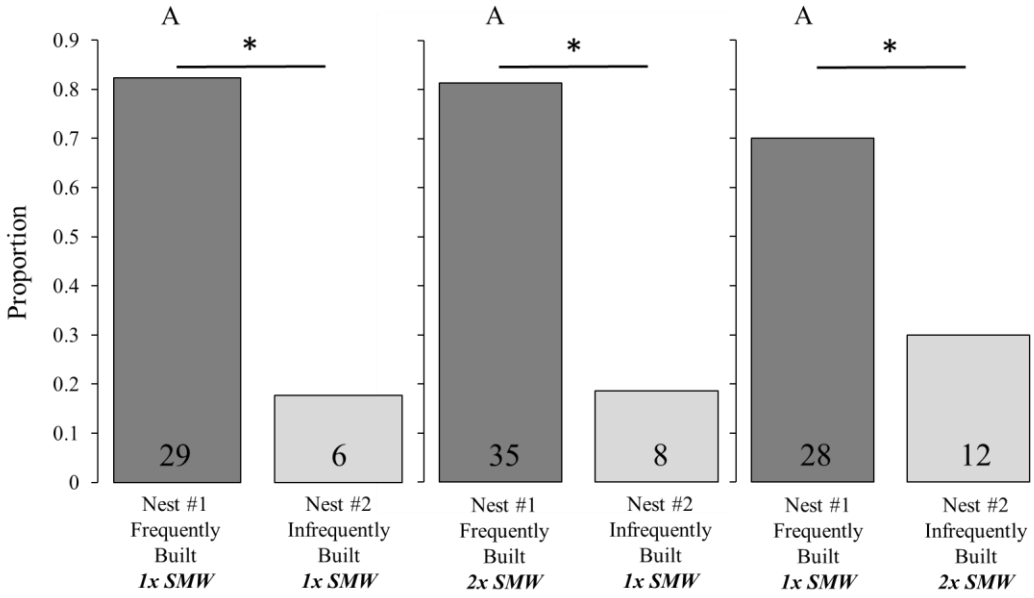
Nest Location Impacted Female Behavioral Responses Differently Across Experimental Sites

At the upstream site, nest location but not pheromone concentrations influenced female preferences. Across all treatments, there was no difference in the proportion of females entering the frequently built nest compared to the infrequently built nest (χ^2 (2) = 2.22, p = 0.33, Figure 4.2A). Within each treatment, females preferred to enter the frequently built nest regardless of pheromone concentration applied (Figure 4.2A); control (observed proportion = 0.83, expected proportion =0.50, 95% CI = 0.66-0.93, p < 0.001), 2x the pheromone in the frequently built nest (observed proportion = 0.83, expected proportion =0.50, 95% CI = 0.67-0.92, p < 0.001), and 2x the pheromone in the infrequently built location (observed proportion = 0.70, expected proportion = 0.50, 95% CI = 0.53-0.83, p=0.02). No interaction between nest location and female retention was observed at this site as retention times matched concentration gradients. Females spent equal time at each nest during control trials (χ^2 (1) = 0.96, p = 0.33, Figure 4.2B), and spent more time at the nest with higher pheromone concentrations: 2x pheromone concentration in the frequently built nest (χ^2 (1) = 19.79, p < 0.001, Figure 4.2B) and 2x pheromone concentration in the infrequently built nest (χ^2 (1) = 3.52, p = 0.06, Figure 4.2B).

At the downstream site, interactions between nest location and pheromone concentration were observed and impacted both female preferences and retention. The proportions of females entering the frequently built nest were different across treatments (χ^2 (2) = 9.67, p < 0.01, Figure 4.3A). Females preferred the infrequently built nest during control trials (observed proportion = 0.83, expected proportion =0.50, 95% CI = 0.66-0.94, p<0.001, Figure 4.3A) and when 2x the pheromone concentration was present in this nest (observed proportion = 0.83, expected proportion =0.50, 95% CI = 0.64-0.94, p<0.001, Figure 4.3A), but 2x the pheromone concentration in the frequently built nest evened out female preferences between nesting

locations (observed proportion = 0.48, expected proportion =0.50, 95% CI = 0.31-0.66, p=1, Figure 4.3A). Female retention times matched the patterns observed for female preferences. Females spent more time on the infrequently built nest during control trials (χ^2 (1) = 11.79, p < 0.001, Figure 4.3B) and when 2x the pheromone concentration was present in this nest (χ^2 (1) = 19.96, p < 0.001, Figure 4.3B), but when 2x the pheromone concentration was applied in the frequently built nest, retention was no different between nesting locations (χ^2 (1) = 0.08, p = 0.77, Figure 4.3B).

A)



B)

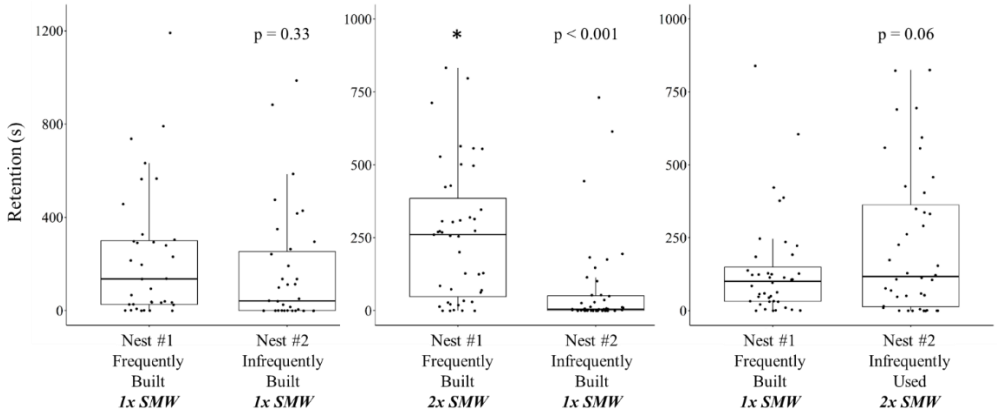
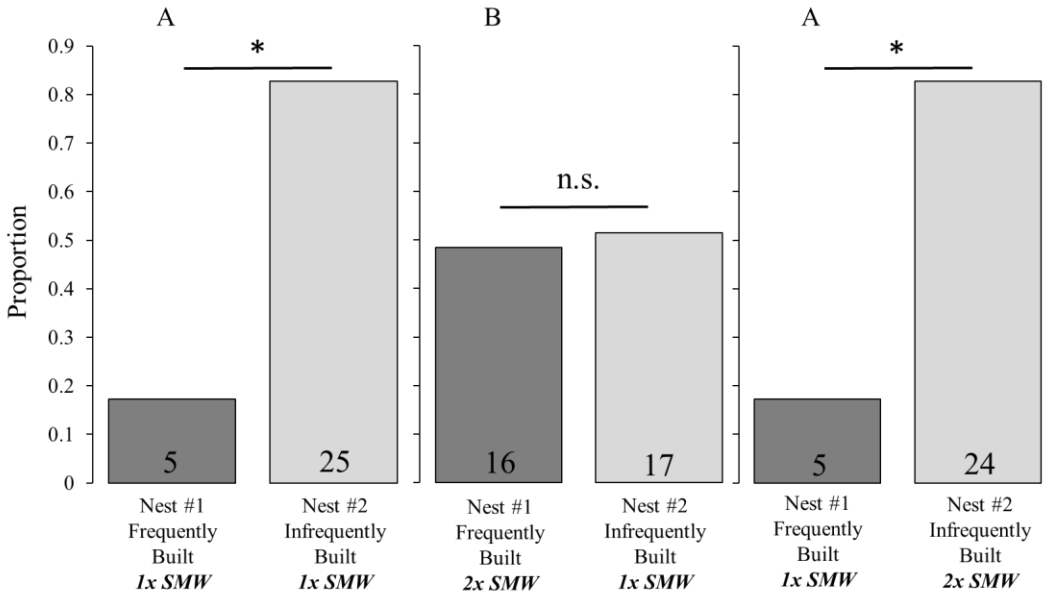


Figure 4.2. At the upstream experimental site, females always preferred the frequently built nest, and female preferences and retention times were not influenced by an interaction between nest location and pheromone concentration. A) Proportion of females entering the frequently or infrequently built nest location first across three separate pheromone treatments. Pheromone treatments included a control (equal pheromone concentrations in each nest), 2x the pheromone concentration in the frequently built nest, and 2x the pheromone concentration in the infrequently built nest. The n within each bar represents the total number of females choosing that nesting location first. Lines above bars represent comparisons within each treatment level, and letters represent comparisons across all treatment levels. Asterisks indicate differences within each treatment (exact binomial test, $\alpha = 0.05$) and different letters indicated differences across treatments (mixed-effects logistic regression with binomial distribution with Tukey's HSD, $\alpha = 0.05$). The frequently built nest location was preferred regardless of the

Figure 4.2 (cont'd)
pheromone concentration (control, $p < 0.001$; 2x SMW in the frequently built nest, $p = < 0.001$; 2x SMW in the infrequently built nest, $p = 0.02$, exact binomial tests), and no difference was observed in female nest entry when compared across all treatments ($p=0.33$, mixed-effects logistic regression with binomial distribution). **B).** Boxplots of paired female retention times (s) within 1 m^2 PIT nests across three pheromone treatments. Each point represents an individual observation, and the boxplots display the median, interquartile range, the maximum (Q3 + 1.5*IQR), the minimum (Q1 – 1.5*IQR), and any outliers. Female retention matched pheromone concentration gradients (increased pheromone = increased retention) where no differences were observed during the control ($p=0.96$, mixed effects linear model) but increased retention was observed when 2x the pheromone concentration was present in the frequently built nest ($p<0.001$, mixed effects linear model) and the infrequently built nest ($p=0.06$, mixed effect linear models

A)



B)

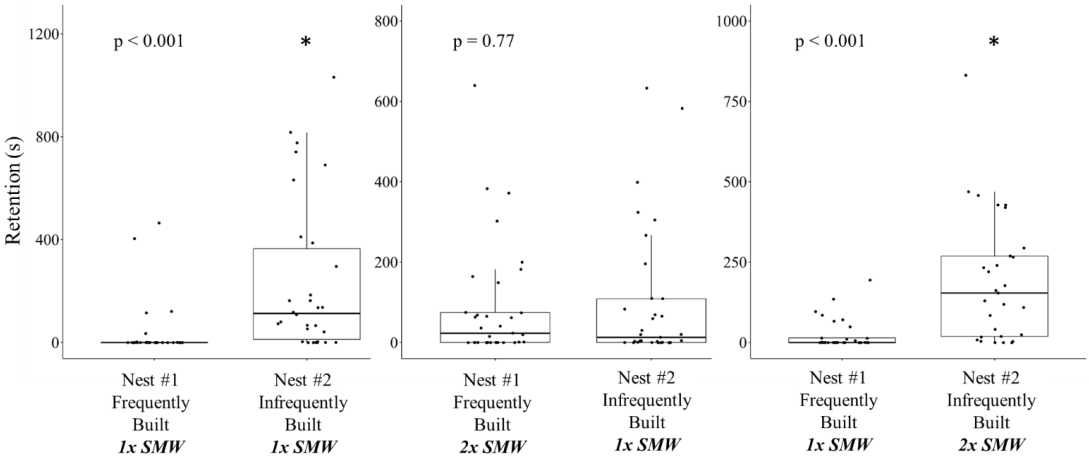


Figure 4.3. At the downstream experimental site, the infrequently built nest was preferred, and female preferences and retention times were influenced by interactions between nest location and pheromone concentration. A) Proportion of females entering the frequently or infrequently built nest location first across three separate pheromone treatments. Pheromone treatments included a control (equal pheromone concentrations in each nest), 2x the pheromone concentration in the frequently built nest, and 2x the pheromone concentration in the infrequently built nest. The n within each bar represents the total number of females choosing that nesting location first. Lines above bars represent comparisons within each treatment level, and letters represent comparisons across all treatment levels. Asterisks indicate differences within each treatment (exact binomial test, $\alpha = 0.05$) and different letters indicated differences across treatments (mixed-effects logistic regression with binomial distribution with Tukey's HSD, $\alpha = 0.05$). The infrequently built nest was preferred during control trials ($p < 0.001$, exact binomial

Figure 4.3 (cont'd)
test) and when 2x the pheromone concentration was present in this nest ($p < 0.001$, exact binomial test), but the preference for this nesting location was evened out when 2x the pheromone concentration was applied in the frequently built nest ($p=1$, exact binomial test). Unlike the upstream location, female nest preferences were not consistent across treatments ($p<0.01$, mixed-effects logistic regression with binomial distribution). **B).** Boxplots of paired female retention times (s) within 1 m^2 PIT nests across the three pheromone treatments. Each point represents an individual observation, and the boxplots display the median, interquartile range, the maximum (Q3 + 1.5*IQR), the minimum (Q1 – 1.5*IQR), and any outliers. Female retention matched trends in nest entry, with increased retention on the infrequently built nests during control trials ($p < 0.001$, mixed effects linear model) and when 2x the pheromone was applied in this location ($p < 0.001$, mixed effects linear model). However, 2x the pheromone concentration in the frequently built location evened out retention between nesting locations ($p=0.77$, mixed effects linear model).

Dispersal of Pheromone Plumes

At the upstream site, dye dispersal and concentration have large areas of overlap between the frequently built and infrequently built nesting locations, largely in part to a large log jam present from ~5-15 m downstream of nesting locations that guides the river current at a diagonal towards the left bank (Figure 4.4A). This appears to create largely overlapping pheromone plumes with minimal changes in concentration gradients (< than an order of magnitude). At the downstream site, dye dispersal and concentration create two distinct plumes until ~35-40 m downstream from nesting locations where they eventually mix (Figure 4.4B). When compared to the frequently built nest, the pheromone plume from the infrequently built nest remains more concentrated all the way down to the release cages, and for large portions (5-35 m) is two to three orders of magnitude more concentrated.

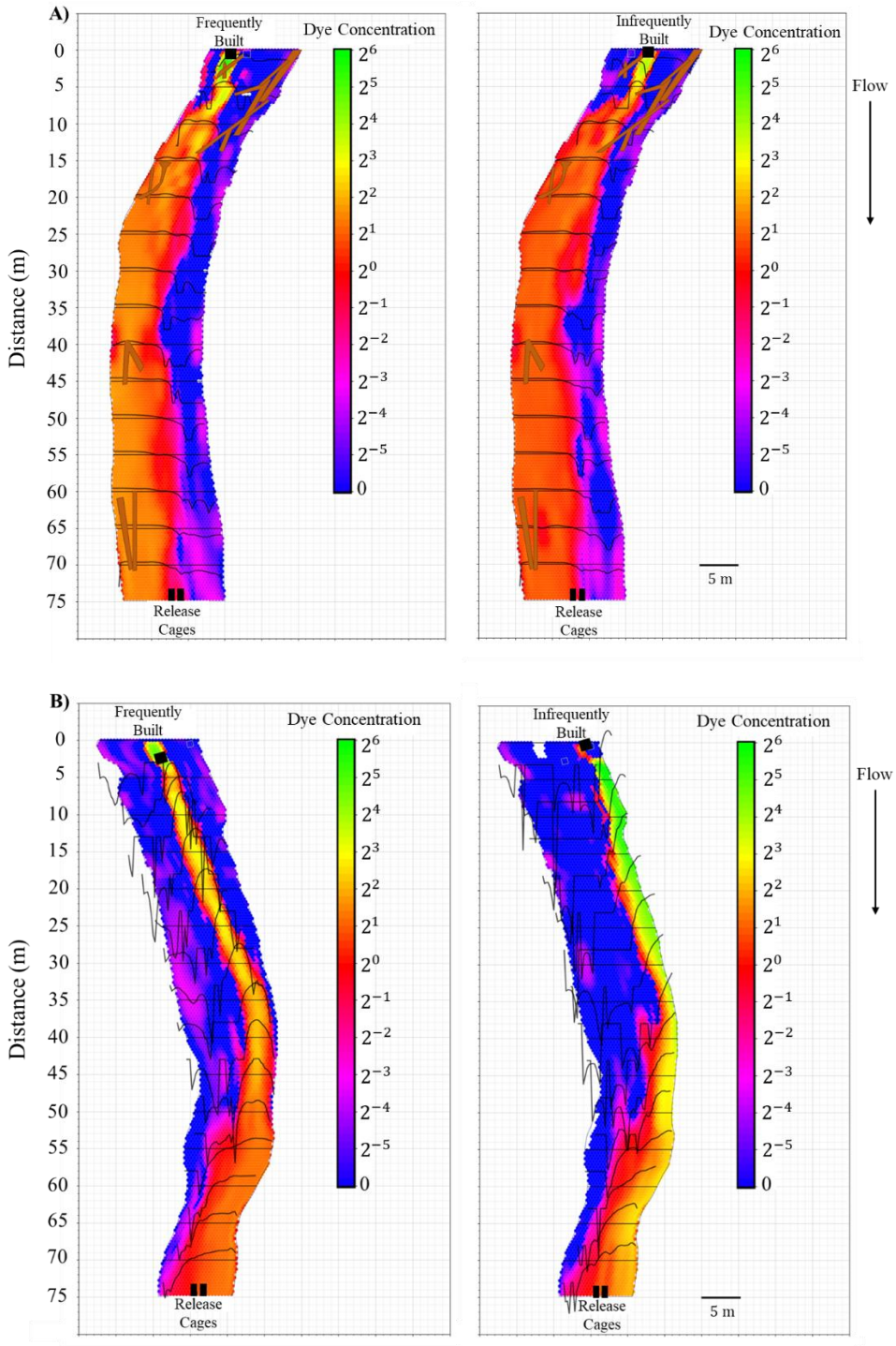


Figure 4.4. Relative dye concentrations for nesting locations at the upstream (A) and the downstream (B) experimental sites. Dye concentrations to depict pheromone dispersal were generated in Python 3.11 using the matplotlib 3.7 library. Sampling points were taken across horizontal river transects at each experimental site. Transects were 2 m apart for the first 20 m and then 5 m apart for the remaining stream distance. Twelve dye measurements were taken at

Figure 4.4 (cont'd)
each transect; one at each bank and then one in each center of 10 equally sized sections across the transect width. Dye concentrations were measured using a DataBank Datalogger with a Cyclops-7 rhodamine dye sensor (Turner Designs, San Jose, CA, USA). In order to generate concentration gradients, dye values were interpolated at any given location (z) with coordinates (x_0, y_0). This was done by 1) interpolating the river edges as b-splines and using estimated edges to calculate a normalized x-value (x_1), 2) creating a new vertical b-spline using the x_1 value as an input for every horizontal transect, and 3) the desired value for z is then found using the vertical b-spline. Mesh points to generate these images were sampled in a hexagonal grid with a radius of $\sqrt{3}/8$ meters, and linear color scaling was used to generated gradient colors. At each 5 m transect, black lines that cross the stream width horizontally display dye concentration trends within that transect. Trees, nests, and release cages were added manually from estimating positions on hand-drawn maps. Nest locations and release cages are represented by solid black boxes at the upstream and downstream ends, and these are not drawn to scale.

DISCUSSION

By combining visual observations collected during the spawning season with behavioral experiments that manipulated a signal attribute and signaling location simultaneously, we investigated how the interactive effects of a male's nest location and pheromone signal influenced female preferences for mates. In support of our hypothesis that nest location could impact male fitness, we found 1) large variation and skew in nest use and the total number of females across nesting locations during the spawning season, 2) nest location alone or an interaction between nest location and pheromone concentration impacted female mate preferences and retention times, with distinct patterns for each behavioral parameter across experimental sites, and 3) indirect evidence that pheromone plume dispersal may explain some of our behavioral results. Taken together, our results highlight the dynamic nature of animal communication by showing that interactions between the environment and signal attributes can differ across fine and broader scales which may have distinct influences on receiver responses and consequently mate choice.

A major focus of sexual selection literature has been on signal attributes and their impacts on mate choice and preferences, which represents only one dimension of complexity in animal communication networks (Bradbury and Vehrencamp, 2014; Hutton et al., 2015; Patricelli and Hebets, 2016). The effects of environment and signal attributes are often studied independently despite likely interactions existing between them, and we often lack information on how receivers respond in these dynamic scenarios (Echeverri et al., 2021; Hutton et al., 2015). In sea lamprey, higher concentration pheromone signals are preferred by females (Buchinger et al., 2017; Fissette et al., 2020), but we found a male's nesting location impacts how and whether females express this preference. At two experimental sites, a higher pheromone concentration did

not, by itself, directly result in female preferences for the location with a higher pheromone concentration, but instead relied solely on nesting location (upstream site) or an interaction between nest location and pheromone concentration (downstream site). Overall, female preferences for male mates were influenced to a greater degree by male signaling location than a specific signal attribute and are consistent with ideas that interactions between the environment and signaling impact communication channels via courtship success (Hebets et al., 2008), influencing signal properties (Halfwerk et al., 2017; Röhr and Juncá, 2013; Simpson and McGraw, 2018), and impacting female preferences for mating sites (Warner, 1987). These results highlight how signal attributes, signaling location, and receiver responses are dynamic across environmental contexts (Echeverri et al., 2021; Endler and Basolo, 1998), and the interactions between them can be equally if not more important than individual characteristics of any one variable.

Interestingly, female behavior was not consistent across experimental locations, and behavioral results only matched predictions at one of two experimental locations. The upstream site matched predictions with no interaction between pheromone concentration and nest preference being observed; females preferred the frequently built nest location over the infrequently built nest regardless of the pheromone concentration applied. However, retention times between nests matched the concentration gradient (higher concentration = higher retention). Males, regardless of pheromone concentration, conceivably benefit from building a nest in the preferred location as it greatly increased opportunities in accessing mates ($\geq$ 70% female preference across all treatments). However, the influence of concentration on retention may have consequences for maintaining spawning groups that often consist of one male and multiple females (Johnson et al., 2015). Females can move among nests and failing to initially

attract a mate does not necessarily mean a male will not have mating opportunities (Johnson et al. 2015). Increased retention and consequently increased proximity to potential mates could influence mating opportunities as solitary chemosensory cells (Daghfous et al., 2020), tactile cues (Chung-Davidson et al., 2013), or electroreception (Chung-Davidson et al., 2004) may be utilized along with pheromones to modulate spawning behavior. Behavioral results did not match predictions at the downstream site (frequently built nest location was never preferred) and could have been impacted by changing river conditions in the time between nesting observations and behavioral experiments, which occurred 2-4 week apart. Nevertheless, behavioral results still provided evidence that nest location impacted female behavior. The infrequently built nest location was preferred by females, but 2x the pheromone concentration evened out preferences and retention times between nesting sites, showing that an interaction between pheromone concentration and nest location existed, which was not observed at the other site. Here, a male with a preferred signal, despite being in a less preferred location could only even out female choice. Unlike the upstream site, female retention times matched the trends observed for female preferences. Overall, males with preferred signals only had a significant advantage in attracting more females when signaling from a preferred nest location, but when located in a less preferred location, increased pheromone concentrations, at best, only evened out female preferences. These results suggest that multiple axes of variation, which can differ spatially within the same environment, influence female behaviors in response to signal attributes and signaling location.

The sensory drive hypothesis posits that the environment shapes communication networks via influences on signal detectability, transmission, and/or efficacy (Cummings and Endler, 2018; Endler, 1992; Endler and Basolo, 1998). Chemical signaling occurs in environments that vary dynamically across space and time, and the physical processes influencing chemical

transport and consequently signal transmission and perception in one location may vary distinctly from a nearby location. Chemical plume distributions and concentration gradients, which are influenced by heterogeneous flow dynamics in aquatic environments (Moore and Crimaldi, 2004; Webster and Weissburg, 2009; Weissburg, 2000), influence behavioral responses to chemical stimuli (Atema, 1995; Moore et al., 1991; Page et al., 2011; Zimmer-Faust et al., 1995). Sea lamprey utilize odor-conditioned rheotaxis to locate pheromone sources (Choi et al., 2013; Johnson et al., 2012b) and concentration gradients influence female behavior (Buchinger et al., 2017; Fissette et al., 2020; Johnson et al., 2009). Stream mapping and fluorescent dye tests provide indirect evidence that signal transmission through the environment impacted female behavior. At the upstream site where pheromone concentration did not influence female preferences, a large logjam upstream near the nest locations appears to similarly direct each pheromone plume downstream which creates large overlaps in pheromone distribution and concentration gradients. Females likely orient upstream similarly regardless of where experimental treatments are applied and enter the first nest encountered, which was the more frequently built nest during the spawning season and preferred regardless of pheromone concentration during experiments. At the downstream site, the preferred nest has a more concentrated pheromone plume that extends all the way down to the release site. Females likely navigated differences in concentration gradients between nests during control trials and when 2x the concentration was applied in the infrequent nesting location (~80% preference) but did not distinguish between the plumes when 2x the concentration was applied in the less preferred nest (~50% preference). These results suggest the possibility that males may be under sexual selection for nest building location as proposed via the sensory drive hypothesis.

Conflicts between natural and sexual selection (Kokko et al., 2003; Magnhagen, 1991; Pomiankowski, 1987) may impose additional costs for male and female sea lamprey during reproduction. These could relate to predation risk (Boulêtreau et al., 2020), limited energy stores (William and Beamish, 1979), limited reproductive window (Applegate, 1950; Dhamelincourt et al., 2021), suitable habitat for nest construction (Applegate, 1950; Manion and Hanson, 1980), social contexts influencing nest construction (Dhamelincourt et al., 2021), and embryo survival (Rodríguez-Muñoz and Tregenza, 2009; Smith and Marsden, 2009). For males, tradeoffs between these variables and maximizing signal efficiency may impact choice of nesting locations, and for females, tradeoffs between these and signal preferences may impact mate choice. For example, a nesting location with flow parameters for excellent signal transmission could be located in an area more prone to predation (shallow depth or lack of cover) or be poor habitat for embryo survival (unsuitable substrate or lack of egg retention, Smith and Marsden, 2009). Observation and behavioral results from the downstream experimental location provide some indirect evidence for this. The frequently built nest at this location was one of four nests built most frequently during the spawning season and accrued the largest female total, but behavioral results conflicted with our predictions, and females showed preferences for a nesting location only built once during the spawning season. A combination of signal transmission and tradeoffs impacting sea lamprey behavior is also plausible because 1) only two experimental sites were used, 2) these sites likely account for a small range of conditions encountered during reproduction, and 3) despite being preferred in behavioral experiments, the preferred nest at the downstream location was only built once and may not have been an optimal paired location for behavioral experiments.

In conclusion, our results highlight the importance of integrating variation in signaling behavior, signal attributes, and the environment to assess their dynamic impacts on receiver responses and communication networks more broadly. Our results that male nest locations had a larger impact on female mate preferences than pheromone concentration alone suggests fitness costs may be incurred and impose selective pressures on male signaling behavior. Altogether, our study adds to the growing body of literature demonstrating the importance of environmental variables on communication and demonstrates how incorporating interactions between multiple variables influencing communication networks are needed to better understand the evolution and function(s) of signals and signaling behavior.

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