IN-STREAM BEHAVIORAL RESPONSES OF FEMALE SEA LAMPREYS TO PHEROMONE COMPONENTS

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ABSTRACT

IN-STREAM BEHAVIORAL RESPONSES OF FEMALE SEA LAMPREYS TO PHEROMONE COMPONENTS

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Interference with sea lamprey (*Petromyzon marinus*) chemical communication may offer effective and benign methods to manage their populations in the Great Lakes where they are destructive predators of large fishes. Previous studies showed that a mating pheromone, putatively consisting of 3-keto petromyzonol sulfate (3kPZS) and 3-keto allocholic acid (3kACA), is excreted by spermiated males and directs ovulated females to spawning nests. Synthesized 3kPZS elicits preference responses in ovulated females in a natural stream, but it is unknown if 3kPZS is the only component of the mating pheromone and whether female responses to synthesized 3kPZS are sufficiently strong to merit its use in management. I hypothesized that 3kPZS would elicit robust upstream movements in ovulated females, directing them into traps and luring them away from natural male odorants. In this dissertation, in-stream behavioral tests showed that ovulated females responded with robust upstream movement directly to the source of synthesized 3kPZS concentrations ranging from 10^{-10} to 10^{-14} molar (M) and in diverse stream conditions. Ovulated female responses to 3kPZS are sufficiently strong to support utility in management where nearly 50% of ovulated females were captured in 3kPZS-baited traps and high concentrations of 3kPZS lured females away from and disrupted orientation to a natural pheromone source. Given that 3kPZS induced ovulated females to migrate upstream, and that it was recently discovered that sea lamprey larvae release

3kPZS, responses of pre-ovulatory females to 3kPZS were re-evaluated at night. Contrary to previous studies, 3kPZS induced strong preference responses in pre-ovulatory females not differing from that elicited by larval migratory pheromone over long distances. 3kPZS may not function specifically as a mating pheromone component, but as a pheromone component that induces directed migration in spawning-phase females regardless of maturity. Therefore, 3kPZS may have greater impacts on sea lamprey management than previously conceived, as it could potentially be used to modify the behavior and distribution of females during the entire migratory period, in addition to the spawning period. However, when 3kPZS and natural mating pheromone were compared directly, it was clear that additional pheromone components were released by males to retain females on nests and induce mating behaviors. 3kACA, previously hypothesized to retain ovulated females on nests, was extensively tested in streams, but it did not modify ovulated female behavior. A new in-stream bioassay was developed to confirm that XAD7HP resin extracted unidentified pheromone components that induced mating behaviors, and that spermiated males release all behaviorally active pheromone components through the head region. Future identification of additional pheromone components from XAD7HP extract using the in-stream bioassay will enable mating pheromone components to be fully characterized and all potential mating pheromone-based management tactics to be realized. Synthesized 3kPZS must be tested in management contexts containing wild lampreys to confirm its utility for sea lamprey control.

To my dad who told me to work hard and be happy…

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TABLE OF CONTENTS

CHAPTER 5

APPENDIX

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

PHEROMONES AS TOOLS IN FISHERIES MANAGEMENT: HOW FAR ARE WE, WHERE DO WE NEED TO GO, WHAT CAN WE LEARN FROM INSECTS?

AN INTRODUCTION TO THE RATIONAL AND OBJECTIVES OF DISSERTATION

ABSTRACT

In nearly every fish species investigated, chemical cues have been implicated to play a role in migration, maturation, or reproduction. Although pheromones influence critical life history events in fishes, they have never been used in a management context to enhance restoration of native species or control nuisance species. In contrast, hundreds of insect pheromones are employed to monitor, mass-trap, and disrupt reproduction in pest species. Insect pheromone control programs are successful when pheromone components are identified, characterized, and pheromone tracking mechanisms are known. Only about 20 putative fish pheromones have been chemically identified and even fewer have characterized behavioral functions. Furthermore, little is known about the mechanisms fish use to orient to, and track, pheromone plumes. The sea lamprey (*Petromyzon marinus*) is a species which provides examples of how pheromones could improve the management of nuisance nonindigenous fishes through improved monitoring, mass trapping, and mating disruption. Sea lamprey pheromones may also be used to aid fish restoration. Fisheries biologists should strive to identify and characterize additional fish pheromones to reveal their full potential to improve the management of invasive fishes and restoration of valued fishes. In this dissertation, the male sea lamprey mating pheromone (3kPZS) was further characterized to enable its future deployment in sea lamprey management. The dissertation describes behavioral responses and orientation of females to synthesized pheromone components in natural streams. The dissertation also describes new methods to isolate and characterize fish pheromones using in-stream bioassays.

Introduction.

Pheromones are chemical cues released into the environment by individual organisms that elicit specific, adaptive behaviors or physiological responses in conspecifics (Wilson and Bossert 1963; Karlson and Luscher 1959). Pheromones are acclaimed for their specificity, potency, and when used in management, their benign ecological impacts (Silverstein 1981; Marx 1973). Pheromones have been implicated in nearly every fish species investigated; however, uses of pheromones to manipulate fish physiology or behavior in fisheries management contexts have been nonexistent. In insects, pheromones have been identified in more than 2,000 species (Hwang 1997; Mayer and Mclaughin 1991) and more than 300 pheromones are currently used worldwide in integrated pest management systems (Hwang 1997) to monitor population density, mass trap, and disrupt reproduction (Carde and Minks 1995; Ridgway et al. 1990; Silverstein 1981). Fish pheromone research has advanced significantly during the past decade, and recent studies on cyprinid (Zielinski et al. 2003), salmonid (Young et al. 2003; Vermeirssen and Scott 2001), and agnathan species (Johnson et al. 2005; Sorensen et al. 2005; Li et al. 2002) demonstrated that pheromonal cues regulate critical aspects of fish life history. This makes pheromones promising tools for the control of invasive fishes and the restoration of native fishes.

Before discussing applications of pheromones in fisheries management, it would be useful to define the types of pheromones that have been characterized. Pheromonal cues are widely used in nature from humans to microbes to warn conspecifics of immediate danger (alarm pheromones), aggregate conspecifics (aggregation pheromones), aid in migration (migratory pheromones) and facilitate reproduction (mating pheromones) (Wyatt 2003 and therein). Most commonly studied are mating pheromones, which are emitted by mature individuals of one sex to induce a specific physiological or behavioral response in mature conspecifics. Mating pheromones can be categorized as priming or releaser pheromones. Priming pheromones prepare the receiver for mating behavior by inducing a hormonal cascade in the endocrine system. Releaser pheromones immediately alter the behavior of the receiver by eliciting a preference response or by inducing specific reproductive behaviors (Wilson and Bossert 1963). Pheromones fundamentally differ from visual, auditory, and tactile cues in that odors rely on a physical medium (air or water) to transmit signals to a receiver. Chemical cues can travel around obstructions and can persist after the signaler is gone, but require that the receiver integrate behavioral responses to the chemical cue based on to the dynamics of the transport medium.

The objective of this review was to develop ideas and research goals to advance the use of pheromones in fisheries management by discussing the current understanding of fish pheromones and by contrasting fish pheromones with established concepts in insect pheromones. First, I reviewed the structure and function of pheromones in fish, described how fish pheromones differed from insect pheromones, and discussed implications for management. Second, I contrasted the dynamics of pheromone plumes in air and water, discussed why fish and insects may use different mechanisms of tracking pheromone plumes, and why understanding pheromone tracking may be important when developing management schemes. Third, I illustrated possible applications of pheromones in fisheries management using the sea lamprey as a model.

Possible applications of fish pheromones will be developed by using lessons learned from pheromone-based insect control. Last, I proposed research objectives to advance the use of pheromones as tools in fisheries management and more specifically in sea lamprey control.

Pheromone systems in fish and insects.

The best-characterized examples of pheromonal communication are in the Class Insecta (Wyatt 2003). In insects pheromonal communication is commonly used to facilitate mate finding. Mature females often release species-specific compounds that elicit upwind flight by mature males, luring them toward females (Ridgway et al. 1990). Female insects may even release pheromones that induce landing and male copulation attempts (Bradshaw et al. 1983). Insect mating pheromones are typically specific mixtures and ratios of two or more long-chain, unsaturated alcohols, acetates, or aldehydes, ranging from 10 to 32 carbons (Figure 1-1A; Silverstein 1981). Rarely does a single chemical component induce a robust behavioral response, but such cases have been reported in the Japanese beetle (*Popillia japonica*) (Tumlinson et al. 1977), the gypsy moth (*Lymantria dispar*) (Bierl et al. 1970), and the silkworm (*Bombyx mori*) (Butenandt and Hecker 1961). Pheromone signals consisting of multiple components likely communicate more information than a single component pheromone, such as the identity, location, and sexual status of the signaler (Linn et al. 1987).

Pheromones have been implicated to mediate critical life history events in many freshwater fishes (Stacey et al. 1996; Stacey and Cardwell 1995; Sorensen 1992; Liley and Stacey 1983; Liley 1982), but the putative chemical identity of migratory and mating pheromones have been discovered in fewer than 20 species. Unlike insects, almost all

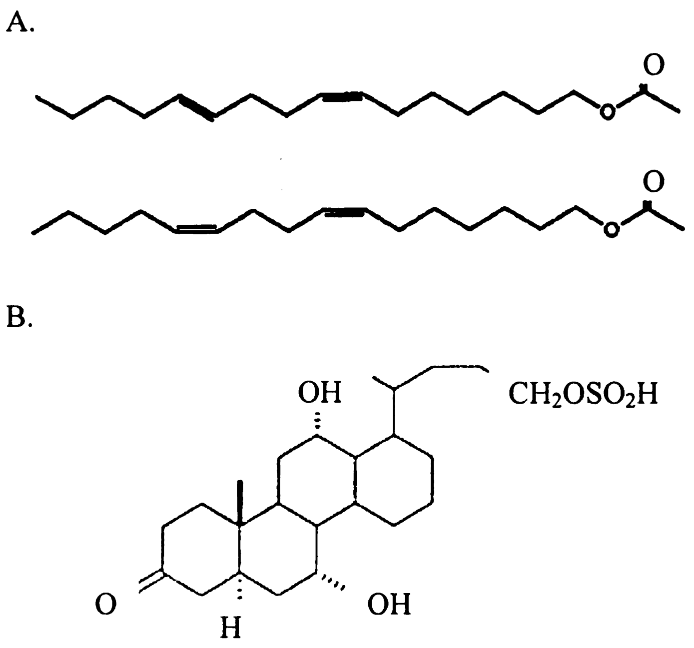

Figure 1-1. Chemical structure of an insect and fish pheromone. A) Long chain acetate pheromone components of the pink bollworm moth (*Pectinophora gossypiella*), (Z, E)-7-11-hexadecadien-1-ol acetate and (Z, Z)-7-11-hexadecadien-1-ol acetate (Gossyplure). B) Sulfated bile acid mating pheromone released by sexually mature male sea lamprey (*Petromyzon marinus*), 7α, 12α, 24-trihydroxy-5α-cholan-3-one 24-sulfate (3kPZS).

fish pheromones identified to date are steroids or bile acids (Figure 1-1B; Sorensen et al. 2005; Li et al. 2002; Sorensen and Stacy 1998). Recently, the amino acid L-kynurenine has been shown to function as a mating pheromone in masu salmon (*Oncorhynchus masou*) (Yambe et al. 2006). Fish pheromones differ from insect pheromones in chemical structure because of the medium into which they are released. Fish pheromones need to be polar to dissolve in water, whereas insect pheromones must be volatile to be evaporated. Like insects, fish are hypothesized to use mixtures of pheromone components because they contain more information about the identity of the signaler than a single component pheromone (Sorensen et al. 1998b).

Perhaps the best-characterized fish pheromone model is the goldfish (*Carassius auratus*), in which mixtures of steroids function as priming and releaser pheromones (Sorensen et al. 1995; Stacey et al. 1989; Sorensen et al. 1988; Dulka et al. 1987). Female goldfish, immediately prior to ovulation, release 4-pregnen-17,20ß–diol-3-one (17,20ßP), 4-pregnen-17,20ß–diol-3-one-20-sulfate (17,20ßP-S) and androstenedione (AD), which act as priming pheromones that trigger a gonadotropin II surge in males and increase sperm production (Sorensen et al. 1998b; Sorensen 1995; Stacey 1989). Ovulated female goldfish release a mixture of prostaglandin F2α (PGF2α) and 15keto-prostaglandin F2α (15K-PGF2α), which acts as a releaser pheromone that triggers male courtship behavior (Sorensen et al. 1989; Sorensen et al. 1988). Spermiated male goldfish also release mixtures of steroids into the water, primarily consisting of androstenedione, which increase male to male aggression (Sorensen et al. 2005). Similar steroid glucuronides and prostaglandins have been implicated as priming or mating pheromones in many other fishes (Table 1-1). Urine and feces are typically the sources

Table 1-1. Fish species hypothesized to use pheromones for migration, to synchronize spawning (priming), or reproduction (releaser).

M = males, F = females.

Species	Action	Responder	Releaser	Reference
Anguilla anguilla- European eels	Releaser	M/F	M/F	Huertas et al. 2006
Anguilla rostrata - American eels	Mating	M	F	Sorensen and Winn 1986; Briand et al. 2002
Barilius bendelisis - Ham	Releaser	M	F	Bhatt and Sajwan 2001
Carassius auratus - Goldfish	Releaser	M	F	Dulka et al. 1987; Poling et al. 2001
Carassius auratus - Goldfish	Mating	M	M	Sorensen et al. 2005a
Carassius auratus - Goldfish	Mating	M	F	Sorensen et al. 1988, 1989
Claria gariepinus - African catfish	Mating	F	M	Resink et al. 1989
Clupea harengus pallasi - Pacific herring	Mating	M/F	M	Stacy, Hourston 1982; Sherwood et al. 1991
Cyprinus carpio - Common carp	Releaser	M	F	Stacey et al. 1994
Gobius niger - Black goby	Mating	F	M	Colombo et al. 1980
Gymnocephalus cernuus - Eurasain ruffe	Releaser	M	F	Sorensen et al. 2004
Ictalurus punctatus - Channel catfish	Mating	M	F	Timms and Kleerekoper 1972
Misgurnus anguillicaudates - Cobitide loach	Mating	M	F	Kitamura et al. 1994
Neogobius melanstomus - Round goby	Mating	F	M	Murphy et al. 2001; Zielinski et al. 2003
Oncorhynchus masou - Masu salmon	Mating	M	F	Yambe et al. 2006
Oncorhynchus mykiss - Rainbow trout	Releaser	M	F	Scott et al. 1994; Vermeirssen et al. 1997
Oreochromis mossambicus - Tilapia	Mating	M/F	M/F	Silverman 1978
Petromyzon marinus - Sea lamprey	Migration	M/F	Larvae	Sorensen et al. 2005b
Petromyzon marinus - Sea lamprey	Mating	F	M	Li et al. 2002
Pterophyllum scalare - Anglefish	Mating	F	M	Chien 1973
Salmo salar - Atlantic salmon	Releaser	M	M/F	Waring et al. 1996; Olsen et al. 2001
Salmo trutta - Brown trout	Releaser	M	F	Moore et al. 2002
Salvelinus alpinus - Artic char	Mating	F	M	Sveinsson and Hara 1995, 2000
Salvelinus fontinalis - Brook trout	Releaser	M	F	Essington and Sorensen 1996

of pheromones in fish (Yambe et al. 1999; Sorensen et al. 1998b), but the sea lamprey mating pheromone is released from the gills (Siefkes et al. 2005). An excellent survey of hormonal pheromones in fishes can be found in Stacey et al. 1996.

Bile acids have also been hypothesized to function as fish pheromones. Mixtures of novel bile acids have been shown to function as sea lamprey migratory and mating pheromones in two-choice mazes (Sorenson et al. 2005; Li et al. 2002). Larval sea lampreys residing in natal streams release a mixture of at least three bile acids; petromyzonamine disulfate, petromyzosterol disulfate, and petromyzonol sulfate. These bile acids have been hypothesized to function as migratory pheromone components to guide sexually immature adults to suitable streams (Sorenson et al. 2005). Furthermore, at the onset of spermiation, males release at least two bile acids, 3-keto petromyzonol sulfate (3kPZS) and 3-keto allocholic acid (3kACA), which likely function as mating pheromone components that lure females to spawning nests containing mature males (Yun et al. 2003; Li et al. 2002). In the sea lamprey migratory and mating pheromone systems, one compound has been shown to induce the majority of the behavioral activity (migratory: petromyzonamine disulfate; mating: 3-keto petromyzonol sulfate), but all components are likely needed to induce a full-suite of behavioral response. In teleost fishes, no specific bile acids have been characterized as pheromones, but Zhang et al. 2001 demonstrated that taurocholic acid is highly stimulatory to the olfactory organ of lake char (*Salvelinus namaycush*) and Vermeirssen and Scott 2001 showed that the bile of ovulating female rainbow trout has pheromonal activity.

Many fish species employ migratory and mating pheromones to complete critical aspects of their life history (Table 1-1). Improved understanding of these chemical cues may lead to new methods to enhance reproduction in valued fish species and decrease recruitment in invasive species.

How multiple components of a pheromone function together?

Nearly all characterized pheromone systems in fish and insects use mixtures of compounds to confer a message to the receiver. When developing pheromone-based management strategies, it is advantageous that all pheromone components be characterized so that the most efficacious technique is developed with the needed behavioral potency. For example, if an individual component of a pheromone elicits a specific behavioral or physiological response, such as attraction (Bradshaw et al. 1993), then only the component responsible for attraction may need to be developed for a trapping management application, thereby saving significant amounts of money during registration, synthesis, and application. However, if pheromone components function synergistically as one signal to elicit all behavioral or physiological responses (Linn et al. 1987), then all components need to be deployed for effective pheromone-based management.

In insects there are two competing hypotheses describing how pheromone mixtures elicit long distance attraction and short-range reproductive behaviors. The most commonly accepted hypothesis is the synergistic blend hypothesis, which views any multi-component pheromone as one synergistic chemical signal that induces all aspects of attraction, courtship, and mating (Baker et al. 1981; Baker and Carde 1979). Linn et al. 1987 provided evidence supporting the synergistic blend hypothesis when they demonstrated that male oriental fruit moths (*Grapholita molesta*) are attracted at greater distances to a three component blend of pheromones than to the major component alone. This makes sense physically because downwind transport of major and minor components occur together as filaments causing all components to be present in the same ratio at all distances downwind from the source (Baker 1986). An alternative hypothesis is that minor pheromone components are not important for long range attraction, but rather eliciting reproductive behaviors near the odor source. Bradshaw et al. 1983 provided evidence for this hypothesis when they demonstrated that separate components of the female pine beauty moth (*Panolis flammea*) mating pheromone elicited separate attraction, landing, and copulatory behaviors in males. It is possible that pheromone mixtures may induce behavioral responses as described in both hypotheses depending on the ecological context in which a species uses the pheromone.

Understanding how fish pheromone mixtures function is beneficial when developing effective management applications. At present few researchers have described how pheromone mixtures induce behavioral and physiological responses in fishes. However, one study showed that the male goldfish olfactory organ can discriminate among steroid components of the female pheromone and each component can elicit different degrees of aggression, nudging, and chasing in males (Poling et al. 2001). Similar questions are yet to be addressed in other fishes.

Species specificity.

The vast majority of insect pheromones are species specific and components are released in precise ratios. Many insect pheromone components are also stereo-specific, meaning that only the correct stereo-isomer elicits a positive behavioral response from the receiver (Stevens 1998). The stereochemistry of each component likely adds another layer of complexity to the signal to maintain species specificity. For example, in the Japanese beetle, the opposite enantiomer of the mating pheromone is used by the sympatric Osaka beetle (*Anomala osakana*) and acts as an antagonist that abolishes the attraction of the Japanese beetle to its own pheromone (Nikonov and Leal 2002; Ladd 1982). For most species, like the scarab beetle (*Aromala octiescostata*), the opposite stereoisomer of a chiral mating pheromone is not detected by the olfactory organ and therefore is not attractive or repulsive (Leal 1999). The specificity of insect pheromone systems make them ideal for management tactics aimed at the benign manipulation of a single species.

Fish pheromones may not be strictly species specific because fish employ steroidal products as pheromones, which tend to be highly conserved among related species (Fine et al. 2004; Sorensen et al. 2003; Olsen et al. 2000; Sorensen et al. 1998a). Indeed, current evidence suggests that fish pheromones are not as species specific as insect pheromones. Bile acids released as putative migratory pheromone components by sea lamprey larvae (PADS, PSDS, PZS) are also released by several different species of *Petromyzontidae* and may be a conserved pheromone in this genus (Fine et al. 2004). The sea lamprey migratory pheromone does not appear to benefit the sender (larvae) and, therefore, may not be under strong selective pressure to be species specific (Fine et al. 2004). Species specific fish pheromones may be expected in highly sympatric species whose reproductive behavior relies strongly on pheromonal cues (Sorensen et al. 1998a; Sorensen et al. 1998b). The specificity of fish pheromones needs further investigation to prevent possible deleterious effects to non-target species through pheromone-based management tactics.

Orientation to pheromone plumes in water and air.

Deployment of pheromone-based management tactics is dependent on understanding the mechanisms fish use to locate pheromone sources (chemorientation). An understanding of chemorientation informs researchers what strategies may be effective and in which habitats pheromone-based techniques will be most efficacious. To locate a pheromone source, fish must track and orient to a pheromone plume. The distribution of the pheromone plume is determined by the dynamics of the carrier medium. Pheromone plumes in flowing media are made up of unpredictable filaments of odor that are variable in concentration through time and space (Finelli et al. 1999; Murlis 1986). In no-flow conditions, diffusion generally results in predictable concentration gradients radiating out from the source. Few studies have investigated animal orientation to aquatic plumes in no-flow conditions; therefore this discussion will be limited to plumes in flowing water and moving air.

In a river or tidal estuary, odors released by fish are disseminated into the water and transported down-current. As the distance downstream of the source increases, odor filaments become more widely dispersed and intermittent (Zimmer-Faust et al. 1995). River or tidal current is remarkably less turbulent than atmospheric winds because the viscosity of water is much greater than air (Vogel 1994; Denny 1993) and water is confined by the width and depth of the river channel (Zimmer-Faust et al. 1995). For example, turbulent eddy diffusivities in estuarine tidal creeks are 100 to 1,000 times lower than those of atmospheric gases in grasslands and forests occupied by insects (Murlis et al. 2000; Zimmer-Faust et al. 1995). Reduced turbulence in rivers means that odors tend to spread vertically instead of laterally, resulting in vertically elongated, thin, and sharply delineated plumes (Zimmer et al. 1999; Zimmer-Faust et al. 1995) when compared to atmospheric plumes. Differences in pheromone plumes between water and air may allow fish to more efficiently track odorants, thereby enhancing pheromone-based management.

An understanding of insect pheromone plume dynamics and tracking behavior provides clues about how fish may locate pheromone sources. Insect pheromones are volatilized and immediately begin to diffuse out of the still air near the source and into the wind where turbulence generated by mechanical forces and buoyancy rip the plume into random filaments of odor separated by pockets of clean air (Murlis 1986). Changes in wind direction lead to unpredictable wandering of the plume over the landscape. Because of the highly intermittent nature of atmospheric plumes, flying insects encounter bursts of odor that are highly variable in concentration and frequency. This makes it unlikely that insects are capable of integrating olfactory input over time and space to obtain precise information about the location of the pheromone source (David 1986; Murlis 1986; Murlis and Jones 1981).

In general, insects have been shown to use a mechanism called optomotor anemotaxis to track highly unpredictable pheromone plumes (Baker 1986; Kennedy 1940). Optomotor anemotaxis is an innate response, in which pheromone stimulation induces flight, orientation is determined by the direction of the wind, and overland progression is gauged by visual cues (Baker 1986; Preiss and Kramer 1986; Kennedy

1940). For example, when a male encounters a filament of female pheromone, the first programmed response is upwind flight, which is determined by mechanoreceptors. Flight speed is held above wind speed by tracking the movement of the ground and surrounding features (Preiss and Kramer 1986). If the odor is lost, the male will begin a self-steered program of zigzags (casts) (Bell and Tobin 1982; Kennedy 1978). Casting functions to allow the male to more accurately determine wind direction and increases the probability of reencountering the plume (Baker 1986; Kennedy 1986). When a new filament is contacted, another surge of upwind movement will occur (Vickers 1999). This program of cast-surge-cast continues until the male is able to locate the female visually (Vickers 1999). It is plausible that fish living in highly turbulent hydrodynamic environments use similar orientation mechanisms.

No studies to date have directly addressed orientation of fish to pheromone plumes. Certainly, more research is needed to advance the use of pheromones in fisheries management. Studies have described chemorientation to food odors in several aquatic organisms such as seastar (*Asterias forbesi*) (Moore and Lepper 1997), blue crabs (*Callinectes sapidus*) (Zimmer-Faust et al. 1995; Weissburg and Zimmer-Faust 1993), crayfish (*Oreonectes rusticus*) (Keller et al. 2001; Moore and Grills 1999), and brown bullheads (*Ameiurus nebulosus*) (Sherman and Moore 2001). Studies on aquatic chemorientation to food odors may provide clues as to how fish orient to pheromones. The following discussion describes chemorientation of aquatic organisms to food odors.

In flowing water, aquatic organisms with two or more chemosenory organs, have been shown to orient to odor plumes using a combination of rheotaxis and tropotaxis. Rheotaxis directs upstream locomotion toward the source using the direction of water-flow, and tropotaxis directs lateral movements by comparing concentration differences between chemoreceptors on two points of the body. For example, when blue crabs encounter an odor plume, they immediately turn into the current and walk upstream (rheotaxis), but lateral movements across the stream are directly related to the presence or absence of the odor (tropotaxis) (Zimmer-Faust et al. 1995). Similarly, American lobsters (*Homarus americanus*) progress upstream toward the source of the plume using simultaneous comparisons of odor intensity (Weissburg 1997).

Aquatic organisms with a single chemosensory organ have been shown to track plumes using a combination of rheotaxis and kinesis; they move upstream in the presence of an attractant (rheotaxis) and move side-stream or downstream in the absence of the attractant (kinesis). A combination of rheotaxis and kinesis may be used because it may be impractical to orient to concentration gradients based on successive samples in different places from one olfactory sensor (klinotaxis). Female sea lampreys may employ rheotaxis and kinesis to locate male mating pheromones. Johnson et al. 2006 showed that when a mating attractant was applied to the stream, females moved directly upstream and rarely cast from side-to-side; but when the odor was turned off, females stopped upstream progression and exhibited significantly more downstream and side-stream casting movements, perhaps as a means to reencounter the plume. This simple mechanism of fish chemorientation may be practical because plumes in flowing water are more confided and less turbulent than atmospheric plumes.

Several studies have demonstrated that aquatic organisms track odor plumes most efficiently in the hydrodynamic conditions of their natural habitats. For example, Sherman and Moore 2001 reported that brown bullheads, which prefer habitats with no flow, located an odor source 100% of the time in no-flow conditions and only 57% of the time in flow conditions. In contrast, blue crabs, which prefer habitats with moving water, successfully located injured clams (*Mercenaria mercenaria*) in flowing water, but oriented randomly and searched unsuccessfully for injured clams in still water (Weissburg and Zimmer-Faust 1993). These studies demonstrate that orientation to pheromone sources may vary significantly among different hydrodynamic environments, and highlights the importance of understanding chemorientation before the application of pheromone-based management techniques in various habitats.

Uses of pheromones in fisheries management.

The use of pheromones in fisheries management has great promise to advance the restoration of desirable fish and the control of undesirable fish. For example, chemical cues may be used to guide migratory fish up fish ladders, undesirable species into traps, or aggregate fish on spawning grounds for removal or to promote reproduction. For fisheries biologists, much can be learned from the development and application of pheromones in integrated insect control programs where they are used to monitor populations, mass trap, and disrupt reproduction (Howse et al. 1998; Ridgway et al. 1990; Silverstein 1981; Marx 1973). Similarly, pheromones may be used to control nuisance fishes through population monitoring, mass trapping, and mating disruption. Alternatively, pheromones could aid fish restoration through improved monitoring, migration, and mating facilitation. Pheromone-based management techniques should not be considered a universal remedy and must occur within the context of integrated management programs (Silverstein 1981).

The sea lamprey, an exotic ectoparasite and predator of Great Lakes salmonid species (Smith and Tibbles 1980), is an emerging model of how fish pheromone systems may be utilized to manage deleterious species. The combined use of migratory and mating pheromone components in sea lamprey management, with the already established techniques of chemical control, trapping, and sterile male release, may greatly aid integrated sea lamprey management in the Great Lakes and become the first use of pheromones in vertebrate pest management. In the following discussion, the sea lamprey will be used to illustrate how migratory and mating pheromones could be applied to improve fisheries management. Relevant lessons learned during the development of pheromone-based insect control programs will be highlighted.

Monitoring. Pheromones may be used to monitor fish populations in two ways: 1) measuring pheromone concentrations in the water to determine the presence and relative abundance of a species or 2) using pheromone-baited traps to determine whether or not a species is present. Sea lamprey pheromones may be used to monitor populations of larvae by measuring the amount of migratory pheromone present in streams, while accounting for streamflow, temperature, and time of year (Fine and Sorensen 2005; Sorensen et al. 2005; Twohey et al. 2003). Pheromone-based monitoring, integrated with electofishing assessment techniques, may reduce cost and improve the larval sea lamprey abundance estimates needed to identify streams requiring chemical treatments.

Mating pheromones may also be used to monitor adult sea lamprey populations by measuring mating pheromones released into stream water by mature males (Twohey et al. 2003). A monitoring system similar to that described for larval sea lamprey could detect the presence and abundance of mature males by measuring pheromone concentrations downstream of spawning grounds. Pheromone-baited traps could be used to detect the presence or absence of mature females in Great Lakes' tributaries.

Pheromone-based monitoring techniques in insect control programs, when compared to traditional monitoring techniques, require minimal labor, are more sensitive, and are species specific (Wall 1990; Carde and Elkinton 1984). Airborne pheromone concentration has not been used to estimate insect abundance because wind direction and atmospheric turbulence cause pheromone concentrations to be highly variable. Pheromone-baited traps have been used to detect the presence of pest species, collect individuals for mark-recapture surveys, and assess populations to plan future pesticide treatments (Lopez et al. 1990). A noted disadvantage of monitoring with pheromones-baited trap is that it is difficult to establish a predictable relationship between trap-catch and population density (Wall 1990) because the area sampled by each trap and trap capture efficiency can vary greatly due to variation in temperature, wind, humidity, and background sources of pheromones emitted by conspecifics (McNeil 1991). Monitoring fishes in riverine systems with traps may prove more efficacious than in insects because the area sampled would be confined by the depth and width of the channel, and unidirectional flow. Despite the limitations, pheromone-baited traps are widely used to determine the distribution of pest insects, and they have been responsible for large reductions of insecticide use by indicating when and where treatments are necessary (Silverstein 1990; Marx 1973).

Mass-trapping. Pheromone-baited traps could be used to remove large numbers of fish for control or relocation. In the case of the sea lamprey, trapping with migratory pheromones (PADS, PSDS, PZS) and mating pheromones (3kPZS and 3kACA), when integrated with a sterile male release program, may provide a multifaceted strategy to reduce the reproductive potential of sea lamprey populations. Migratory pheromones may be used by control agents to increase the trap captures of male and female sea lampreys on their migration upstream (Sorensen et al. 2003). Removal of males and females would reduce the reproductive potential of sea lamprey populations prior to spawning and would provide more animals for sterile male release programs. Traps baited with mating pheromones may be used to mass-trap mature females just prior to spawning, further reducing the reproductive potential of sea lamprey populations and increasing the effectiveness of sterile male programs. Mass-trapping applications could be especially important in Great Lakes' tributaries where many streams are inhabited by sea lampreys, but densities of larvae are too low to warrant lampricide treatment (Li et al. 2003).

Insect mass-trapping programs have been most successful when male mating pheromones are used to remove females. Removal of females results in a proportional reduction in viable eggs. For example, the male boll weevil (*Anthonomus grandis*), a destructive pest of American cotton (*Gossypium hirsutum*), releases pheromones that are attractive to both males and females (Bradley et al. 1968; Cross and Mitchell 1966). In the 1980s and 1990s, the boll weevil pheromone was used by control agents to monitor and mass-trap female weevils in an integrated system of control, which lead to the eradication of the pest from six states of the southeast United States (Brazzel et al. 1996).

However, in most insect species female mating pheromones attract males. When only males are attracted to the pheromone, mass trapping operations are rarely successful at economically feasible costs. To significantly reduce reproduction by trapping males, approximately 90% need to be captured (Campion 1984). To achieve a 90% capture rate, trap densities must be very high, all components of the pheromone mixture must be characterized, and competition from natural sources of pheromone must be low (Lander 1990).

Mating disruption. The objective of mating disruption is to reduce reproduction by using high concentrations of point source pheromones to prevent pheromone communication (Silverstein 1981; Marx 1973). In sea lamprey management, synthesized mating pheromone components (3kPZS, 3kACA) might effectively disrupt mate finding and reproductive behaviors when applied at high concentrations to streams (Twohey et al. 2003). An inability to use pheromone communication to find a mate may reduce reproductive success, especially in the sea lamprey, which are believed to be blind at maturation, and semelparous spawners (Applegate 1950).

In insects the most successful uses of pheromones for control have been through mating disruption (Carde and Minks 1995). Supernormal pheromone concentrations are typically applied using slow release pheromone formulations which are spread throughout the treatment area. Disruption of pheromone communication in insects has been shown to function by 1) olfactory sensory adaptation/habituation, 2) following false-trials of pheromone, 3) camouflage of naturally occurring plumes, and 4) sensory imbalance by the release of partial pheromone mixtures or non-natural ratios (Wyatt 2003). Several disruption mechanisms may work in synchrony to reduce reproduction. Carde et al. 1998 described mating disruption in the pink bollworm (*Pectinophora gossypiella*) as a combination of false-trial following, camouflage of natural plumes, and habituation. When developing mating disruption programs, researchers should determine what concentration of pheromones are required, how many pheromone point sources are needed, and which biological mechanism(s) are responsible for mating disruption. Like mass trapping, mating disruption is most effective when population densities are low and all components of the pheromone are characterized (Carde and Minks 1995). Mating disruption may be more efficacious in aquatic systems because water is less turbulent and more confined that the atmosphere, making application more direct, easier, and cheaper.

Characteristics of successful pheromone control programs. In general, successful applications of pheromones in insect control have the following characteristics: 1) all pheromone components were identified, 2) the behaviors elicited by pheromone components were fully characterized, 3) the target organism occurred at low densities in the treatment area, and 4) pheromone-based techniques were integrated with other effective methods of control (Jones 1998; McNeil 1991; Silverstein 1990).

Using pheromones to restore valued fish species.

Fish pheromones may not only offer use in controlling pest species, but may be used to restore species in decline. For example, migratory and mating pheromones may be used by other lamprey species whose populations are threatened (Sorensen et al. 2005). The Pacific lamprey (*Lampetra tridentata*), a culturally valued fish species (Close et al. 2002), has been extirpated from much of its historical range in the Columbia River Basin due to dam construction. In this case, migratory pheromones could be used to direct lampreys to fish ladders or to tributaries with no dams. Mating pheromones could be used to direct mature Pacific lampreys to highly productive spawning riffles, and to synchronize spawning activity. Similarly, if pheromones are identified in Pacific Salmon species (*Onchorhynchus* spp.), they could be used to direct migration, maturation, and reproduction of this valued resource in the Columbia River Basin.

Pheromones may also be used in fish propagation to synchronize spawning of broodstock and increase milt quality and quantity. Many economically valuable salmonid species are cultured for fish restoration or for human consumption. Studies have shown that Atlantic salmon (*Salmo salar*) (Waring et al. 1996), brown trout (*Salmo trutta*) (Olsen et al. 2000), and rainbow trout (Vermeirssen et al. 1997) likely release priming pheromones that synchronize spawning and increase milt production in males. If identified, priming pheromones may be a cost effective, natural method of synchronizing spawning and improving milt production and quality without handling or transferring fish.

The possibilities of using pheromones to achieve management goals are as diverse as the management goals themselves. It is clear that pheromones influence major life history events in fishes and may become useful additions to the tool box of fisheries managers. More research must be focused on fish pheromones to advance the use of this potentially potent and environmentally benign method of managing fisheries.

Future research objectives.

Pheromones need to be identified and characterized in more fish species so that their full potential to aid fisheries management may be realized. A current limitation of using pheromones in fisheries management is that the chemical identities of most pheromones are unknown or have not been investigated. The first step in developing pheromone-based management techniques is to identify and synthesize all components of

the pheromone mixture (McNeil 1991). This will allow investigators to characterize how fish behaviorally and physiologically respond to pheromones. In the sea lamprey and goldfish, the function of pheromones have been described quickly once pheromone structures were identified (Sorensen et al. 2005; Yun et al. 2003; Li et al. 2002; Poling et al. 2001), thus shedding light on how pheromone-induced behaviors can be manipulated to achieve management objectives (Johnson et al. 2006; Wagner et al. 2006; Johnson et al. 2005; Siefkes et al. 2005). Furthermore, the specificity of fish pheromone systems needs additional investigation to prevent deleterious effects to non-target fishes.

Research should focus on characterizing each component of the pheromone mixture and describing orientation strategies through an understanding of plume dynamics. The most common reason that pheromone-based management has failed in insects is that there was inadequate knowledge of the insect's chemical communication system and behavior (McNeil 1991; Ridgway et al. 1990). Efficient traps and effective mating disruption programs can only be designed after pheromone-induced behaviors are characterized and understood. For example, it is important to understand how fish track pheromone plumes in different hydrodynamic conditions, because it will directly impact how traps and slow-release pheromone formulations are designed, and in what habitats pheromone-based techniques are efficacious. Furthermore, it is not known whether fish locate pheromone sources by continuously monitoring pheromone concentrations using klinotaxis, tropotaxis, or if they use a presence/absence rheotaxical kinesis response by simply swimming upstream in the presence of the odorant. The last step, before full-scale management application, is to conduct comprehensive field tests to determine whether the pheromone can achieve the desired objectives under varying population densities and environmental conditions.

Overview of dissertation.

The goal of my dissertation is to further characterize the male sea lamprey mating pheromone to permit its deployment in sea lamprey management. In this review and dissertation, I broadly discussed pheromones as chemical signals that modify animal behavior. I also commonly experimented on and discussed specific compounds that putatively comprise the behaviorally active components of the pheromone mixture. In the next chapter, I develop my understanding about how to conduct animal behavior research in the field by critically reviewing aquatic chemical ecology experiments conducted in nature. Surprisingly few studies have been conducted in field environments. When field studies were conducted, results obtained in natural environments were often different from, and even contradictory to what was reported in laboratory constructs. Therefore, it is critical that sea lamprey pheromone research with a management goal be conducted in natural streams. Starting in Chapter 3, I work in a natural stream to test the efficacy of synthesized 3kPZS as a control agent to lure females into traps, draw females away from a natural mating pheromone source, and disrupt female orientation to a natural mating pheromone source. At the same time behavioral responses of females to synthesized 3kPZS were described over a 1000 fold range of concentrations and in different stream environments. Detailed in-stream tracking of female movement and 3kPZS plume distribution allowed movement patterns of females to be described and characterized.

In Chapter 4 the research took an unexpected turn when it was discovered that 1) 3kPZS, the putative mating pheromone component, was present at ecologically relevant quantities in extracted migratory pheromone and 2) the putative migratory pheromone components identified in lab constructs did not elicit behaviors in natural streams. Therefore, a field study was conducted to test the hypothesis that 3kPZS also functions as a migratory pheromone component to guide immature females up streams. Pre-ovulatory female movements in a spawning stream were monitored at night over 250 m when exposed to synthesized 3kPZS and synthesized migratory compounds (PADS, PSDS, PZS). Results showed that 3kPZS induced preference responses in pre-ovulatory females and suggest that 3kPZS may be used to manipulate the migratory behavior of immature females.

Direct comparisons of the natural pheromone released by mature males and synthesized 3kPZS in Chapter 3 revealed that mating pheromone components in addition to 3kPZS retained females on spawning nests, whereas 3kPZS alone failed to do so. For a successful pheromone control program, the chemical identity and behavioral responses of animals to the pheromone must be well characterized. The goal of Chapter 5, therefore, was to initiate efforts to identify and characterize all ecologically relevant mating pheromone components. First, ovulated female responses to 3kACA, a putative mating pheromone component, were tested. Then, a new in-stream bioassay was developed to determine whether traditionally used pheromone extraction procedures captured and eluted all behaviorally active pheromone components from spermiated male washings. Future research testing extracts in the field bioassay will enable all male mating pheromone components to be identified and characterized, thereby revealing the full use of mating pheromone in sea lamprey management.

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CHAPTER 2

UNDERSTANDING BEHAVIORAL CHEMICAL ECOLOGY OF MOTILE AQUATIC ORGANISMS IN NATURAL OR QUASI-NATURAL ENVIRONMENTS

ABSTRACT

A review of research in chemical communication of motile aquatic organisms was conducted and revealed an abundance of studies that described chemically mediated behaviors in laboratory microcosms, but research in natural field conditions had received considerably less attention. Research in laboratory settings can be highly productive and allow for controlled experiments; however, laboratory tanks and flumes often lack the physical, physiological, and social contexts associated with natural environments. Field experiments can be a critical step in confirming discoveries made in the laboratory and often implicate the ecological significance of chemical signals. When laboratory experiments have been repeated in the field, different and sometimes contradictory conclusions are drawn. Examples include studies of sea lamprey pheromones and alarm odorants in fishes. This chapter reviews field research conducted on crustaceans and fishes concerning aquatic pheromones, alarm signals, and chemorientation to food odorants, highlighting the following topics: 1) contradictory results obtained in lab and field experiments, 2) how environmental and physiological context influences behavior, 3) the challenges and constraints of field research, and 4) recent field studies in chemical communication and the techniques that made them successful.

INTRODUCTION

Sensory modalities are the critical first steps by which organisms determine the nature of their environment and behave accordingly. For humans, it is in our nature to perceive how visual, auditory, gustatory, and tactile cues influence animal behavior because those senses are the ones with which we are most intimate. But given our relatively poorly developed sense of olfaction (Kleerekoper, 1969), it often escapes our imagination that chemical communication is the most potent and widely employed mechanism of information transfer in nature (Fisher et al., 2006). Among fishes, the most highly developed sensory modality is olfaction (Kleerekoper, 1969), where chemical signals influence behaviors concerning habitat selection (Mathis & Smith, 1992), predator avoidance (vonFrisch, 1938), migration (Sorensen et al., 2005), maturation (Scott et al., 1994; Dulka et al., 1987), mating (Li et al., 2002) and paternal care (Neff, 2003). Crustaceans also have exquisitely sensitive chemosensory systems which aid to synchronize egg hatching (Pettis et al., 1993), locate food (Moore & Grills, 1999), locate suitable habitat (Diaz et al., 2003), determine social hierarchies (Moore & Bergman, 2005; Breithaupt & Eger, 2002) and facilitate reproduction (Stebbing et al., 2003; Hassler & Brockmann, 2001). The potency of chemical signals to direct behaviors of aquatic organisms can be astounding, for example, where synthesized pumping pheromones of the mud crab (*Rhithropanopeus harrisii*) elicit responses at sub-picomolar concentrations (Pettis et al., 1993), and pheromones of the sea lamprey are effective at 10^{-14} molar (M) (Sorensen et al., 2005).

Aquatic environments demand dependence on chemical signals because high turbidity, low light availability, and complex habitats obstruct reliance on visual cues. Furthermore, aquatic environments are especially amenable to efficient transmission of chemical signals and odor plume navigation because turbulence and eddy diffusivities typically are 100 to 1,000 times less than in terrestrial environments (Zimmerfaust et al., 1995). Studies of aquatic chemical communication offer multitudes of unique opportunities to understand how animal behavior and physiology are mediated by chemical signals.

The vast majority of our understanding of chemical communication in aquatic organisms has been gleaned from experiments conducted in laboratory environments. Laboratory studies can quickly and inexpensively determine whether chemical cues may be of behavioral or physiological significance. This was demonstrated in 1969 when Losey published one of the first influential laboratory studies concerning fish pheromones. In small laboratory aquaria (45 L), Losey showed that non-nest guarding males of the *Hysoblemmius* family were attracted to odors from males that were actively defending nests. Since that time, approximately 90% of all studies conducted on chemical communication in motile aquatic organisms have been laboratory exercises. This is especially apparent when it is considered that the domesticated goldfish (*Carassius auratus*) is the most extensively studied model system of pheromonal communication in fishes. Because of the inherently artificial nature of laboratory settings, it is also highly relevant to understand how chemical cues influence animal behavior in natural environments. The ecological relevance of chemically-mediated behaviors can only be inferred when experiments are conducted in natural environmental contexts, and animals are in a natural physiological state. Experiments in nature are best suited to determine whether odorants can be exploited as agents of human management to restore or control populations (Corkum & Belanger, 2007).

Laboratory (lab) experiments fall in the middle of what I perceive to be a field-to-lab-to-field model. To explain further, many questions in chemical ecology originate from field observations. Given behaviors observed in the field, scientific inquiry is moved into the lab where experiments are comparable, reproducible, relatively inexpensive, easy to set-up, and can often be conducted year-round. Lab studies are particularly useful to determine whether an organism uses chemical communication, as well as which individuals release and respond to the odorant (see numerous publications by G. Brown and M. Wisenden). Labs are also amenable to experiments aimed at identifying the physiological and neurological mechanisms behind pheromone-induced behaviors. Researchers who study animal models in the lab, however, infrequently return to the field to replicate results in a context where chemical signals have ecological significance. This issue may be prevalent because field environments challenge the design and execution of experiments, and they can be physically taxing to work in. In this chapter, a subset of field studies in aquatic chemical ecology will be reviewed that highlight experimental approaches that have proven successful in natural environments.

Interestingly, in the cases in which lab experiments have been replicated in the field, results have often been different and even contradictory to those obtained in the lab. There are several reasons to suspect that behavioral responses of animals in the lab may be different than those in nature. As a consequence of insufficient space and water, lab environments tend to be overly simplistic: lacking natural contexts of flow, water chemistry, physical structure, food, and social interactions. For instance, flumes and Y-mazes artificially slow and straighten flow (Zimmer et al., 1999), and small aquaria create unnatural contact with chemical stimuli (Pearson, 1977; Mackie, 1972).

This chapter will further illustrate the need for field verification of lab results by discussing examples of field research that yielded results contradictory to lab research. The goal of comparing studies is not to take sides and claim that one study was better than the other, but instead to highlight that the results are indeed different. Furthermore, possible explanations will be provided as to why lab and field results may differ in light of how chemically-mediated behaviors are dependent on environmental, physiological, and social contexts. Finally, constraints of field research will be described and examples will be provided as to how obstacles have been overcome. Successful and informative field studies will be reviewed and the new techniques and technologies employed in them will be discussed.

CONTRADICTORY RESULTS OBTAINED IN LABORATORY AND FIELD STUDIES

Contradictions between lab and field results have occurred under two circumstances: either behaviors elicited in the lab do not represent the full suite of chemically-mediated behaviors induced in the field, or robust behaviors elicited in the lab were not observed in the field. Instead of reviewing many such examples, studies of sea lamprey pheromones and Ostariophysan alarm odorants will be used to illustrate the two circumstances under which lab and field results may differ. A critical idea to highlight using these two examples is that the field-to-lab-to-field model was very useful in understanding the ecological significance of lab research. In the two examples below, original hypotheses were developed from field observations, laboratory experiments were designed to test hypotheses and characterize behavioral responses, and then field experiments aimed to replicate lab results revealed the ecological significance of lab discoveries.

Sea lamprey pheromone-induced behaviors are more diverse in natural environments.

Chemical communication in the sea lamprey (*Petromyzon marinus*) has been studied for the past 25 years and provides many examples to illustrate the use of field research in chemical ecology. Before continuing, a brief description of sea lamprey life history is needed. The sea lamprey, native to the Atlantic Ocean, invaded the upper Laurentian Great Lakes in the 1930s and 1940s and became a vicious predator of lake trout (*Salvelinus namaycush*) and lake whitefish (*Coregonus clupeaformis*) causing the collapse of these and other ecologically valuable fish populations (Smith & Tibbles, 1980). Sea lamprey larvae are born in streams where they feed on algae and detritus. When approximately 150 mm in length (age 3 to 7 years), larvae metamorphose into parasitic adults and migrate downstream into the ocean or Great Lakes where they parasitically feed on bodily fluids of large fish. In the spring, after spending a year and a half as parasites, they detach from their hosts and migrate up freshwater streams, spawn, and die (Applegate, 1950). Sea lamprey chemical ecology is of great interest to researchers because successful reproduction is dependent on chemical cues. In the Great Lakes, manipulation of chemical cues may provide an environmentally benign control technique for this destructive invader (Li et al., 2003).

The first indication that sea lampreys use odorants to coordinate mate finding and reproduction came from field reports of French fishermen who captured high numbers of "ripe" females (ovulated) in traps baited with "roped" males (spermiated) (Fontaine, 1938). To confirm these and other reports of female attraction to sexually mature males (Adams et al., 1987a; Adams et al., 1987b; Teeter, 1980), a two-choice maze was constructed on the bank of a natural spawning stream and supplied with stream water (Figure 1-2). Maze experiments confirmed ovulated females did indeed show preference and search responses to the washings of spermiated males (Li et al., 2002; Siefkes et al., 2005). Furthermore, a putative pheromone component, 3-keto-petromyzonol sulfate (3kPZS), was identified in the washings of spermiated males. 3kPZS and spermiated male washings induced statistically similar preference behaviors in ovulated females in the two-choice maze. Given these results, it was concluded that 3kPZS was the male sea lamprey mating pheromone that induced preference and search responses in ovulated

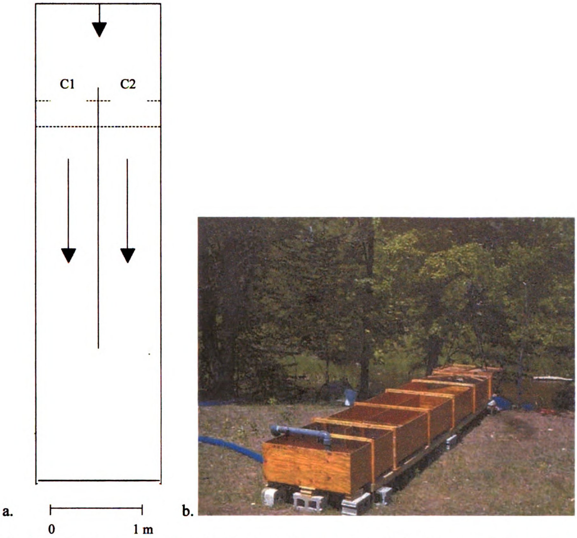


Figure 1-2. Sea lamprey two-choice maze constructed on the bank a natural spawning stream. Using the maze it was determined that spermiated males release a pheromone, 3kPZS, which is highly attractive to ovulated females. However, it was unable to determine that additional pheromone components induce near-source spawning behaviors in ovulated females likely because the maze was too small and lacked natural substrate and flow. **a.** Water is pumped into the maze at the upper arrow and flows down the page where the maze is split into two chambers. A test odorant is added to the maze at C1 or C2 and the time a sea lamprey spends in each side of the maze before and after odorant application is recorded. The functional length and width of the maze is 4.27 by 1.22 m. Reprinted from *Animal Behaviour*, Vol. 70, Siefkes,M.J., Winterstein, S.R., Li, W., Evidence that 3-keto petromyzonol sulphate specifically attracts ovulating female sea lamprey, *Petromyzon marinus*, 1037-1045., 2005, with permission from Elsevier. **b.** Photograph of the maze presented in color.

females (Li et al., 2002).

To confirm stream-side maze results, experiments were replicated in a natural stream, where an island divides the river into two channels (Figure 2-2). When working in the natural two-choice maze it was discovered that, contrary to lab results, 3kPZS did not elicit the same preference and search response in ovulated females as spermiated male washings (Siefkes et al., 2005). When male washings were applied to the stream, ovulated females were lured from 70 m downstream to the exact point of odorant release and remained there for up to an hour. But females were only lured to within 1 m of a 3kPZS source of similar concentration, where they briefly paused and then continued moving upstream (Siefkes et al., 2005). Results from in-stream experiments were inconsistent with the conclusion derived from maze experiments, and demonstrated that pheromone components other than 3kPZS are needed to elicit near source attraction and retention in ovulated females.

Even more intriguing are the contradictory published results concerning the release mechanism(s) of the male sea lamprey mating pheromone. Teeter 1980 and Adams 1987 present laboratory maze data showing that behaviorally active components are released through the urogenital fluid. However, similar maze data collected 15 years later by Siefkes et al. 2003 showed that females strongly prefer anterior washings from males and that females are not attracted to posterior washings. These authors also showed that 3kPZS is present in anterior washings, and proposed a mechanism by which gill granular cells facilitate 3kPZS release. Is it possible that all three laboratory studies are correct? If laboratory mazes are too small to allow lampreys to display a full repertoire of pheromone-induced behaviors, 3kPZS could be released via the gills where

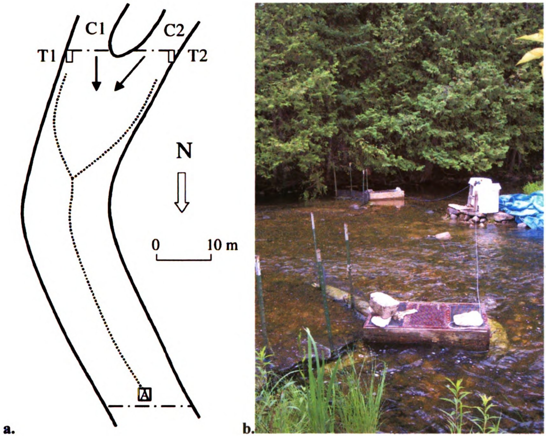


Figure 2-2. Section of the Ocqueoc River MI, USA, used for field tests of sea lamprey mating pheromones. Sea lamprey historically spawned in this section of stream but several km downstream a lamprey barrier was constructed to prevent lampreys from moving upstream. **a.** The experimental site is divided into two channels (C1 and C2) by an island. A pheromone is applied to one channel and control odorant to the other channel to create a natural two choice maze (T1 and T2). Ovulated females are released 70 meters downstream from an acclimation cage (A) and tracked upstream. **b.** Stream photograph of the design in **a**. Figure 2-2b is presented in color. The capture rate of ovulated females in traps (T1 and T2) baited with male pheromones or control solvent was determined.

it functions to induce upstream rheotaxis, while other unidentified pheromone components, released in the urogenital fluid, induce near source attraction and retention. Field experiments were conducted in Chapter 5 to test this hypothesis.

In addition to the mating pheromone, sea lamprey have also been hypothesized to use bile acids released by conspecific larvae as cues indicating suitable spawning streams (Bjerselius et al., 2000). Evidence that a migratory pheromone is used as an indicator of suitable spawning streams originated from field observations that numbers of migratory adults entering streams treated the previous year with the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) drop drastically (TFM kills about 95% of larvae) (Moore & Schleen, 1980). To confirm this field observation, Teeter 1980 showed in a maze that migratory sea lampreys were attracted to larval lamprey washings. In 1995, Li et al. identified petromyzonol sulfate (PZS) and found it to be highly stimulatory to the olfactory organ of sea lampreys as measured by electro-olfactogram (EOG). During 2000, Bjerseluis et al. tested PZS as well as allocholic acid (ACA), another putative migratory pheromone, in a two-choice maze and concluded that PZS and ACA were important components of the migratory pheromone. A year later, however, Vrieze and Sorensen concluded from maze studies that little of the activity found in the migratory pheromone can be explained by PZS and ACA. Instead, Vrieze and Sorensen reported that migrating sea lampreys strongly prefer blank stream water (without larval pheromones) over Lake Huron water. Unfortunately, all experiments prior were conducted in Lake Huron water or well water. Most recently it has been reported that two newly identified bile acids, petromyzonamine disulfate (PADS) and petromyzosterol disulfate (PSDS), along with PZS, constitute the sea lamprey migratory pheromone (Sorensen et al., 2005). However, none of the putative migratory pheromone components have been shown to elicit behavioral responses in an experiment conducted in nature (Chapter 4, Wagner et al. unpublished data). Additional contradictory results between two-choice maze experiments and field experiments will be revealed in Chapter 4.

Alarm odorants elicit more robust responses in the lab than in the field.

Results generated from lab and field studies of Ostariophysan alarm odorants (*Schreckstoff*) have been among the most controversial in chemical ecology research. The term *Schreckstoff* was coined by von Frisch in 1938 who, using field observations, described an "alarm pheromone" released by injured European minnows (*Phoxius phoxinus*) that elicited fright responses in conspecifics (von Frisch, 1941; von Frisch, 1938). Since von Frisch, over 100 published aquaria studies concerning *Schreckstoff* collectively show that Ostariophysan fishes contain epidermal club cells which, when ruptured (Pfieffer, 1974), release an odorant, that induces alarm responses in conspecifics. In certain contexts heterospecifics also show an alarm response (Brown & Godin, 1997; Brown et al., 1995).

The function of *Schreckstoff* was not largely debated until Magurran et al. 1996 published field data and commentary contending that *Schreckstoff* not be termed an alarm "pheromone" (Magurran et al., 1996). Magurran et al. 1996 used an underwater camera to observe the behavior of European minnows before and after exposure to conspecific skin extract (Schreckstoff) and muscle extract (control). When exposed to Schreckstoff, minnows failed to elicit stereotypical alarm responses such as dashing, hiding, freezing, or leaving the observation area. The only thing alarming in the paper was that their field results were contradictory to what was observed in the lab (Smith, 1997). After Magurran et al. 1996, alarm odorant researchers were more likely to conduct field experiments or include a quasi-field component with laboratory studies to account for possible discrepancies in alarm responses in the lab and field (Friesen & Chivers, 2006; Golub et al., 2005; Pollock et al., 2005; Wisenden et al., 2004a; Wisenden et al., 2004b; Mirza & Chivers, 2001; Brown et al., 2000).

In their commentary, Magurran et al. 1996 state that, because the alarm response of European minnows to Schreckstoff was contingent on "other factors", that it was not in fact a "pheromone". The authors highlight that aquaria fishes are confined and satiated and therefore reported alarm responses to Schreckstoff may be artifacts of the physical (confinement) and physiological (satiated) context. I agree, and from personal experiences with confined two-choice mazes, I understand how erroneous data may arise due to experimentation in unnatural contexts. However, I do not agree that, because an odorant does not elicit a response in all contexts, it is not a pheromone. In fact, when reviewing the literature I found no pheromone that elicited a behavioral response in all individuals in all environments. It appears that behavioral responses of fish are modified more by context than by a specific odor. The natural circumstances surrounding chemically-mediated behaviors should be of great interest to chemical ecologists, for this indicates how and when animals use chemical cues to communicate in their environment.

Given my experiences and those of others (Sorensen et al., 2005; Magurran et al., 1996), I believe that contradictory results obtained from lab and field studies have their origin in the physical, physiological, and social contexts of experimental systems. In the next section, studies will be reviewed that demonstrate how context influences behavioral responses to chemical stimuli. The aim is to review important contexts to consider when designing aquatic chemical ecology experiments in the lab and field.

PHYSICAL, PHYSIOLOGICAL, AND SOCIAL CONTEXT INFLUENCES CHEMICALLY-MEDIATED BEHAVIORS

The published literature demonstrates that three interrelated contexts influences animal responses to chemical cues in nature. The physiological state of an animal is influenced by its physical environment, defined by chemical, biological, and structural characteristics. Ultimately, the physiological context of an animal determines whether a behavioral response is elicited, and the degree to which a response is shown. Experiments conducted in natural conditions are expected to yield ecologically relevant results because animals are in natural physical and social environments and thus a natural physiological context. Experiments conducted under laboratory conditions may not yield ecologically relevant results because the physical environment may be inadequate, resulting in experimental animals that are in unnatural physiological states. When conducting lab and field research, experimenters should consider the physical, social, and physiological contexts to ensure results are ecologically informative.

Physical context.

Where pheromones are used in natural environments to control insect pests (Howse et al., 1998), it is no surprise that physical contexts such as temperature, wind speed, photoperiod, and time of day strongly influence the release rate and responsiveness of individuals to pheromones (Shorey, 1973). Similarly, it is becoming evident that responsiveness of aquatic animals to chemical cues can be influenced by unnatural contexts of light (Bjerselius et al., 2000), sound (Moore & Waring, 1999), temperature (Jaensson et al., 2007; Brown, 2002), water chemistry (Lurling & Scheffer, 2007; Diaz et al., 2003; Vrieze & Sorensen, 2001), hydrodynamics (Keller & Weissburg, 2004; Weissburg & Zimmer-Faust, 1994), and habitat complexity (Golub et al., 2005). Proper physical contexts not only represent a prerequisite for successful chemical communication, but they are also necessary for the completion of most life history events such as migration, which may be influenced by temperature, stream discharge, photoperiod, tidal cycle, salinity, and oxygen levels (Smith, 1985; McKeown, 1984; Northcote, 1984). Here studies are reviewed that highlight how physical contexts mediate responsiveness to chemical cues in aquatic environments.

Complexity of the aquatic environment. Water temperature is a commonly understood component of an organism's environment and is typically accounted for in experimental design. But chemical ecologists also need to consider how physical cover and complexity (or lack of it) can influence chemically-mediated behaviors. For example, in a field observation experiment using snorkeling techniques, Golub et al. 2004 documented variation among pumpkinseed fish (*Lepomis gibbosus*) responses to alarm odorants in habitats of different complexities. In weedless environments, pumpkinseeds showed less robust responses to alarm odorants than those exposed to alarm odorants in weedy environments. It is likely that pumpkinseeds do not show alarm responses in weedless environments because they are better able to use visual cues. Substrate can also strongly influence behavioral responses to pheromones, especially if chemically-mediated behaviors, such as reproduction, are depended on a specific substrate type. For example, the sound of redd cutting in riffle habitats triggers male Atlantic salmon parr (*Salmo salar*) to up-regulate their reproductive systems in lab environments (Moore &

Waring, 1999). In the two-choice maze used for sea lamprey research, substrate type was one of the flaws in the design (Figure 1-2; Li et al. 2002) because it did not contain the rocky substrate needed for spawning (Li et al., 2002; Applegate, 1950).

Water source and chemistry. Another commonly overlooked context in aquatic systems is the water itself, where clarity, chemistry, flow, and source can influence animal responsiveness to chemical cues. Aquatic organisms often prefer water originating from specific habitat types. Juvenile blue crabs can distinguish between estuarine water and water collected from the open ocean and respond differently to chemical cues presented in different water types (Diaz et al., 2003). Migratory sea lampreys searching for spawning streams show a strong preference for stream water (without pheromones) over water from the Great Lakes, and responses to migratory pheromones are synergized when tested in stream water (Vrieze & Sorensen, 2001). Water clarity has also been shown to affect fish responses to alarm cues, where more robust responses occur in turbid water than in clear water, presumably because turbidity obstructs visual cues (Hartman, 2000).

Water pH has been shown to influence responses of fish to chemical cues. In several studies, pheromones are more stable and have higher detection thresholds in water with slightly basic pH (Leduc, 2004; Bhatt et al., 2002; Brown, 2002; Brown et al., 2001a; Brown et al., 2000). This is the case for male three-spined stickleback (*Gasterosteus aculeatus*) pheromones, which are more strongly preferred by females when presented in water that is slightly basic rather than slightly acidic (Heuschele & Candolin, 2007). Fathead minnow (*Pimephales promelas*) responses to alarm odorants in laboratory conditions are abolished at a pH less than 6.0 (Brown, 2002; Brown et al., 2000). The mechanism by which an increase in pH enhances behavioral responsiveness is still unresolved, but one possibility is that basic water shifts the ionic phase of the chemical cue, making its new three-dimensional structure more assessable to olfactory receptors (Heuschele & Candolin, 2007). This mechanism has been implicated in insects where pheromone-binding proteins of the olfactory organ increase the pH of pheromone components, thereby facilitating binding to receptors (Zubkov, 2005). Alternatively, acidic water may shift the chemical structure of odorants through protination, rendering them unavailable to receptor sites as hypothesized for putative nitrogen oxide alarm odorants (Leduc, 2004; Brown et al., 2000). Water is an important environmental context, and when possible experiments should be conducted with water collected from natural habitats.

Researchers should be alert, however, because even in natural waterways chemical communication can be abolished by industrial pollutants and eutrophication. Humic acid, pesticides, and heavy metals have been shown to reduce fish responsiveness to odorants. For example, in eutrophic lakes and rivers, levels of humic acid can be as high as 200 mg/L (Steinberg, 2003) and in goldfish (*Carassius auratus*) concentrations of humic acid between 1 mg/L to 1000 mg/L have been shown to reduce the availability of steroidal mating pheromones in the olfactory epithelium (Hubbard et al., 2002). In the case of the goldfish, steroidal pheromones are primarily hydrophobic and may become absorbed into the core of humic acid hydrophobic microvesicles (Mesquita et al., 2003; Hubbard et al., 2002). High concentrations of humic acid are the hypothesized mechanism for the recent hybridization of two swordtail fish species *(Xiphophorus* *birchmanni and Xiphophorus malinche)* in eutrophic streams of northern Mexico (Fisher et al., 2006).

Other olfactory disruptants that have been measured at environmentally relevant concentrations in natural waters include heavy metals and pesticides. Copper and cadmium at 10 ug/L and 2 ug/L, respectively, reduce alarm odorant responses in Colorado pikeminnows (*Ptychocheilus lucius*) (Beyers and Farmer 2001) and fathead minnows (Carreau & Pyle, 2005) in lab environments. Cadmium also reduces alarm odorant responses in rainbow trout (Scott, 2003). Herbicides, atrazine and simazin, disrupt male Atlantic salmon response to female priming pheromones individually (atrazine and simazin at 1.0 ug/L) or in a synergistic fashion when mixed together (Moore & Lower, 2001; Moore & Waring, 1998). The insecticide diazinion disrupts antipredator behaviors and homing in chinook salmon (*Oncorhynchus tshawytscha*) at 10.0 ug/L (Scholz et al., 2000) and pyrethroid insecticide cypermethrin disrupts the pheromone-mediated endocrine system in male Atlantic salmon (Moore & Waring, 2001) and brown trout (*Salmo trutta*) at 0.004 ug/L (Jaensson et al., 2007). Researchers may also unintentionally reduce olfactory sense in experimental fish by using an anesthetic such as quinaldine, phenoxyethanol, or 3-aminobenzoic acid ethyl ester (MS-222) (Losey & Hugie, 1994). In most cases, mechanisms of chemical communication disruption are unresolved, but researchers should beware that pollutants in natural waters may influence responses to chemical cues. For more information on olfactory disruptants, see the recent review by Lurling and Scheffer 2007.

Hydrodynamics. In addition to water source and chemistry, the movement of water in the experimental system can affect animal responses to chemical cues. Whether water is lentic or lotic can directly influence the ability of bullheads (*Ameiurus nebulosus*) to locate fish gelatin (a food odorant). In still water, 100% of bullheads located gelatin, whereas only 56% were successful in moving water (Sherman & Moore, 2001). In the sea lamprey, responsiveness to migratory pheromones has been showed to be dependent on water velocity (Bjerselius et al., 2000), where flow direction may be used to orient towards sources of migratory and mating pheromones via chemically-mediated rheotaxis (Bjerselius et al., 2000). Similarly, blue crabs and crayfish (*Orconectes virilis*) use water flow to orient to food odorants using rheotaxis (Weissburg & Zimmer-Faust, 1994; Hazlett et al., 2006).

If a species responds to odorants in moving water, minor changes in the hydrodynamics (movement of water over a substrate) of the experimental system can alter behavioral responses. The hydrodynamic environment determines the spatial and temporal distribution of an odorant and ultimately determines how an organism encounters an odorant plume. Several studies have evaluated orientation responses of crustaceans to food odorants in different hydrodynamic environments. Weissburg and Zimmer-Fuast 1994, showed that blue crabs orient to food odors more efficiently in smooth-turbulent flows than in high-turbulent flows, where crabs moved more slowly and made more frequent stops. However, just the opposite result was reported in crayfish (*Onconectes rusticus*), where orientation to food odors is more efficient on cobble substrate (more turbulence) than on sand (less turbulence) (Moore & Grills, 1999). How an odorant is applied to the hydrodynamic environment also influences crustacean orientation efficiency. Blue crabs orient most efficiently to odors applied at high fluxes (concentration of odorant applied per unit time) and low pulse rates (2.5 sec verse 4.0

sec) (Keller & Weissburg, 2004). Little research has been conducted to determine how hydrodynamics influence behavioral responses of fish to odorants, but changes in flow and substrate composition likely influence fish in similar ways as they do crustaceans. Because behavioral responses of animals change according to the hydrodynamic context, the hydrodynamics of experimental systems should be controlled and described so that results may be comparable among studies (read last section of this chapter for more information) (Moore & Grills, 1999).

Physiological context.

Often directly influenced by the physical environment, the physiological context of an animal ultimately determines whether a behavioral response is elicited by a chemical cue. Responsiveness to chemical cues can be influenced by the collinear effects of age, size, sex, maturity (Li et al., 2002; Dulka et al., 1987), hormone concentrations (Bhatt et al., 2002; Yambe & Yamazaki, 2001; Carolsfeld et al., 1997a; Cardwell et al., 1995), stress (Carolsfeld et al., 1997a; Scott et al., 1994), and hunger (Chivers, 2000; Brown, 1996; Smith, 1981). The ecological relevance of an odorant is best understood when experimental animals are tested in an environment in which physiological processes are natural. Furthermore, results of chemical ecology research can be better interpreted if the physiological context of animal subjects are described and controlled for. Here, studies are briefly reviewed to highlight how physiological context influences responsiveness to chemical cues.

Size and hunger influence responsiveness to alarm odorants. Responses to alarm odorants have been shown to be dependent on the size of the fish, and some species undergo an ontogenetic shift such that alarm odorant responses are only elicited in young animals (Golub et al., 2005; Harvey & Brown, 2004; Golub & Brown, 2003; Mirza & Chivers, 2002). For example, small largemouth bass (<50 mm) (*Micropterus salmoides*) respond with an antipredator response to heterospecific alarm odorants, but large bass (>50 mm) respond with feeding behaviors (Brown et al., 2002; Brown et al., 2001b). A similar ontogenetic shift has been described in signal crayfish (*Pacifastacus leniusculus*), where only juveniles show an alarm response to predatory eel odorants (Stebbing, 2004). Studies of blue crab show that stage I and stage IV-V instars respond with opposite behaviors to the same visual and chemical cues (Diaz et al., 2001). It makes sense that small fishes would be most alerted by alarm odorants because they are at greatest risk of attack.

Hunger also mediates the degree of antipredator response when fish are exposed to alarm odorants. The first study to evaluate the effect of hunger on responsiveness to alarm cues was conducted by Smith in 1981 when he found that Iowa darters (*Etheostoma exile*), after fasting for 12 h, responded to a mixture of food and alarm odorants with a feeding response, but darters responded to the same odorant with an alarm response when satiated. Similarly, alarm responses in fathead minnows and reticulate sculpins (*Cottus perplexus*) are abolished after 24 h of fasting (Brown, 1996). Hunger of experimental fish may explain contradictory results obtained in lab and field studies of alarm odorants (Magurran et al. 1996), where in the lab fish are often fed to satiation, but in the wild food resources may be limiting.

Age, sex, and maturity. Studies of the sea lamprey clearly illustrate how chemically-mediated behaviors are dependent on the age, sex, and maturity of experimental animals. During sea lamprey migration, both males and females are extremely sensitive and attracted to migratory pheromones released by larvae (Sorensen & Vrieze, 2003), but sea lampreys become less responsive to migratory pheromones when the migration is complete and they begin to mature sexually. When fully mature, lamprey do not respond to migratory pheromones (PZS and ACA) (Bjerselius et al., 2000), but at that time, spermiated males construct spawning nests and release a mating pheromone component (3kPZS) that is highly attractive only to ovulated females (Siefkes et al., 2005). Therefore, different ages and sexes of sea lamprey release and respond to chemical cues throughout their life history. Similar sex and maturity effects have been reported in the goldfish, where females sequentially release mixtures of pheromones throughout the ovulation cycle to prime males and induce courtship behaviors. Details will not be considered here because goldfish pheromones have been reviewed elsewhere (Sorensen & Stacey, 2004; Stacey, 2003; Stacey et al., 2003; Kobayashi et al., 2002; Sorensen et al., 1998; Sorensen, 1992).

Consideration should be taken when working with a species with alternative mating strategies because dominate and sneaker (jack) males may differ in pheromone release and response. For example, dominate male black gobies (*Gobius niger*) respond aggressively to the ejaculate of other dominate males, but not to the ejaculate of sneaker males, likely because sneaker male ejaculate is pheromonally inconspicuous (Locatello et al., 2002). Even though sneaker males of most species typically do not release pheromones, evidence suggests that sneaker males in most cases are able to detect and respond to conspecific pheromones. For example, Yambe et al. 2006 used "jack" masu salmon (*Oncorhynchus masou*) as bioassay subjects to identify female mating pheromones because small jacks were easy to work with in the lab.

Sexual maturity is linked to increases in sex steroid concentration in animals with associated mating systems. Sex steroids function to physiologically ready an animal for mating, including enabling mature animals to detect and respond to mating pheromones. Carolsfeld 1997b showed in Pacific herring (*Clupea pallasii*) that individuals with high sex steroid concentrations were most likely to respond to spawning pheromones. In masu salmon and rainbow trout, responsiveness to pheromones was linked to sex steroid concentration when immature male parr, which do not naturally respond to female priming and mating pheromones, did indeed respond when treated with methyltestosterone (Yambe et al., 2003; Yambe & Yamazaki, 2001). Similarly, injection of androgens into male *Barilius bendelisis* increased responsiveness to female mating pheromones by activating male olfactory receptors (Bhatt et al., 2002). Overall, very little is known about the mechanism by which sex steroids act on the central nervous system and olfactory organ to modify responsiveness to chemical cues, but work in this area has just begun in the sea lamprey and the mechanism likely involves the hypothalamic-pituitary-gonadal axis (Chung-Davidson et al., in prep).

Given the importance of sexual maturity in responsiveness, it is critical that the reproductive state and, when possible, sex steroid concentrations of experimental animals be described in mating pheromone experiments. Some field studies reviewed would have been more instructive if maturity of experimental animals had been carefully considered. For example, Young et al. 2003 conducted a field experiment to determine whether traps baited with mature male and female brook trout (*Salvelinus fontinalis*) captured a higher proportion of animals than unbaited traps. Indeed, they found that traps baited with mature males captured more mature males than unbaited traps or traps baited with females. However, their in-stream experiments were conducted before most brook trout had spawned (inappropriate physiological context) and in a stream section containing no suitable spawning habitat (inappropriate environmental context). Their results would have been more informative and likely more convincing if their experiments had been conducted during the mating season on spawning riffles.

Temporal variation in responses to chemical cues. Even if experimental animals are of the correct age, sex, and maturity, they may only respond to chemical cues at certain times of the day. Again, this point is clearly illustrated in the sea lamprey where migratory adults are nocturnal and only respond to migratory pheromones at night (Bjerselius et al., 2000). However, sexually mature sea lampreys are arrhythmic (Applegate, 1950), spawning night and day, and accordingly mature females respond to the mating pheromone night and day (Johnson et al., 2005). In the goldfish female priming pheromone, 17α, 20β-dihydroxy-4-pregnen-3-one (17,20, βP), robustly induced sexual arousal in males in the early morning when females are likely to be releasing 17,20, βP. However, males exposed to 17,20, βP in the afternoon showed lower levels of arousal (Defraipont & Sorensen, 1993). When planning behavioral studies, the life history of the experimental species should be carefully considered so that trials can be conducted at times when the species naturally responds to the chemical cue.

Stress. The level of stress experienced by experimental animals should always be of concern in any animal behavior research. In most species, stress decreases responsiveness because increased cortisol concentrations lower sex steroids, which act to reduce behavioral responses. Transport of animals from the field to the lab may be particularly stressful, and this presents a potential problem for lab experiments in which animals are obtained from the wild. This has been demonstrated in bull frogs (*Rana catesbiana*) (Licht, 1983), painted turtles (*Chrysemys picta*) (Licht, 1985), brown trout (*Salmo trutta*) (Pickering, 1987), and rainbow trout (Scott et al., 1994; Pottinger, 1992). Interestingly, Pacific herring respond more robustly to spawning pheromones when held in aquaria with shallow water (shallow water stressor). Spontaneous spawning may even occur when water is being drained from the tank (Carolsfeld et al., 1997b). Pacific herring naturally spawn in shallow turbulent rock reefs, so spontaneous spawning may be a natural response to a natural physical context. Regardless of whether stress increases or decreases responsiveness to chemical cues, the stress levels of experimental animals should be controlled so they are similar to those naturally encountered under field conditions.

Social context, experience, and learning.

In addition to physical and physiological contexts, social environment is an important context that influences responses to chemical cues (Ferrari et al., 2005). In goldfish, the effects of social context on male response to female priming pheromones 17,20 β-P and prostaglandin F2α (PGF) have been investigated. When in social isolation, the male endocrine system is only primed by exposure to 17,20 βP (Fraser, 2002). But the male endocrine system is primed by PGF when males are able to interact socially with females (Sorensen, 1989). In crustaceans, social context influences the release of mating pheromones and odorants in reproductive males (Breithaupt & Eger, 2002).

Female Chinese mitten crabs (*Eriocheir sinensis*) only release mating pheromones after physical interaction with a male (Herborg et al., 2006).

Experience and learning by experimental animals has also been shown to alter behavioral responses of fish to alarm odorants. Several fish species have been observed to exhibit learned predator recognition to alarm odorants. Simultaneous exposure of an individual to a novel odorant and a stressful event, like chasing with a net, causes the individual to associate the odorant with danger (Kelley & Magurran, 2003; Chivers et al., 1995). Hatchery reared fish have been trained to recognize odors of natural predators, but the efficacy of this technique to reduce mortality of stocked fish has yet to be demonstrated (Mirza & Chivers, 2000; Brown & Smith, 1998). Zebrafish (*Brachydanio rerio*) have even been trained to associate red light with predation threat by simultaneously exposing them to alarm odorants and red light (Hall & Suboski, 1995b; Hall & Suboski, 1995a). Recognition of odorants can last up to a month, and such recognition has been demonstrated in field contexts (Pollock et al., 2003). Given these examples, the previous experiences of experimental animals should be carefully controlled, and test subjects should not be used more than once in any experiment.

My literature search revealed that no chemical cue induces the same response in all individuals in all contexts. Responses to chemical cues are dependent on specific physical, physiological, and social contexts, all of which are interrelated and dependent on each other (Mikheev et al., 2006; Diaz et al., 2003; Hassler & Brockmann, 2001). If experiments cannot be conducted in the field, the most important contextual mediators of chemically-induced behaviors should be replicated and reported in the literature. The best option would be to conduct experiments at the time and location in nature where chemically-mediated behaviors occur.

CONTRAINTS OF EXPERIMENTS IN FIELD ENVIRONMENTS

Given how important it is to conduct chemical ecology experiments in natural contexts, one would expected that most research would be conducted in the field and would yield ecologically relevant results. But this is not the case because field research is constrained by the same physical and physiological contexts that are needed to elicit chemically-mediated behaviors. In the next section, major experimental and environmental factors that constrain field research will be discussed by reviewing field studies that yielded meaningful results despite the constraints imposed by nature.

Experimental constraints.

Observation of animals. One of the greatest constrains of experiments conducted in aquatic environments is the inability to observe behaviors because water is often turbid, deep, fast flowing, and structurally complex. Even if behaviors can be observed, care must be taken that observation techniques are noninvasive and do not alter animal behavior. In clear lakes or streams visual observation techniques have been used to record responses of fish to chemical cues (Johnson et al., 2006; Johnson et al., 2005; Johnsen, 1980). For example, the upstream movements of sea lamprey orienting toward mating pheromones have been documented by tracking individuals with radio telemetry and recording their movements on stream maps (Figure 3-2) (Johnson et al., 2006). Disadvantages of this technique are that data cannot be reviewed, some animals may be lost, and it requires a great deal of manpower. For example, during in-stream experiments two technicians were required to track one lamprey, and even then only half

Figure 3-2. Photograph illustrating two technicians manually tracking an ovulated female sea lamprey as it moves upstream towards a trap baited with spermiated male washings. One technician uses telemetry to locate the lamprey while the other technician records observed behaviors. This technique is labor intensive, requiring two technicians to track a lamprey. Image is presented in color.

of the lampreys that moved upstream to the pheromone were observed (Johnson et al., 2006). Other studies have used brief focal observations of individuals to obtain behavioral data (Siefkes et al., 2005; Liley et al., 1993), but observation times were short and behavioral data were lost. Perhaps the most adventurous observation technique employed to date has been snorkel surveys, in which fish were manually observed by divers. Snorkeling techniques have yielded several informative behavioral studies concerning alarm odorants and parental care in fishes (Golub et al., 2005; Neff, 2003), but require much manpower, and divers may disturb natural fish behavior.

To avoid the drawbacks of directly observing behaviors, video cameras have been exploited to record behaviors in experimental sites spanning less than 10 m. Cameras, however, are of little use if animals need to be tracked over long distances and are especially susceptible to malfunction when used in harsh weather. For example, Finelli et al. 2000 monitored blue crab foraging behavior in a small tidal creek with a camera mounted above the water, but 206 of 323 observations had to be discarded because of poor quality.

In many instances, pseudo-measures of behavior are collected when it is not feasible to continuously observe animal behaviors. This is especially true in studies of alarm odorants in fishes, where numbers of fish (or lack of fish) captured in traps baited with alarm odorants are taken as a pseudo-measure of alarm response (Pollock et al., 2005; Wisenden et al., 2004a; Pollock et al., 2003; Mirza & Chivers, 2001; Chivers et al., 1995; Mathis et al., 1995; Wisenden et al., 1994; Mathis & Smith, 1992). Another example is described in Wagner et al. 2006 in which sea lampreys were tagged with passive integrated transponders (PIT tags and antennas, Oregon RFID) to determine when they moved past defined stream locations while orienting towards migratory pheromones (Wagner et al., 2006). PIT tagging systems are a pseudo-measure of behavior because they only inform us about when lampreys passed a particular stream location and do not record the speed, direction of movement, or other pheromone-induced behaviors. It is essential that pseudo-measures of behavior correlate with the chemically-mediated behavior of interest in order to draw informed conclusions. With determination, creativity, and new technology, researchers can overcome obstacles of observing animals in field studies and can adequately describe chemically-mediated behaviors.

Experimental animals. When designing field experiments it is important to consider the source and history of experimental animals. Most field studies use animals naturally present in the environment as test subjects (Olsen et al., 2006; Carton & Montgomery, 2003; Brown et al., 2001b; Finelli et al., 2000; Zimmerfaust et al., 1995). This approach ensures that responses to chemical cues are relevant in natural populations. However, using wild animals limits experimental design because it is difficult to identify individuals, obtain physiological data on test subjects (size, age, sex, maturity), evaluate social interactions, control for past experiences, ensure sample size will be adequate (Scholz et al., 2000), and determine whether the same animal was tested more than once (pseudo-replication). Some studies attempt to avoid pseudo-replication by making experimental sites hundreds of meters apart (Brown et al., 2001b). Other researchers, like Zimmer et al. 1999, evaluated the likelihood of pseudo-replication by estimating the density of the experimental species in the field site using mark-recapture techniques.

To avoid the constraints of experimenting on animals naturally present in the environment, sea lampreys have been released into experimental streams during experiments. To do this, wild lampreys were captured in traps and allowed to naturally mature in holding cages (1 m^3) located in spawning streams. Once fully mature, females were tagged and then released into the experimental stream segment (Johnson et al., 2006; Wagner et al., 2006; Johnson et al., 2005; Siefkes et al., 2005). The advantages of releasing test subjects into the wild are that a known number of animals can be released from a known location, sex and maturity of released animals can be controlled, physiological characteristics can be described, and individuals can be uniquely marked. The disadvantages of introducing animals are that it can be labor intensive, it requires additional holding facilities, and test subjects may be stressed prior to experimentation. Furthermore, if experimental animals are released in groups, pseudo-replication can occur if individual animals within the group do not behave independently. In some studies, individuals released in groups have been shown to behave independently and have been treated as an individual replicate (Johnson et al., 2006; Siefkes et al., 2005). If groups of animals are released, researchers must determine whether individuals behaved independently within the group, and whether individuals tested on different trial dates respond differently.

Chemical identity of odorants unknown. Perhaps the most critical experimental constraint for field research is that the chemical identity of pheromones are largely unknown and thus synthesized copies are unavailable (Young et al., 2003). When synthesized components are unavailable, experiments are constrained because odorants must be extracted from animals and the molar concentration of the active component cannot be determined to ensure it is sufficiently high to overcome background odorants (Friesen & Chivers, 2006). Odorant extracts also contain other compounds in addition to the active components, making it impossible to determine which compound(s) induced the behavior. When synthesized odorants are available, sufficient quantities and precise concentrations of a single compound can be applied to lakes or rivers for hours or even days. If synthesized odorants are not available large scale experiments can not be conducted. Consider the difficulty of obtaining sufficient odorants from skin extracts to test whether alarm odorants could exclude an invasive species from a harbor in the Great Lakes for a week (Maniak et al., 2000). Clearly, large scale field experiments are more feasible when conducted with synthesized odorants.

Even when synthesized pheromone components are available, as with sea lamprey migratory (PADS, PSDS, PZS) and mating pheromones (3kPZS, 3kACA) (Sorensen et al., 2005; Li et al., 2002), government agencies like the United States Environmental Protection Agency (EPA) regulate application to natural waters. According to the Federal Insecticide, Fungicide, and Rodenticide Act, USA (FIFRA; www.epa.gov/lawsregs/laws/fifra.html), sea lamprey pheromones are considered pesticides because they may be used in lamprey control operations. Therefore experimental user permits (EUP) are required to apply synthesized pheromones at concentrations as low as 10^{-14} M. Packaged together with EUPs are requirements to collect data according to Good Laboratory Practices (GLP; www.fda.gov/ora/compliance_ref/bimo/glp/default.htm). Compliance with strict GLP data management and recording requirements are oxymoronic considering that experiments are conducted in the field, not a laboratory. The application process to obtain an EUP is extensive. However, in the case of the sea lamprey, once permits were obtained and field protocols established, great advances in the understanding of pheromone communication have been made by conducting experiments with synthesized odorants in natural streams (Siefkes et al., 2005).

Distribution of chemical cues in natural systems. To understand the behavioral mechanisms underlying chemorientation, odorant concentration and distribution must be known and correlated to the location and orientation of experimental animals. It is difficult to determine how odorants mix and disperse in the environment even if synthesized copies of an odorant are available. Hydrodynamics in natural systems constantly change and are extremely difficult to measure at biologically relevant spatial and time scales. A few studies have successfully measured the distribution of odorants in field conditions at small spatial scales using dyes and electrochemical tracers such as dopamine (to be reviewed in the next section) (Finelli et al., 2000; Finelli et al., 1999; Zimmer et al., 1999; Zimmerfaust et al., 1995), but over distances of tens to hundreds of meters the concentration of an odorant at every location at biologically relevant time scales has not been determined. Estimates of odorant concentration and distribution over stream distances of hundreds of meters can be obtained with large scale dye tests (Figure 4-2; Chapter 3), but these dye tests only display a static picture of odorant distribution and cannot be used to correlate odorant concentration with instantaneous orientation behaviors.

Environmental Constraints.

Field site location. In addition to the numerous experimental constraints of conducting field work, environmental constraints such as field site suitability, unpredictable weather conditions, and short field seasons limit study design. For

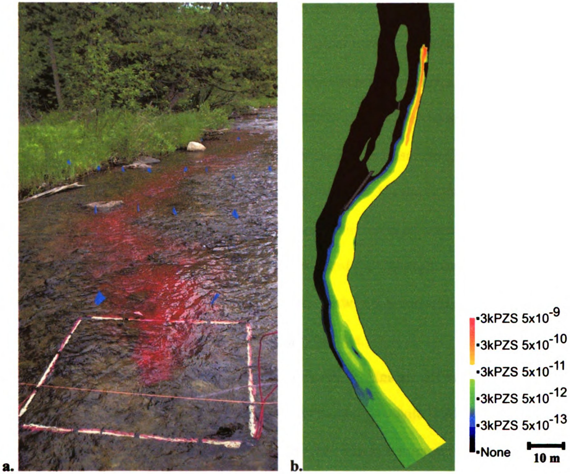


Figure 4-2. Application of rhodamine dye to determine the distribution and concentration of male sea lamprey mating pheromone in the Ocqueoc River, MI, USA. Average dye concentration was determined at locations marked with blue flags (a.). Theoretical dye concentrations were modeled between sampling points and maps were produced to visualize plume distribution and concentration in the stream (b.). Images are presented in color.

example, it can be challenging to obtain a field site that is easy to access, located near research facilities, and containing the environmental contexts needed for the display of chemically-mediated behaviors. A potential problem with any field site is that background odorants and pollutants present in the water unknowingly influence behavioral responses of animals (Lurling & Scheffer, 2007). Sea lamprey researchers, to avoid potentially confounding background pheromones, release experimental animals into streams without a natural population of sea lamprey. However, this reduces the number of streams that can be used as field sites and limits experimental design because only one sex can be tested to prevent establishment of the test organism. Field sites located on private land are preferred because researchers can conduct studies with less fear that vandals will steal equipment or destroy habitat.

Variable weather. Even when all experimental plans are off the ground and working well, sudden extreme weather conditions can ruin whole field seasons. High and low temperatures, droughts, floods, and storms can destroy equipment, experimenters, and the physical contexts needed to induce chemically-mediated behaviors. Furthermore, the environmental conditions necessary for research may only last one or two weeks when working with reproductive pheromones, limiting the number of experiments that can be conducted. It is important that field researchers consider all possible weather related problems and plan experiments according. A now humorous setback encountered during a sea lamprey pheromone experiment in my dissertation research occurred when a person upstream of the field site blew out a beaver dam, which washed away experimental animals and equipment. Floods were anticipated, but not a sudden flood without a drop of rain!

Obstacles of field research conclusion.

Experiments in nature offer natural environments, which in turn produce natural behaviors that implicate the ecological significances of chemical cues. But experiments in nature are difficult to conduct because conditions are highly variable and difficult to monitor. Even if field experiments yield conclusive results, it is often difficult to explicitly state the mechanisms by which results were obtained because of an inability to observe animal behavior, describe environmental conditions, or determine how much of which odorant was in the water. Therefore, it is often considered that field experiments are risky because meaningful data may not be obtained. Conversely, lab experiments can pose equal amounts of risk because results may be artifacts of laboratory contexts. Several field studies have overcome the constraints and uncertainties of nature through the use of innovative designs and technologies. These studies are among the most influential papers in aquatic chemical ecology. In the final section of this chapter, several of these studies will be reviewed and the techniques that enabled them to be successful will be discussed.

ENABLING TECHNOLOGIES FOR FIELD STUDIES OF AQUATIC CHEMICAL ECOLOGY

Ecological significance of alarm odorants revealed with underwater cameras.

One of the most influential studies in alarm odorants was conducted by Magurran et al. 1996 who showed that European minnows do not respond to alarm odorants in a field environment. Previous to that study, unobtrusive observation of minnows in lakes and streams was nearly impossible, and it was only through the utilization of new (at that time) underwater video technology that offered Magurran et al. 1996 a viewing window to observe fish behavior. Magurran et al. 1996 spurred debate and stimulated additional field studies that utilized underwater cameras to investigate fish responses to alarm odorants. Wisenden et al. 2004 conducted experiments on blacknose dace (*Notropis heterolepis*) in three Minnesota lakes, essentially replicating the techniques of Magurran et al. 1996. Wisenden found that dace responded to alarm odorants with the same intensity as a model predator, but the intensity of the alarm response was less robust than observed in laboratory experiments. In a similar underwater camera study, Friesen and Chivers 2006, found that fathead minnows, finescale dace, and brook stickleback did indeed elicit an antipredator response when exposed to alarm odorants taken from conspecifics and prey guild members. Collectively, field studies using underwater camera technologies have defined the ecological significance and context dependence of responses to alarm odorants.

Pheromone identification and PIT tagging technologies allow management scale tests of sea lamprey pheromones.

Recent advances in the extraction (Fine et al., 2006) and identification of sea lamprey migratory (PADS, PSDS, PZS) and mating pheromone components (3kPZS, 3kACA) (Hoye et al., 2007; Dvornikovs et al., 2006; Sorensen et al., 2005; Yun et al., 2003; Li et al., 2002) have given researchers the ability to conduct large scale field experiments testing the efficacy of pheromones to control sea lamprey (Johnson et al., 2006; Wagner et al., 2006; Johnson et al., 2005; Siefkes et al., 2005; Sorensen et al., 2003). The chemistry techniques used to identify sea lamprey pheromones have also been used to identify mating pheromones in the masu salmon (*Oncorhynchus masou*) (Yambe et al., 2006) and are becoming more widely used among chemical ecologists. New chemistry techniques will likely soon allow for measurement of mating and migratory pheromone concentrations directly from lake and stream water samples (Yun et al. in preparation; (Scott & Ellis, 2007). This would greatly enhance experimental power by enabling researchers to non-invasively confirm pheromone application rates, determine background concentrations of pheromones in experimental waters, and investigate pheromone release rates of fishes in natural environments.

New tracking technologies have also greatly enhanced sea lamprey field experiments by helping to overcome the constraints of the short spawning season and the difficultly of monitoring lamprey behaviors over long distances. Initially, radio telemetry was used to track individual radio-tagged lampreys (Johnson et al., 2006; Siefkes et al., 2005). Although useful in pilot studies, the use of radio telemetry was limited by cost (one tag cost ~ $200, receiver costs ~ $2,000), labor requirements, and only one lamprey could be tracked at a time (Figure 3-2). Recently, radio-frequency identification tagging technology (RFID, Oregon RFID) (Wagner et al., 2006) as been used and has increased the number of animals which can be experiment on. Passive integrated transponders (PIT tags) are externally mounted on lampreys and if a PIT-tagged lamprey passes through a magnetic field generated by a PIT antenna (loop of copper wire) within the stream (Figure 5-2), a unique identifying number and time is transmitted to a data logger. PIT antennas have been constructed to span stream channels 30 m wide and up to eight antennas have been used simultaneously. Antennas are highly adaptable and can be built to monitor lamprey movements over miles of stream, or they can be used to monitor small scale movements in and out of pheromone-baited spawning nests. RFID technology requires less labor than radio tracking and can monitor nearly limitless numbers of individuals. However, PIT tag data only describe when animals passed through specific stream locations and does not yield information about specific behaviors.

Mechanisms of crustacean chemorientation mechanisms revealed with motion sensing and odorant plume monitoring.

Given the difficultly of tracking fish movements and determining odorant distribution in lakes or streams, it is not surprising that researchers to date have not determined how instantaneous changes in pheromone concentration influence fish chemorientation. However, several influential field studies on crustacean chemorientation have reported orientation behavior in relation to odorant plume dynamics quantified at time scales relevant to crustacean chemosensory organs (~10 Hz)

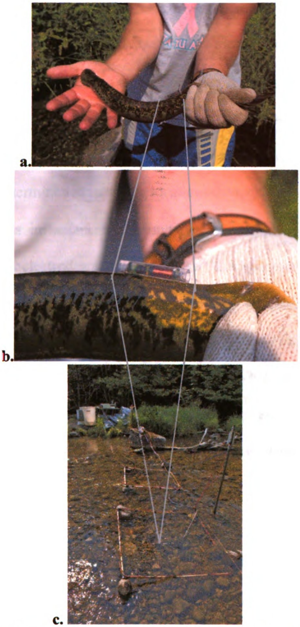
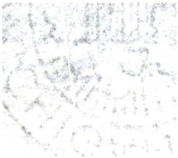


Figure 5-2. **a.** Passive integrated transponder (PIT tag, 23 mm) externally mounted on ovulated female sea lamprey. **b.** The PIT tag is housed in silicon tubing and the tubing is sutured to the back of the lamprey. **c.** If a PIT-tagged lamprey passes through the magnetic field generated by a PIT antenna constructed around a pheromone-baited spawning nest (copper wire attached to 1 m^2 PVC piping), the time and location will be transmitted to a data logger. Images are presented in color.



(Finelli et al., 2000; Zimmerfaust et al., 1995). For example, Zimmer-Faust et al. 1995 described the attraction of predatory blue crabs to food odorants originating from hard crabs (*Mercenaria mercenaria*) in a tidal creek. They recorded blue crab movements using mounted video cameras and then digitized movements of crabs that approached the food odorant. Odor plume dynamics were traced by mixing fluorescein (dye tracer) and dopamine (electrochemical tracer) with the odorant. Time-averaged fluorescein concentrations were determined at sampling locations throughout the field site. At the same sampling locations, instantaneous dopamine concentrations were recorded at 10 Hz using carbon fiber microelectrodes and a data logger (Medsystems Corp. IVEC-10). General flow characteristics were described with an electromagnetic flow meter (Marsh-McBirney), and shear velocities and Reynolds numbers were calculated (Denny, 1993; Denny, 1988). Integrating the behavioral data, odor plume data, and turbulence calculations, the authors concluded that blue crabs in tidal creeks move upstream in the presence of a food odor (rheotaxis), and that side-stream movements are determined by the fine scale distribution of the odor plume (chemotaxis). They conclude that chemotaxis orientation is feasible in tidal creeks because turbulence eddy diffusivities are over a hundred times less than that of forests where moths orient to pheromone plumes with stimulus-triggered optomotor amenotaxis (Murlis et al., 2000; Kennedy, 1974).

Two additional chemorientation field studies have been conduced in tidal creeks (about 2 m width) using odorant plume quantification techniques similar to Zimmer-Faust et al. 1995. Finelli et al. 2000 further explored orientation mechanisms of blue crabs in physically and chemically altered odorant plumes. They found that search efficiency and success varied significantly among crabs exposed to various water flows, odorant compositions, and odorant release rates. These results illustrate that odor release rates and flow conditions must be quantified and controlled for experimental results to be comparable to those from other studies. A second field study by Zimmer et al. 1999 found that orientation success of estuarine mud snails was more dependent on the physical dynamics of the plume than the chemical composition (amino acid composition or concentration); thus highlighting the importance of both chemical factors and hydrodynamic conditions when conducting experiments.

To date, electrochemical tracing techniques and animal motion sensing have only been applied on small scales and in environments with low turbulence. No large scale field studies have been conducted using these technologies. Furthermore, dye and electrochemical tracing techniques only sample from point locations and do not instantaneously measure plume dynamics in the whole experimental system. In the laboratory, planar laser-induced fluorescence (PLIF) has been developed to provide an instantaneous, quantitative, and non-intrusive description of dye distribution in lab flumes (Jackson et al., 2007; Weissburg et al., 2003; Koehl et al., 2001; Webster & Weissburg, 2001). It will be advantageous for future field studies to integrate animal motion sensing with PLIF technologies to understand chemorientation at finer scales and over longer distances.

CONCLUDING REMARKS

Based on the papers reviewed here, about 90% of the current literature on aquatic chemical ecology is founded on experiments conducted in the lab. Here a few examples of contradictory results obtained between lab and field studies have been highlighted to emphasize the need to confirm lab findings in the field. Furthermore, it has been illustrated how chemically-mediated behaviors are directly related to the physical environment of the experimental system and the physiological context of test animals. It is advantageous for researchers to consider the contexts that influence animal behavior in their natural environment even when designing lab experiments. This approach will make it easier to conduct subsequent experiments in the field. Recent technological advances in chemical identification, animal observation, and odorant plume modeling now offer willing researchers the ability to conduct highly controlled and descriptive field experiments. More chemical ecologists need to step out of the lab and embrace the risk and reward of experiments in nature for chemical ecology to continue to advance and yield solutions to human provoked problems and needs.

Accordingly, my dissertation exclusively reports results of sea lamprey mating pheromone experiments conducted in natural streams. PIT tagging and dye tracing technology is used in Chapters 3 and 4 to conduct a series of sea lamprey pheromone field experiments to confirm or refute laboratory two-choice maze results and reveal the efficacy of 3kPZS to control sea lampreys. In Chapter 5 a new field bioassay is developed which will enable researchers to isolate and characterize sea lamprey mating pheromone components exclusively in field environments.

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CHAPTER 3

A SYNTHESIZED PHEROMONE INDUCES UPSTREAM MOVEMENT IN FEMALE SEA LAMPREYS AND SUMMONS THEM INTO TRAPS

Johnson, N.J., Yun, S-S., Thompson, H.T., Brant, C.B., and Li, W. In press. A synthesized pheromone induces upstream movement in female sea lampreys and summons them into traps. Proceedings of the National Academy of Sciences, USA.

ABSTRACT

Female insect pheromone blends induce robust tracking responses in males and direct them into traps. In vertebrates, pheromones that induce strong and precise tracking responses in natural habitats have rarely been described. Here it is demonstrated in the sea lamprey (*Petromyzon marinus*), a vertebrate invader of the Laurential Great Lakes, that a synthesized component of the male mating pheromone, 7α, 12α, 24-trihydroxy-5α-cholan-3-one 24-sulfate (3kPZS), when released into a stream to reach concentrations of 10^{-14} ,10^{-13}, 10^{-12} ,10^{-11}, or 10^{-10} molar (M), triggers robust upstream movement in ovulated females drawing about 50% into baited traps. Experiments conducted in diverse stream segments demonstrate the level of behavioral response was not affected by habitat conditions and is effective over hundreds of meters (m). 3kPZS is equally effective at luring ovulated females as the whole pheromone blend released by males between 10^{-14} M and 10^{-11} M. 3kPZS diverts ovulated females away from and disrupts orientation to male washings when applied at concentrations higher than washings. Indeed, a single pheromone compound is able to redirect female sea lampreys away from a natural pheromone source and lure them into traps, which should be more effective than targeting males when applied in population control. These findings may spur the discovery of other potent and environmentally benign agents to combat biological invasion; a process accelerated by globalization, exacerbated by climate change, and costing the global economy US$ 1.4 trillion of damage annually.

INTRODUCTION

Pheromones are naturally occurring chemical signals and thus environmentally benign agents for pest control. In insect species, blends of synthesized female pheromones have been used for decades to lure males into traps (1) and disrupt reproduction (2). Vertebrate pests have never been controlled with pheromones. Studies of pheromone-mediated behavior of vertebrates in natural habitats have been limited both because relatively few vertebrate pheromones have been chemically identified (3) and because field research on this topic is often constrained by the ecology and behavior of vertebrates (4). Nonetheless, studies of the sea lamprey (*Petromyzon marinus*), an ancestral vertebrate and destructive invader of the Laurentian Great Lakes (5), indicate that spermiated males release a pheromone, 7α, 12α, 24-trihydroxy-5α-cholan-3-one 24-sulfate (3kPZS) (6), that induces predictable movements in ovulated females in spawning streams (7). The sea lamprey offers a uniquely advantageous model for determining possible applications of pheromones in vertebrate pest control.

Odors, both in air and water, occur as turbulent plumes where intermittent discrete packets and filaments of odorants at high and intermediate concentrations are interspersed with regions of below threshold concentrations (8, 9). These plumes are typically mixtures containing more than a single biologically relevant compound (10). Temporal information present in odor plumes appears to be used by moths (8) and crustaceans (11) to assess the direction and perhaps the distance of the odor source.

For the sea lamprey, odor plumes of 3kPZS released by mature males also challenge ovulated females with a comparable level of temporal and spatial complexity.

Lamprey spawning riffles are often separated over kilometers by stream segments that have highly variable fluid dynamics. An orientation strategy that relies on concentration gradients (chemotaxis) may not be effective for ovulated females to track a 3kPZS plume because sea lampreys are monorhinic and move scented water into and out of the olfactory capsule through a single nostril with each respiratory cycle (12). This limits odor plume sampling to a few "sniffs" per second and restricts ovulated females to sequential sampling of odor plumes (klinotaxis). This sampling rate appears to be too slow to obtain reliable measurements of the dynamic properties in turbulent odor plumes (9) to permit chemotaxis. I hypothesized that ovulated females could move close to a source of 3kPZS by simply swimming upstream when the odorant is detected and searching when it is lost; that is chemically-mediated upstream movement.

If this is true, 3kPZS may be highly effective at luring ovulated females into specific stream locations. Therefore, the main objective in studying the behavioral response of ovulated females to 3kPZS was to assess the potential utility of 3kPZS in control of sea lamprey in the Laurentian Great Lakes (5). Over 3,000 insect pheromones have been identified (13) and mixtures of female pheromones are commonly used as agents to enhance male trapping efficiency and disrupt reproduction as benign alternatives to traditional pesticide treatments (14). 3kPZS offers an ideal model for developing pheromone-based vertebrate pest control because, unlike mixtures of insect pheromones that attract males, 3kPZS alone modifies the behavior of ovulated females in their natural habitat (7). Further, 3kPZS is the first synthesized vertebrate pheromone in which an experimental user permit from US Environmental Protection Agency has been issued to allow application in a natural stream. The overall goal of the study was to describe the behavioral processes by which ovulated females locate 3kPZS in order to reveal the efficacy of 3kPZS as a control agent.

RESULTS

Synthesized 3kPZS induces upstream movement in ovulated females and lures them into traps.

Ovulated females in spawning streams encounter variable fluid dynamics and spermiated male populations (15). Therefore, I postulated that ovulated females would be able to locate sources of 3kPZS that vary in concentration, and subsequently, be guided into 3kPZS-baited traps. To test this prediction, I observed whether ovulated females were able to locate the exact source of 3kPZS by baiting traps with 3kPZS, recording ovulated female capture rates and tracking ovulated female movements; thereby also revealing the utility of 3kPZS as a sea lamprey control agent. Experiments were conducted in a natural spawning stream divided into two channels by an island (Ocqueoc River, MI, USA) (16). A sea lamprey trap was placed in each channel and one trap randomly received 3kPZS to achieve a concentration of 10^{-11}, 10^{-12}, 10^{-13}, or 10^{-14} M and the other trap received methanol as a control for the solvent in which the pheromone was dispersed in the treatment traps (Table 1-3 and herein; Figure S1). Concentration was calculated assuming complete mixing with stream discharge, which was confirmed to occur 70 m downstream by dye tests. I released ovulated females 70 m downstream and found that traps baited with 3kPZS 10^{-11}, 10^{-12} and 10^{-13} M did not differ in capture rate (logistic regression; $X^2 = 1.75$, df = 2, p = 0.417) and captured 46% of ovulated females released and 68% of ovulated females that moved upstream. Even at 10^{-14} M, 3kPZS-baited traps captured 25% of ovulated females released and 75% of ovulated females that moved upstream, which was more than the control trap

Table 1-3 3kPZS-induced upstream movement directs females into traps. Distribution is the number of ovulated females that moved upstream and were captured when one trap was baited with control solvent and the other trap was baited with 3kPZS, and when both traps were baited with control solvent (Control). All ovulated females were captured in 3kPZS-baited traps when 3kPZS was applied to the stream. Orientation summarizes behaviors of ovulated females that were captured in 3kPZS-baited traps: Time, mean (s.d.) time to swim from the release cage and enter trap; Rests, mean (s.d.) number of rests; Downstream, mean (s.d.) number of downstream movements; and Sidestream, mean (s.d.) sidestream movements taken by each ovulated female while moving upstream toward the 3kPZS-baited trap. Distribution data were evaluated with logistic regression and orientation behaviors evaluated with general linear models. 3kPZS treatments that share a letter were not significantly different ($\alpha = 0.05$). Orientation statistics were not evaluated for control and 3kPZS 10^{-14} M treatments.

Treatment		**Distribution**		**Orientation**				
Trap Bait (M)	**n**	**Upstream (n)**	**Captured (n)**		**Time (min)**	**Rests**	**Downstream**	**Sidestream**
3kPZS 10^{-11}	25	19 A	11 *AB*		17.7 (21.9) A	3.9 (5.1) A	0.4 (0.8) A	1.8 (2.6) A
3kPZS 10^{-12}	25	18 A	14 *A*		20.8 (24.0) A	4.9 (5.3) A	0.0 (0.0) A	0.5 (0.7) A
3kPZS 10^{-13}	24	13 AB	9 *AB*		11.3 (9.3) A	2.8 (2.2) A	0.2 (0.4) A	2.0 (2.9) A
3kPZS 10^{-14}	24	8 B	6 *BC*		NA	NA	NA	NA
Control	22	11 AB	1 *C*		NA	NA	NA	NA
	X^2	12.29	18.64	**F-value**	0.38	1.15	0.01	1.12
	df	4	4	**$^{NDF}/_{DDF}$**	2/27	2/23	2/23	2/23
	p-value	0.015	0.001	**p-value**	0.689	0.334	0.99	0.345

(binomial distribution, $p = 0.012$).

Behavioral observations show that ovulated females oriented towards 3kPZS-baited traps by swimming directly upstream (Figure 1-3). Ovulated females captured in traps baited with 3kPZS at 10^{-11}, 10^{-12}, or 10^{-13} M did not differ in time taken to swim upstream into the trap after leaving the release cage (mean = 18.1 min, range 2.7 to 84.8 min), the number of rests (mean = 3.8, range 0 to 16), number of downstream movements (mean = 0.2, range = 0 to 2), or number of sidestream movements (mean = 1.3, range = 0 to 7) (Table 1-3). Only 17% of ovulated females exhibited two or more sidestream movements while moving upstream toward the 3kPZS-baited trap. Ovulated females located the exact release point of 3kPZS even when concentrations varied 1000 fold.

I further reasoned that ovulated females would not become adapted to 3kPZS even after prolonged exposure in 3kPZS plumes during their directed upstream movement over long distances and in diverse river habitats. This hypothesis was tested by recording ovulated female responses to 3kPZS over a 650 m distance at two experimental sites in the Ocqueoc River. One segment was located on the sea lamprey spawning riffle used in previous experiments and the other site was located several km downstream of the spawning riffle which was characterized by slow deep flow and sandy bottom (run) (Figure S2). At each experimental site, ovulated females were released 650 m downstream of a trap baited with 3kPZS to reach 10^{-12} M and a trap baited with control solvent.

3kPZS induced directed upstream movement over 650 m in both environments. In the riffle and run stream segments, the proportion of ovulated females moving upstream and entering within 1 m of 3kPZS-baited traps did not differ, showing that

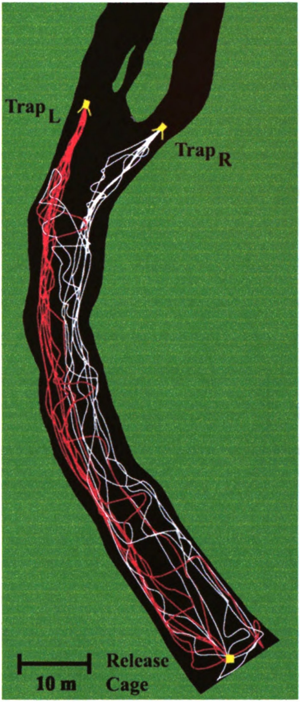

Figure 1-3 3kPZS-baited traps capture all females when compared to unbaited traps. Observed movements of individual ovulated females trapped when 3kPZS was applied at 10^{-11} M, 10^{-12} M, or 10^{-13} M in a randomly selected trap and when control solvent was applied in the other (Trap$_L$ and Trap$_R$). Red lines illustrate ovulated females entering the left trap when the left trap was baited with 3kPZS. White lines illustrate ovulated females entering the right trap when the right trap was baited with 3kPZS. Green illustrates ground and black illustrates river. Figure displayed in color.

3kPZS induced equally strong migrations in both habitats (Fisher's exact; $p = 0.738$). More ovulated females moved upstream and entered within 1 m of the baited traps when 3kPZS was applied to the stream than when control solvent was applied to both traps (Table S1 and herein; Fisher's exact; riffle: $p < 0.001$; run: $p = 0.005$). However, the proportion of ovulated females captured in 3kPZS-baited traps was greater in the riffle habitat than in the run habitat (Fisher's exact; $p = 0.002$) and the time for ovulated females to enter within 1 m of the 3kPZS-baited trap was longer in the riffle stream segment than the run segment (Wilcoxon rank-sum test; $p = 0.027$, $z = -2.21$, $df = 30$). Slower swimming speeds of ovulated females in the riffle may be due to fast water velocity and the inefficiency of anguilliform swimming. Similarly, high water velocity through the trap at the riffle site may have caused ovulated females to swim with great effort into the trap funnel resulting in higher capture efficiency. 3kPZS elicits long upstream migration in both environments, but the trapping techniques used in this study may be most efficient if traps are placed in riffle environments.

Role of 3kPZS as a component of the pheromone mixture.

Given that most characterized pheromones are mixtures which only elicit strong responses when all components are present (17), it was interesting to observe that 3kPZS alone induced robust upstream movements over long distances and ranges of concentrations. It has been hypothesized that spermiated male washings (SMW) contain additional pheromone components (18) that induce near source search behaviors in ovulated females (7). Thus, I wished to confirm the role of 3kPZS-mediated upstream movement when placed in the context of SMW by directly comparing responses of ovulated females to synthesized 3kPZS and SMW over long and short distances. SMW were used instead of live spermiated males to provide an unequivocal test of whether additional pheromone components induce near source search behaviors. Previously, it was found that behavioral responses of ovulated females to spermiated males or their washings in a two-choice maze do not differ (7). In a natural stream, traps baited with spermiated males and SMW both capture large proportions of ovulated females (16, 19). These results are not surprising since ovulated females are blind (15) and naris-plugged ovulated females are not able to locate spermiated males over long or short distances (19). Therefore, by comparing 3kPZS and SMW, I also evaluated the potential utility of 3kPZS in redirecting ovulated females away from natural sources of pheromone, and thus a potential mate.

At the spawning riffle segment, a lamprey nest was constructed in each river channel 45 m upstream of the confluence of the channels (Figure S3a). In one nest SMW was applied to reach an in-stream natural 3kPZS concentration of 7.5×10^{-13} M. In the other nest (other channel) synthesized 3kPZS was applied at 0.7, 1.0, 1.3 or 3.3 times the concentration of natural 3kPZS in SMW. Females were released 250 m downstream and had to choose which channel to enter 45 m downstream of the odor sources. Surprisingly, when applied at equal 3kPZS concentrations, synthesized 3kPZS and SMW attracted equal proportions of ovulated females, and at merely 3.3 times the concentration of 3kPZS in SMW, synthesized 3kPZS attracted 84% of responsive ovulated females (Table 2.3). Notably, nest observations show that ovulated females spent 10 fold more time in nests baited with SMW than nests baited with 3kPZS (Table 2-3).

Table 2-3 Female preference for synthesized 3kPZS and natural pheromone mixture. Distribution of ovulated females when presented with nests baited with synthesized 3kPZS or spermiated male washings (SMW; natural pheromone) and median time spent within 0.5 m of the nest (Retention). Data from two experimental designs are presented; when ovulated females choose an odor source from 45 m downstream (Treatment: 45 m; Supporting Fig. 3a online) and when the odorants were 1.25 m apart (Treatment: 0 m; Supporting Fig. 3b online). Distribution data were evaluated with logistic regression and retention data were evaluated with a general linear model. NA = data not applicable.

Treatment		**Distribution**			**Retention**		
Direct Comparison	n	3kPZS (n)	SMW (n)	p-value (df;X^2)	3kPZS (sec)	SMW (sec)	p-value (NDF/DDF;F-Stat)
45 m: 0.7 X 3kPZS vs. SMW	38	1	24	<0.001 (1; 20.81)	60	1849	0.051 (1/23; 4.27)
45 m: 1.0 X 3kPZS vs. SMW	41	9	15	0.078 (1; 3.11)	152	618	0.010 (1/21; 8.00)
45 m: 1.3 X 3kPZS vs. SMW	36	12	8	0.315 (1; 1.01)	8	447	0.001 (1/18; 14.29)
45 m: 3.3 X 3kPZS vs. SMW	36	16	3	0.035 (1; 4.45)	80	419	0.015 (2/16; 5.52)
0 m: 1.3 X 3kPZS vs. SMW	21	0	19	<0.001 (1; 29.06)	NA	439	NA
0 m: 3.3 X 3kPZS vs. SMW	40	12	13	0.583 (1; 0.30)	30	342	<0.001 (1/23; 19.11)
45 m: 10^{-11} M 3kPZS vs. SMW	60	18	10	0.184 (1; 1.76)	123	530	0.035 (1/26; 4.94)
45 m: 10^{-12} M 3kPZS vs. SMW	38	11	10	0.247 (1; 1.34)	197	874	<0.001 (1/19; 25.33)
45 m: 10^{-13} M 3kPZS vs. SMW	60	11	14	0.488 (1; 0.48)	208	297	0.097 (1/23; 3.00)
45 m: 10^{-14} M 3kPZS vs. SMW	83	9	11	0.276 (1; 1.19)	70	64	0.717 (1/18; 0.19)

The distribution of ovulated females between channels baited with 3kPZS and SMW show that 3kPZS plays a major role in inducing directed upstream movement, but a higher potency of SMW in retaining ovulated females on nests suggests that additional components increase near source retention. To confirm this finding, the near source effects of 3kPZS and SMW were compared by building two spawning nests 1.25 m apart (Figure S3b) and applying SMW to one nest to reach a natural 3kPZS concentration of 7.5 X 10^{-13} M and synthesized 3kPZS to the other at 1.3 or 3.3 times the concentration of natural 3kPZS in SMW. Contrary to results from 45 m comparison experiments, all ovulated females went to the SMW when synthesized 3kPZS was applied at 1.3 times, and equal proportions of ovulated females visited both nests when 3kPZS was applied at 3.3 times (Table 2-3). Again, SMW retained ovulated females about 10 times longer than synthesized 3kPZS.

Why did SMW and 3kPZS equally attract ovulated females to nests over a 45 m distance when applied at 7.5 X 10^{-13} M, but synthesized 3kPZS did not retain females on nests? It is possible that, at this 3kPZS concentration, the pheromone components within SMW that attract and retain ovulated females near nests may not be detected long distances downstream due to lower release rates or olfactory sensitivities (or both). To investigate this possible scenario, 3kPZS and SMW were directly compared at a 45 m distance across a 1000 fold change in concentration. Responses to SMW and 3kPZS were directly compared when the 3kPZS concentration of both sources were equal to 10^{-11}, 10^{-12}, 10^{-13}, and 10^{-14} M, respectively. At each concentration, 3kPZS and SMW triggered equal proportions of ovulated females to move upstream into the baited channels (Table 2-3 and herein), showing that within the range of concentrations tested, 3kPZS is the only pheromone component that influences long distance responses in ovulated females. As expected, retention in the SMW nest was significantly higher than 3kPZS at 10^{-11} M and 10^{-12} M. However, retention in the SMW nest and 3kPZS nest did not differ at 10^{-13} M and 10^{-14} M, perhaps because at extremely low concentrations minor components were not detectable even when ovulated females were on the nest.

3kPZS disrupts female orientation to male pheromone.

Given the dominant role 3kPZS plays in the pheromone mixture to induce upstream movement over various distances, I postulated that high concentrations of 3kPZS can disrupt both near and far source effects of the natural male pheromone blend. An experiment was conducted to test this hypothesis and further confirm that 3kPZS indeed maintains robust movement directly upstream over a wide range of concentrations. At the spawning riffle, SMW was applied to reach an in-stream natural 3kPZS concentration of 10^{-12} M 20 m upstream of the ovulated female release site and a background synthesized 3kPZS source was applied 40 m upstream of the SMW source, so that the synthesized 3kPZS-plume enshrouded the SMW-plume (Figure 2-3). Background concentrations of 3kPZS applied were 0 (vehicle solution), 10^{-12}, 10^{-11}, or 10^{-10} M.

Consistent with the hypothesis, a higher proportion of ovulated females completely missed the enshrouded SMW source while swimming upstream to the 10^{-10} M synthesized 3kPZS source than to the control source (Table 3-3 and herein; logistic regression; $X^2 = 4.24$, df = 1, p = 0.040). To discern the mechanism for this disruptive effect, individual ovulated female movement tracks were compared to the plume

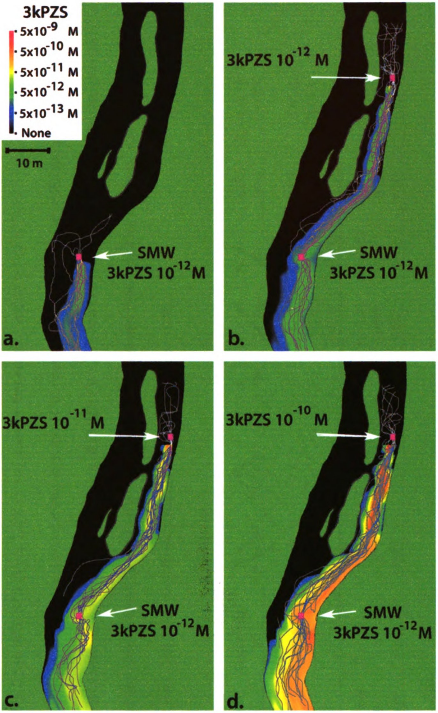

Figure 2-3 Female movement tracks during 3kPZS disruption experiments. Synthesized 3kPZS was released 40 m upstream of a source of spermiated male washings (SMW) with natural 3kPZS at 10^{-12} M. Color scale illustrates estimated 3kPZS molar concentrations from both sources of 3kPZS throughout the stream. Background 3kPZS concentrations achieved when fully mixed with the stream discharge. (a) No 3kPZS background. (b) 3kPZS 10^{-12} M background. (c) 3kPZS 10^{-11} M background. (d) 3kPZS 10^{-10} M background. Figure displayed in color.

Table 3-3 Disruption of female orientation to the natural pheromone enshrouded with 3kPZS. Number of ovulated females released, number entering within 0.5 m of 3kPZS or SMW (Responding), number swimming upstream of SMW (Pass Natural), number that entered within 0.5 m of 3kPZS (Visit 3kPZS), and number that entered within 0.5 m of 3kPZS but did not enter within 0.5 m of SMW (Bypass natural). The time for ovulated females to swim 10 m upstream of acclimation cage (Respond), time spent within 0.5 m of natural pheromone (Natural), time spent within 0.5 m of 3kPZS (3kPZS), and swimming speed from SMW source to 3kPZS source. All times are medians. Binary variables evaluated with logistic regression and time variables evaluated with general linear model. Treatments that share a letter are not significantly different (two-tailed α = 0.05).

Background 3kPZS	Released (n)	Responding (n)	Pass Natural (n)	Visit 3kPZS (n)	Bypass Natural (n)	Respond (sec)	Natural (sec)	3kPZS (sec)	Speed to 3kPZS (cm/sec)
None	37	29 A	6 A	-	1 A	629 A	796 A	-	-
3kPZS 10^{-12}	39	26 A	16 *B*	16 *A*	2 *A B*	1522 A B	820 A B	72 *A*	1.05 A
3kPZS 10^{-11}	39	25 A	20 *B*	20 *A*	4 *A B*	836 A	444 B	178 *A*	0.65 A
3kPZS 10^{-10}	38	28 A	24 *B*	20 *A*	7 *B*	2889 B	358 B	116 *A*	1.06 A
	Test Stat	$X^2 = 2.37$	$X^2 = 19.55$	$X^2 = 1.26$	$X^2 = 7.54$	$F = 3.81$	$F = 3.63$	$F = 2.20$	$F = 2.54$
	df	3	3	2	3	3/99	3/81	2/53	2/52
	p-value	0.499	<0.001	0.534	0.057	0.012	0.016	0.121	0.088

distribution as determined by dye tracing (Figure 2-3). When 3kPZS 10^{-10} M was applied, ovulated females were more widely distributed across the stream and were less likely to track the highest concentration of natural 3kPZS originating from SMW (Figure 2-3; Table S2; Figure S4; ANOVA; F = 5.795, df = 3/20, p-value = 0.005). Furthermore, a higher proportion of ovulated females swam upstream of the SMW source when 3kPZS was applied at 10^{-12}, 10^{-11}, or 10^{-10} M than when control solvent was applied. For those ovulated females that did visit the SMW source, they spent less time within 0.5 m of the SMW release point when 10^{-11} M or 10^{-10} M synthesized 3kPZS was applied upstream (Student's t-test; t-value = -2.81, DF = 81, p = 0.063 and t-value = -2.61, DF = 81, p = 0.011, respectively). When ovulated females moved upstream of the background source of synthesized 3kPZS they exhibited more sidestream and downstream movements (Table S3).

These experiments also confirm that ovulated females display robust upstream movements to 3kPZS over concentrations ranging 100 fold. First, the proportion of females that moved upstream and located a source of SMW or 3kPZS did not differ when 3kPZS concentration varied from 10^{-12} M to 10^{-10} M (Table 3-3). Second, swimming speed and swimming distance (Table S4) from SMW to the 3kPZS source did not differ among 3kPZS concentrations. Third, time spent within 0.5 m of the 3kPZS source did not differ among 3kPZS concentrations (Table 3-3).

DISCUSSION

In natural spawning streams, synthesized 3kPZS applied over a wide range of concentrations lured ovulated females to swim upstream over long distances and subsequently enter traps. Efficient localization of potential mates is essential for sea lamprey to bring their complex life history to fruition in a single spawning event over a few days before senescence (15). The male sea lamprey mating pheromone facilitates mate finding by signaling to ovulated females the location of spawning grounds and individual nests.

3kPZS-mediated upstream movement is sufficient and efficient in directing ovulated females to individual nests. A single source of 3kPZS triggered the same directed response in ovulated females over distances of 70 m and 650 m and concentrations varying from 10^{-10} M to 10^{-14} M. It is adaptive for ovulated females to display 3kPZS-induced upstream movement over highly diverse conditions because flow and male abundance differ greatly within and among spawning streams, causing 3kPZS plumes to vary greatly in intensity, and in temporal and spatial profiles. Ovulated females appear to employ the simple orientation strategy of swimming upstream when 3kPZS is detected and moving back downstream and casting side-to-side when the signal is lost.

Not only did a vertebrate swim up pheromone plumes, but its efficacy in locating the pheromone sources is comparable to those known in insects. This can be attributed in part to the predictability of shallow river pheromone plumes, which are essentially confined in a one dimensional space by the width, depth, and unidirectional flow of water

(20). Moths in a forest environment orient to unpredictable airborne pheromone plumes in a three dimensional space by moving upwind when the pheromone is detected and casting from side to side (optomotor amenotaxis) when the scent is lost (21). Unlike insects, ovulated females oriented toward a single source of 3kPZS by swimming directly into the unidirectional flow (Figure 1-3). In the disruption experiments, where an additional source of 3kPZS was located upstream of SMW, ovulated females that bypassed the SMW moved directly upstream to the background source of 3kPZS and subsequently showed more sidestream and downstream movements when ovulated females bypassed the background 3kPZS source (Figure 2-3). Ovulated females may use casting as a behavior to ensure that they do not overshoot the spawning grounds and bypass possible mates. It is notable that when ovulated females lose the 3kPZS signal, it takes several seconds (many sniffs) to begin casting, suggesting that either there is a significant integration time to recognize that the signal has been lost, or that upstream movement, once triggered by the pheromone, continues for a period governed by an internal mechanism, as proposed for moths (22). Sea lamprey and moths appear to use similar casting strategies, albeit on different temporal and spatial scales, to relocate lost plumes. A distinct difference is that in a one-dimensional environment, when the odor is briefly lost, it may not be advantageous to immediately move sidestream because plume intermittency may be related to distance from the source rather than location within the stream channel.

In many insects, sex pheromones, like most natural odors, are typically blends of components in specific proportions, with two or more being necessary to elicit a behavioral response (10). In particular, robust long distance behavioral responses are sometimes only elicited when a blend of compounds which function as one signal are present (17). 3kPZS alone elicited robust upstream movements over long distances and was equally effective as the whole pheromone blend found in SMW from 10^{-11} M to 10^{-14} M at attracting ovulated females at a 45 m distance (Table 3-3). However, males may excrete additional components which function over short distances to retain ovulated females on the nest. In experiments directly comparing 3kPZS and SMW released into nests separated by 1.25 m, SMW retained ovulated females 10 fold more time than 3kPZS (Table 2-3). Additional evidence that males release additional compounds is gleaned from disruption experiments when 3kPZS 10^{-10} M was applied 60% of responding ovulated females located and were retained at source of SMW even when background synthesized 3kPZS 1 m downstream of the SMW source was 5 times greater than the 3kPZS present in the SMW as determined by dye tests (Figure 2-3). Recently, several compounds have been isolated from larval sea lamprey washings and subsequently shown to modify behaviors of migratory adults in two-choice mazes (23).

Collectively, results show that 3kPZS is a component of the pheromone that functions independently to elicit long distance upstream movements in ovulated females, directing them to nests, and that unidentified components induce near source attraction and retention. The mechanism by which the male sea lamprey mating pheromone coordinates mate finding and reproduction closely resemble the "component" mechanism first described in the pine beauty moth (*Panolis flammea*) (24) and recently described in the red-legged salamander (*Plethodon shemani*) (25) where each component of the pheromone induces separate behaviors such as attraction, landing, or copulation (24). The data collected in this study do not support the "blend" hypothesis (17), in which all pheromone components work as one signal to induce all behaviors. However, these hypotheses should be re-evaluated when all pheromone components are identified.

From an applied standpoint, the data show that a synthesized pheromone modifies the behavior of ovulated females in their natural habitat and demonstrate the possible utility of 3kPZS as the first synthesized vertebrate pheromone control agent. This hypothesis should be further tested by comparing the effectiveness of 3kPZS versus spermiated males in their natural habitat. Capture rates and effective distances of 3kPZS-baited traps were similar to or greater than those reported in insects (14); but unlike mixtures of insect pheromones that attract males, 3kPZS alone induced robust responses in females. These are distinct advantages of 3kPZS because removal of ovulated females will result in a proportional reduction in viable eggs, and thus be more effective than removal of males. A single compound is less expensive to synthesize, easier to apply, and requires less testing to register with regulatory agencies. In addition to trapping, 3kPZS may be used to divert ovulated females away from natural male pheromones or redistribute ovulated females to tributaries not suitable for spawning or survival of offspring. This approach may be highly effective because sea lamprey only use about 6% of streams in the Great Lakes basin to spawn (5), and in any particular stream sea lamprey use a small portion of habitat for spawning (15). Furthermore, an "all or nothing" response to 3kPZS over a wide range of concentrations makes control applications even more efficacious because high concentrations are not required to induce strong responses. In the end, 3kPZS-based techniques may provide an environmentally benign means of managing sea lampreys in the Laurentian Great Lakes, where only 270 g of 3kPZS would be required to activate all currently trapped streams in Lakes Huron, Michigan, and Superior at 10^{-13} M during the 3 week spawning period (5.5 trillion liters of water).

MATERIALS AND METHODS

Behavior tests and permit.

Use of sea lampreys was approved under Michigan State University Institutional Animal Use and Care Committee permit 05/06-066-00. Application of 3kPZS and related bioactive components was approved by the Michigan Department of Environmental Quality and United States Environmental Protection Agency through experimental user permit 75437-EUP-2. Experiments were conducted in the Ocqueoc River, MI, USA (7, 16), in stream segments historical infested with larval and spawning-phase sea lampreys (15), however a barrier several km downstream currently prevents sea lamprey infestation. 3kPZS concentrations were calculated as the final in-stream concentration when completely mixed with the whole stream discharge. Dye tests confirmed that 3kPZS was thoroughly mixed 70 m downstream of the application point. 3kPZS was custom synthesized by Bridge Organics (Vicksburg, Michigan, USA) at purity higher than 95%. A single batch of SMW with a natural 3kPZS concentration of 1.85 mg/L was used in all 3kPZS verse SMW direct comparison experiments in 2007 and a single batch of SMW equaling 3.27 mg/L was used in 2008 direct comparison experiments. A single batch of SMW with natural 3kPZS concentration of 1.5 mg/L was used in all 3kPZS disruption experiments. Ovulated females were fitted with external radio tags (Model 393, Advanced Telemetry System, Isanti, Minnesota, USA) and tracked using direction radio antenna and receiver (Lotek Engineering Incorporated, Newmarket, Ontario, Canada) (7, 16) during 70 m trapping experiments. In all other experiments, ovulated females were fitted with external passive integrated transponders

(PIT tags) and tracked with PIT tag antennas connected to a multiplexer (Oregon RFID, Portland, Oregon, USA). Females were released in groups of three to five for 70 m trapping and in groups of six to 11 for all other experiments. Visual observations of random ovulated females were recorded on stream maps using stream markers as reference points (19).

Behavioral Statistics.

Female behaviors were assumed to be independent as observed from earlier studies (7, 16). Binary data from experiments with more than two treatment groups were evaluated with logistic regression and models showed no evidence of overdispersion or nonlinearities. Binary data from experiments with two treatment groups were evaluated with a nonparametric Fisher's Exact Test. Time variables and orientation behaviors were evaluated with general linear models where time variables were square-root transformed and orientation behaviors were square-root transformed or ln transformed when needed to meet model assumptions of residual heteroscedasticity and normality. Time data in 650 m trapping experiments were evaluated with a nonparametric Wilcoxon rank-sum test because data could not be transformed to meet parametric statistic assumptions. For 3kPZS verse SWM direct comparison and disruption experiments, data were also analyzed with mixed effect logistic regression and mixed-effect general linear models with a random effect of trial date. All statistical results from general linear models are robust to the inclusion of the random effect of trial date, supporting the assumption that a single ovulated female can be treated as an individual sample (7). Statistical results reported are from two-tailed analyses. A listing of the statistical tests and transformations conducted are in Table S5.

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SUPPORTING FIGURES

Figure S1. Sea lamprey traps on the historic sea lamprey spawning riffle, Ocqueoc River, MI, USA. One trap is baited with synthesized 3kPZS and the other trap is baited with control vehicle. Water flows from right to left across the figure. Odorants were administered to traps with peristaltic pumps (located on island) and females were always captured in the trap baited with 3kPZS. Figure presented in color.

Figure S2. Sea lamprey traps in the run stream segment downstream of the historic lamprey spawning ground, Ocqueoc River, MI, USA. One trap is baited with synthesized 3kPZS and the other trap is baited with control vehicle. Water flows from right to left across the figure. Odorants were administered to traps with peristaltic pumps (located on shore) and females were always captured in the trap baited with 3kPZS. Figure presented in color.

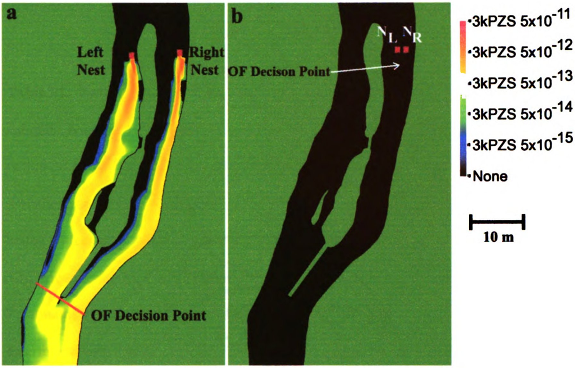

Figure S3. Experimental site and designs for comparison between 3kPZS and SMW. (a) *Direct Comparison 45 m*: One nest was baited with synthesized 3kPZS and the other nest was baited with spermiated male washings (SMW). Odorants were switched each trial. Ovulated females (OF) must choose which channel to enter 45 m downstream. Color scale illustrates final in-stream levels of 3kPZS in molar concentrations. Sandbags were placed to fortify and extend the island downstream (green lines). (b) *Direct Comparison 0 m*: Right channel contains two lamprey nests (N_L and N_R) baited with 3kPZS or SMW. Females (OF) choose which nest to enter immediately downstream. No dye data were available for this experimental set-up. Figure presented in color.

SUPPORTING TABLES

Table S1. Females exhibit upstream movement over long distances and in diverse habitats. Number of ovulated females released 650 m downstream of traps that moved within 30 m, within 1 m, and were captured when one trap was baited with 3kPZS and the other trap was baited with control solvent (3kPZS 10^{-12} M), and when both traps were baited with control vehicle (Control) in riffle and run stream segments (Figures S1 and S2). Mean time (s.d.) for ovulated females to encounter (1 m) the 3kPZS-baited trap. Treatments with the same letter are not significantly different. Distribution statistics determined with Fisher's Exact Test and time statistic determined with general linear model.

Habitat	Treatment (M)	n	30 m (n)	1 m (n)	Captured (n)	Time (min)
Riffle	3kPZS 10^{-12}	41	16 A	15 A	15 A	217 (109) A
Run	3kPZS 10^{-12}	39	19 A	16 A	3 B	140 (130) B
Riffle	Control	33	1 B	0 B	0 B	NA
Run	Control	13	1 B	0 B	0 B	NA

Table S2. Distribution of ovulated females among 3kPZS concentrations during disruption experiments (see supporting methods). The percent time ovulated females were observed in a block of a given rank when four different treatments of background 3kPZS were applied. A rank of 1 was given to the block that contained the highest average 3kPZS concentration of the blocks within that row. (a) When ovulated females were located below the SMW there was a significant interaction between treatment and rank (F = 5.795, df = 3/20, p-value = 0.005); meaning that ovulated females were more likely to enter high ranked blocks only when background 3kPZS treatment was None or 10^{-12} M, but not when 3kPZS treatment was 10^{-11} M or 10^{-10} M. (b) When ovulated females were 0 m to 20 m above SMW there was a significant effect of rank (F = 6.257, df = 1/13, p = 0.027); meaning that in all treatments ovulated females were more likely to enter high ranked blocks. (c) When ovulated females were 20 m to 0 m below the 3kPZS there was a significant effect of rank (F = 234.2, df = 1/13, p<0.001).

a.

Treatment	Rank 1	Rank 2	Rank 3	Rank4	Rank 5	Rank 6	Rank 7
No 3kPZS	31%	29%	17%	10%	3%	4%	4%
3kPZS 10^{-12}	33%	22%	22%	9%	8%	6%	1%
3kPZS 10^{-11}	23%	12%	23%	14%	10%	11%	7%
3kPZS 10^{-10}	20%	13%	20%	18%	10%	11%	9%

b.

Treatment	Rank 1	Rank 2	Rank 3	Rank4	Rank 5
3kPZS 10^{-12}	45%	14%	18%	14%	9%
3kPZS 10^{-11}	38%	5%	31%	20%	7%
3kPZS 10^{-10}	30%	9%	25%	29%	7%

c.

Treatment	Rank 1	Rank 2	Rank 3	Rank4	Rank 5
3kPZS 10^{-12}	45%	25%	21%	7%	2%
3kPZS 10^{-11}	46%	30%	20%	3%	1%
3kPZS 10^{-10}	41%	26%	20%	8%	4%

Table S3. Number of ovulated females that moved sidestream and downstream during 3kPZS disruption experiments. Number of ovulated females observed that moved sidestream and downstream when downstream of SMW and 3kPZS (location: Below SMW), when between SMW and 3kPZS (location: Below 3kPZS), and when upstream of 3kPZS application (location: Above 3kPZS). Data evaluated with logistic regression. Locations that share a letter are not significantly different ($\alpha = 0.05$).

Location	Observed (n)	Sidestream (n)	Downstream (n)
Below SMW	24	11 A	0 A
Below 3kPZS	30	12 A	4 A
Above 3kPZS	39	38 *B*	33 *B*
	X^2	36.94	67.97
	df	2	2
	p-value	<0.001	<0.001

Table S4. Swimming distance of ovulated females during 3kPZS disruption experiments. Average distance (m) that ovulated females swam during 3kPZS disruption experiments in each section of the Ocqueoc River while approaching SMW (45-60 m) or a treatment of 3kPZS (60-80 m and 80-100 m). Statistics were computed based on general linear models where distance is explained by 3kPZS treatment.

Treatment	45-60 m	60-80 m	80-100 m
None	16.9	-	-
3kPZS 10^{-12}	15.9	22.6	21.3
3kPZS 10^{-11}	19	23.4	21.4
3kPZS 10^{-10}	16.8	22.3	22.1
F-Stat	5.53	0.53	0.93
df	3/29	2/29	2/27
p-value	0.004	0.593	0.409

Table S5. Statistical procedures for behavioral data. The statistical test (nonparametric, logistic regression or general linear model (GLM)) and data transformation needed (square-root – sqrt, natural log – ln) to analyze behavioral data collected on ovulated females during 3kPZS trapping, direct comparison, and disruption experiments. Variable names are the same as those used in the data tables listed in the experiment column.

Experiment	Variable	Nonparametric	Logistic Reg.	GLM	Sqrt	ln
Trapping 70 m	Upstream		X			
Table 1-3	Captured		X			
	Time			X	X	
	Rests			X	X	
	Downstream			X		X
Trapping 650 m	30 m	X				
Table S2	1 m	X				
	Captured	X				
	Time	X				
Comparison	Distribution		X			
Table 2-3	Retention			X	X	
Disruption	Responding		X			
Table 3-3	Pass Natural		X			
	Visit 3kPZS		X			
	Bypass Natural		X			
	Respond			X	X	
	Natural			X	X	
	3kPZS			X	X	
	Speed			X	X	
Table S3	Distribution			X		
Table S4	Sidestream		X			
	Downstream		X			
Table S5	Swim Distance			X		

SUPPORTING METHODS

Animals and procedures.

Animals. Sea lampreys were captured in mechanical traps from Lake Michigan and Lake Huron tributaries from May through July in 2005, 2006, 2007, and 2008 and sexed (1). Females were held in cages (~1 m^3) in Lake Huron tributaries until ovulation. Ovulated females were immediately moved to United States Geological Survey Hammond Bay Biological Station for tagging. Tagged ovulated females were placed in an acclimation cage (1 m^3) in the study stream at least 8 h before experiments. Immediately prior to release, ovulated females were pre-exposed to odorants for at least 30 min. Females were captured by hand at the end of experimentation when possible. Females were never used twice for the same experiment.

Odorant application. Stream discharge was measured according to McMahon et al. 1996 (2) with flow meter (Flo-Mate Model 2000, Marsh-Mcbirney, Frederick, Maryland). 3kPZS was prepared for experimentation by diluting the needed amount in 1 ml of methanol and was applied to the stream by diluting the methanol solution of 3kPZS in river water (25 L if experiment was 2.5 h) and pumping the water/3kPZS solution to the stream at a rate of 10 L/h via peristaltic pump (Masterflex 7553-70, Cole-Parmer, Vernon Hills, Illinois, USA). Control solution was 1 ml of methanol dissolved into river water (25 L if experiment was 2.5 h) and pumped at a rate of 10 L/h. Batches of SMW used for direct comparison experiments with synthesized 3kPZS (Table 2-3) were prepared by placing 20 spermiated males in 110 L of aerated water for 24 h. The batch of SMW used for disruption experiments (Table 3-3) was prepared by placing 20 spermiated males in 80 L of aerated water for 8 h. Washings were aliquoted into 1 L bottles and stored at temperatures below -20^0 C until experimentation. Washings were applied to the stream by thawing the needed volume of SMW, mixing it with river water (if the experiment was 2.5 h, total volume needed to be 25 L), and pumping the water/washings solution into the stream at a rate of 10 L/h. Concentration of natural 3kPZS in SMW was determined by LC/MS using a Quattro micro API mass spectrometer (Waters, Milford, MA, USA). Samples were separated on a C18 analytical column (Symmetry, 2.1 x 100 mm, Waters) over 15 min using 80% acetonitrile/0.1% formic acid as mobile phase. Elution of 3kPZS and internal standard (3kPZS-d_5) was monitored using a multiple reaction monitoring (RMR) scanning mode for ion pairs at *m/z* 471.3>96.8 for 3kPZS and *m/z* 476.3>97.8 for 3kPZS-d_5. Calibration curve was established between 10 pg – 2 ng/injection.

Trap Design. Traps identical to those described in Johnson et al. 2006 (3) were used, with the following modifications. Two 1.1 m plastic mesh (1 cm in diameter) leads were extended from the downstream funnel at 45^0 angles. Two sandbags (0.60 m x 0.25 m x 0.25 m) were placed on the upstream side of each lead to deflect water away from the trap and one sandbag was placed in the upstream funnel to slow the velocity of water flowing through the trap (Figure S1 and S2). No block net was placed upstream of the trap. These trap modifications may have aided capture efficiency, but the study did not test the utility of the modifications.

Details for specific behavior experiments.

Female orientation to 3kPZS-baited traps from 70 m. Side of 3kPZS treatment was randomly determined by flipping a coin for each trial. 3kPZS treatments and control treatment were randomized without replacement by drawing numbers out of a hat. Ovulated females were pre-exposed to the odorants for 30 min prior to release. Five trials were conducted for each treatment from 10-June-2005 to 1-July-2005 between 0700 and 1500.

Female orientation to 3kPZS-batied traps from 650 m in riffle and run stream segments: Stream segment descriptions. Stream segments were in the Ocqueoc River, MI, USA. The riffle stream segment was located on a historic sea lamprey spawning riffle (Township 35N, Range 3E, Sections 27 and 34) and was the primary site used throughout this study since it is the natural habitat in which sea lamprey spawn. The riffle segment is characterized by water velocities around 0.60 m/sec, depths between 0.1 and 0.5 m, and a substrate nearly 100% composed of rubble and coarse gravel (Figure S1). The run stream segment was located approximately 10 km downstream of the riffle stream segment (Township 36N, Range 3E, Section 20). At this site, the river is characterized by water velocities around 0.15 m/sec, depths between 0.5 and 1.0 m, and a substrate nearly 100% composed of sand and clay (Figure S2). A more detailed description of the two sites can be found in Applegate 1950 (4).

Female orientation to 3kPZS-batied traps from 650 m in riffle and run stream segments: Experimental design. At both experimental sites a mixture of 3kPZS 10^{-12} M and 7α, 12α-dihydroxy-5α-cholan-3-one-24-oic acid (3-keto allocholic acid; 3kACA) 10^{-13} M was applied to the pheromone-baited trap. The active component applied to the traps was 3kPZS 10^{-12} M because all experiments conducted to date show that 3kACA does not elicit behaviors in ovulated females (Chapter 5). Side of 3kPZS treatment was randomly determined by flipping a coin. Ovulated females were pre-exposed to odorants for 1 h and released for 8 h. Movements into the trapping area were determined with an across channel PIT antenna arrayed 30 m downstream of the traps. Movements near the traps were determined with 2 m^2 PIT antennas arrayed around the outside perimeter of the traps to determine if ovulated females encountered the trap (entered within 1 m of odor source). Traps were checked at the end of the experiment for ovulated females. At the riffle stream section, four 3kPZS treatment trials and three control trials were conducted from 2-June-2006 to 10-June-2006 between 0600 and 1800. At the run stream section, six 3kPZS treatment trials and 2 control trials were conducted from 1-August-2005 to 8-August-2005 between 0700 and 1800.

Female preference for 3kPZS and spermiated male washings at 45m downstream. Sandbags were used to prevent water from mixing between channels through the island and sandbags were used to extend the island 12 m further downstream (Figure S3). It was confirmed that no surface water was mixing between channels by conducting rhodamine dye tests and analyzing water samples with a fluorometer. Across channel PIT antennas were arrayed at the downstream end of each channel to record movements into each channel. Pheromone was pumped into the middle of a man-made sea lamprey spawning nest approximately 0.5 m in diameter, which is a typical size of a lamprey nest in the Ocqueoc River (4). Square PIT antennas (1 m^2) were placed around each nest to record entry and retention within 0.5 m of each odorant.

In the first set of comparisons testing SMW with 3kPZS equal to 7.5×10^{-13} M, females were pre-exposed to the odor for 1 h and released for 3 h. Test odorants were switched between channels for each new trial. The four synthesized 3kPZS treatments were conducted at different times and dates: 3kPZS 5 X 10^{-13} M (0.67 times) was tested 12-June-07 to 15-June-07 between 0800 and 1200; 3kPZS 7.5 X 10^{-13} M (1.0 times) was tested 24-July-07 to 27-July-07 between 1900 and 0300; 3kPZS 10^{-12} M (1.33 times) was tested 7-July-07 to 13-July-07 between 1830 and 0200; and 3kPZS 2.5 X 10^{-12} M (3.3 times) was tested 5-July-07 to 12-July-07 between 1830 and 0100. Four trials were conducted for each 3kPZS treatment. Differences in female retention within 0.5 m of pheromone sources were evaluated with general linear models where retention was explained by the pheromone visited. In the 3.3 times 3kPZS verse SMW experiment, the nest effect was included in the model because it significantly improved model fit (Likelihood ratio test $p = 0.009$; DF =1/17; F-Stat = 8.78); meaning that in this experiment, ovulated female retention times were different between nests, regardless of pheromone treatment.

Direct comparisons of 3kPZS and SMW at 10^{-11}, 10^{-12}, 10^{-13}, and 10^{-14} M were conducted between 14-July-08 and 7-Aug-08 between 2000 h and 0230 h. Four trials were conducted at 10^{-12} M, six trials were conducted at 10^{-11} M and 10^{-13} M, and 10 trials were conducted at 10^{-14} M. The same test system was used as described above, with the exception that ovulated females were only released for 2 h after a 1 h odor exposure period. During 2008 experiments, ovulated females showed a significant preference for the right channel regardless of pheromone treatment (10^{-11} M: $p = 0.002$, $df = 1$, $X^2 = 9.17$; 10^{-12} M: $p<0.001$, $df = 1$, $X^2 = 22.36$; 10^{-13} M: $p<0.001$, $df = 1$, $X^2 = 16.43$; 10^{-14} M: $p = 0.001$, $df = 1$, $X^2 = 6.22$). To confirm that females would enter the left channel if a more preferred odorant was applied, the left channel was baited with synthesized 3kPZS at 2 x 10^{-12} M and the right channel was baited with 3kPZS at 10^{-12} M. Three trials were conducted from 26-July-08 to 28-July-08. Fourteen of 16 responding females entered the left channel baited with 3kPZS 2 x 10^{-12} M, which is significantly greater than the proportion expected to enter the left channel given a 50/50 binomial function ($p = 0.002$).

Female preference for 3kPZS and spermiated male washings when separated by 1.25 m. Four trials were conducted for each treatment from 28-July-07 to 31-July-07 between 1900 and 0430. Test odorants were switched between nests for each new trial. Cross channel PIT antennas were arrayed at the downstream end of each channel and square antennas (1 m^2) were placed around each odorant. Females were pre-exposed to the odor for 1 h and released for 3 h.

3kPZS disruption experiments. 3kPZS treatments were randomized without replacement by drawing numbers out of a hat. PIT antennas were arrayed across the stream channel 20 m and 60 m upstream of the ovulated female release point and square antennas (1 m^2) were placed around each odorant. Four trials were conduced for each treatment from 30-June-2006 to 17-July-2006 between 0700 and 1300.

To quantify ovulated female orientation in relation to estimated 3kPZS concentrations, three grid systems, aligned parallel to streamflow, were overlaid on maps illustrating lamprey movements. The lower grid covered 20 m downstream to 0 m downstream of the SMW release point; the middle grid covered 0 m upstream to 20 m upstream of SMW; the upper grid covered 20 m downstream to 0 m downstream of 3kPZS release point. The size of a square in the grid was approximately 1 m^2. Average dye concentration within each square was calculated. Squares within each row were ranked according to average dye concentration within that block; where 1 rank indicated the highest average dye concentration within that row. In the lower grid, blocks were ranked according to the average concentration of dye originating from the SMW release point.

Using the grid ranking system, ovulated female distribution in the stream was evaluated by numerating the number of times ovulated females entered blocks of given rank. Then the percentage of times ovulated females entered a block of a given rank was calculated by dividing the number of times ovulated females entered a block of a certain rank by the total number of blocks that ovulated females entered. Differences in the percentage of times ovulated females entered a block of a given rank among 3kPZS treatments were evaluated within each ranking grid system using a general linear model where the percent times ovulated females entered a block was explained by the rank of the block, the treatment, and the interaction between rank and treatment.

The distance each lamprey swam in each grid was determined by using an opisometer (ATM, Swiss) to measure the length of each lamprey track on the raw data sheets. Map distances were converted to stream distances using the map scale. Differences in average distance ovulated females swam among 3kPZS background treatments within each grid system were evaluated with a general linear model where swimming distance was explained by treatment.

Dye tests.

3kPZS disruption dye tests. During 3kPZS disruption experiments, dye tests using rhodamine (Turner Designs, Rhodamine WT, Sunnyvale, CA, USA) were conducted to model 3kPZS dilution and distribution in the stream. In the first test, rhodamine was applied at the SMW release point to reach a final in-stream concentration of 1.0 ug/L. In the second test, rhodamine was applied at both the SMW and 3kPZS release points to reach a final in-stream concentration of 1.0 ug/L. After a 10 min dye introduction period, water samples were collected in 5 ml glass vials in transects across the stream at every 0.5 m. Stream transects were located every 5 m when greater than 10 m downstream of a pheromone source and every 2.5 m when less than 10 m downstream of a pheromone source. The florescence intensity of each sample measured at 556 nm was determined in a luminescence spectrometer (Perkin Elmer LSS55, Downers Grove, IL, USA) and rhodamine concentration was estimated using a standard curve (R^2 = 0.9998). Because a test was not conducted when dye was only applied at the 3kPZS application location, the concentration of dye originating from the 3kPZS application location was estimated by calculating the difference in rhodamine concentration between the second and first dye tests at each sampling point. Rhodamine originating from the 3kPZS application location was increased by 10 and 100 times at each sampling point to estimate 3kPZS treatments of 10^{-11} M and 10^{-10} M, respectively.

3kPZS vs. SMW 45 m dye tests (Figure S3a). Three dye tests were conducted with rhodamine as described above: 1) Dye was applied to the pheromone release point in the left channel, 2) dye was applied to pheromone release point in the right channel, and 3) dye was applied to both pheromone release points. Dye was sampled in stream transects and analyzed as described above.

Dye map programming. Dye concentration in the stream was modeled by a Monotonic Piecewise Cubic Hermite Interpolation Polynomial (5). Stream maps, dye distribution maps, and ovulated female movement tracks were produced in Python

(Version 2.4, http://www.python.org/ Copyright © 1990-2006, Python Software Foundation) and Python Imaging Library (Version 1.1.6 http://www.pythonware.com/products/pil/ Copyright © 1997-2006 by Secret Labs AB & Copyright © 1995-2006 by Fredrik Lundh, Publisher: Secret Labs AB) and exported to Photoshop (Version CS2) for final display. Dye map color contours were exponentially scaled to match the back calculated concentration of 3kPZS in the stream.

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CHAPTER 4

IN-STREAM RESPONSES OF PRE-OVULATORY FEMALE SEA LAMPREYS TO PUTATIVE MIGRATORY AND MATING PHEROMONE COMPONENTS

ABSTRACT

On their journey to locate suitable spawning streams and habitat within streams, sexually immature adult sea lampreys (*Petromyzon marinus)* are directed by migratory pheromones excreted by larval lampreys. A mixture of petromyzonamine disulfate (PADS), petromyzosterol disulfate (PSDS), and petromyzonol sulfate (PZS) induce preference responses in migratory sea lampreys in a two-choice maze. When sexually mature, female sea lampreys are directed upstream to spawning nests by a mating pheromone component, 3-ketopetromyzonol sulfate (3kPZS), released by spermiated males. The objective of this study was to test the hypotheses that PADS, PSDS, and PZS direct in-stream migratory behavior of pre-ovulatory females, and that 3kPZS functions as a mating pheromone to direct upstream movement of only ovulated females. In-stream preference responses of pre-ovulatory females were recorded when exposed to three different groups of compounds: a mixture of synthesized PADS, PSDS, and PZS; a mixture of PADS, PSDS, PZS, and 3kPZS; and 3kPZS alone. 3kPZS induced directed upstream movement in pre-ovulatory females that did not differ from that of natural migratory pheromone over long distances. A mixture of PADS, PSDS, and PZS only induced directed upstream movement when spiked with 3kPZS. Results demonstrate that 3kPZS, not a mixture of PADS, PSDS, PZS, directs the upstream migration of pre-ovulatory females. 3kPZS is likely an aggregational pheromone that directs upstream movement of sexually mature and immature lampreys. These results have major implications for pheromone-based management of sea lamprey in the Great Lakes, where

it now appears that 3kPZS may be used to lure sea lampreys into traps during the migratory and spawning period.

INTRODUCTION

Pheromones are used by numerous fishes to elicit specific, adaptive behaviors or physiological responses in conspecifics (Chapter 1). Behavioral responses of fishes to pheromones in lakes or streams have rarely been described because it is challenging to unobtrusively observe fishes (Chapter 2) and few pheromones have been chemically identified and synthesized (Brennan & Zufall, 2006). Thus, most fish pheromone research has been conducted in laboratory contexts. Due to increased complexity in contextual regulation of behavior (Stowers & Marton, 2005; Ziegler, 2005) and motor systems (Mason, 1989; Novotny et al., 1986), fishes may not be expected to always respond naturally to pheromones within the constructs of laboratory conditions (Chapter 2).

Sea lamprey *(Petromyzon marinus)* pheromones have been extensively studied in laboratory contexts (Bjerselius et al., 2000; Adams et al., 1987; Teeter, 1980), but recently synthesized compounds have allowed research to be conducted in natural streams (Siefkes et al., 2005). Larval lampreys residing in natal streams release migratory pheromones that direct immature adult lampreys into suitable spawning streams (Fine & Sorensen, 2005; Moore & Schleen, 1980) and to suitable habitat within a stream (Wagner et al., 2006). Using two-choice maze bioassays, mixtures of novel bile acids have been identified and hypothesized to function as migratory pheromone components (Sorensen et al., 2005). The putative components of the migratory pheromone are petromyzonamine disulfate (PADS), petromyzosterol disulfate (PSDS), and petromyzonol sulfate (PZS). PADS, PSDS, and PZS are potent olfactory stimulants in pre-ovulatory females (Fine & Sorensen, 2008; Sorensen et al., 2005), but have not been extensively tested in natural environments.

Sexually mature male sea lampreys (spermiated) also release a mating pheromone, which induces strong preference responses in sexually mature females (ovulated) in laboratory mazes (Li et al., 2002; Teeter, 1980) and mating behaviors in spawning streams (Chapter 5). Two-choice maze experiments show that 3-ketopetromyzonol sulfate (3kPZS) is the major component of the male mating pheromone that induces preference responses (Li et al., 2002). In natural streams, 3kPZS induced directed upstream movement in ovulated females, but not in pre-ovulatory females (Siefkes et al., 2005).

Recent experimental findings have brought into question the conclusion that 3kPZS is a mating pheromone that specifically induces directed upstream movement in only ovulated females (Siefkes et al., 2005) and that PADS, PSDS, and PZS function as migratory pheromones in streams. First, when reviewing Siefkes et al. 2005, I realized that experiments evaluating responses of pre-ovulatory females to spermiated male washings and synthesized 3kPZS may not have been biologically relevant. Trials were conducted during the day when pre-ovulatory females (nocturnal) are behaviorally inactive (Applegate, 1950). Conducting behavioral assays during the day may have lead to an erroneous conclusion that pre-ovulatory females do not respond to 3kPZS. Second, ovulated females swim directly upstream (migrate) to the source of 3kPZS over long distances (Chapter 3), but when females arrive at a 3kPZS-baited nest, they are not retained (Chapter 3) and do not exhibit mating behaviors as if the nest was baited with spermiated male washings (Chapter 5). It is possible that 3kPZS released by spermiated males may function to guide the migration of mature and immature females to the spawning grounds, whereas additional mating pheromone components retain ovulated females on nests and induce spawning behaviors. Third, pre-ovulatory females have not shown a preference response to the synthesized migratory pheromones (PADS, PSDS, PZS) in streams (M. Wagner, personal communication), causing me to question whether PADS, PSDS, or PZS function as migratory pheromones. Last, during recent analyses of extracted sea lamprey larval washings, it was discovered that larvae release 3kPZS at sufficient quantities to support a potential function as a component of the migratory pheromone (Yun et al., in prep). Previously, it was assumed that only spermiated males released 3kPZS because pre-spermiated and spawning phase female sea lampreys do not release 3kPZS (Li et al., 2002).

Given recent discoveries, it was necessary to reevaluate whether PADS, PSDS, PZS, and 3kPZS induce preference responses in pre-ovulatory females. My objective was to use in-stream experiments to test the hypotheses that PADS, PSDS, and PZS direct upstream migration of pre-ovulatory females, and that 3kPZS specifically functions as a mating pheromone component that does not influence pre-ovulatory female migratory behavior.

METHODS

Experimental animals.

Use of sea lampreys was approved under Michigan State University Institutional Animal Use and Care Committee permit 05/06-066-00. Pre-ovulatory female sea lampreys were captured in Michigan from the Manistique River, a Lake Michigan tributary, in mechanical traps in late May of 2008 by the United States Fish and Wildlife Service. Females were identified by their soft abdomen and were separated from males, which were identified by their dorsal ridge (Vladykov, 1949). Sea lampreys were stored in 1000 L flow through tanks fed with Lake Huron water at ambient temperatures, which ranged from 4 to 10 °C. Lamprey holding facilities were located at the United States Geological Service Hammond Bay Biological Station, Millersburg, MI.

All females used in the field experiment were classified as pre-ovulatory because eggs were not expressed by manual pressure to the abdomen (Vladykov, 1949). To confirm that females were pre-ovulatory, gonads were collected for histological analyses. Twelve females were sacrificed on 3-June-08 and on 12-June-08 and gonadal tissue from the anterior, middle, and posterior sections were collected and fixed in 4% paraformaldehyde. Gonad samples from each lamprey were then transferred to 70% ethanol, and 10 to 30 egg cells from each gonad location were subjected to hematoxylin and eosin staining. The developmental state of oocytes was evaluated by determining whether or not an oocyte nucleus and columnar follicular cells were present. The appearance of a nucleus occurs early in the developmental state of the oocyte, more than 10 days prior to ovulation (Yorke & McMillan, 1980). Columnar follicular cells are critical to ovulation, and develop approximately 1 day prior to ovulation (Yorke & McMillan, 1980).

Test odorants.

Application of PADS, PSDS, and PZS and related bioactive components, and of 3kPZS and related bioactive components, to the experimental stream was approved by the Michigan Department of Environmental Quality and United States Environmental Protection Agency through experimental user permits 75437-EUP-1 and 75437-EUP-2, respectively. PADS, PSDS, PZS and 3kPZS were synthesized by Bridge Organics (Vicksburg, Michigan, USA). Synthesized pheromone components were analyzed for purity using a series of analytical techniques including mass spectrometry, nuclear magnetic resonance, high pressure liquid chromatograph, and thin-layer chromatograph. Purity of synthesized compounds was 95% or higher.

Female responses to synthesized pheromone components were compared to the whole migratory pheromone released by larval sea lampreys (larval washings). Water was collected from tanks containing more than 10,000 larval sea lampreys (larval washings) and was extracted using Amberlite acrylic ester XAD7HP resin (XAD, Sigma-Aldrich, St. Louis, MO; (Fine et al., 2006; Wagner et al., 2006). The migratory pheromone extract was eluted from the resin with methanol. The methanol solution of larval extract was concentrated via roto-evaporation at 35° C. Concentrated larval extract was combined into one batch and stored at -80° C until experimental use. The amounts of PADS, PSDS, PZS, and 3kPZS in the batched larval extract were measured using mass spectrometry (Table 1-4).

Table 1-4. Molecular weights of PADS, PSDS, PZS, and 3kPZS, amount (ug/L) present in larval extract, molar concentration in larval extract, and molar concentration when applied to the stream during positive control trials.

Compound	ug/L in Extract	Molecular Weight	Molar in Extract	Molar Applied to Stream
PADS	371	704	5.30E-07	1.00E-12
PSDS	18	509	3.50E-08	6.70E-14
PZS	295	474	6.20E-07	1.20E-12
3kPZS	198	472	4.20E-07	8.00E-13

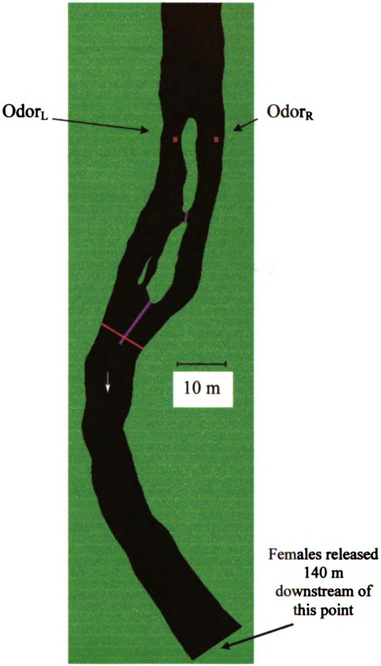
Experimental system.

Experiments were conducted in a 250 m section of the Upper Ocqueoc River about 15 km upstream of a sea lamprey barrier to prevent sea lampreys from infesting the upper river (Siefkes et al., 2005). Historically, the Upper Ocqueoc River supported significant spawning runs of sea lampreys (Applegate, 1950); however, above the barrier, wild sea lampreys have not been observed, but the stream still contains high quality larval and spawning habitat.

The upstream end the experimental stream section is divided into two channels with nearly equal discharge, width, and substrate. Stream discharge in each channel was measured at least once every third day, and after significant precipitation events using a Marsh-McBirney Flow-Mate flow meter (Marsh-McBirney Incorporated, Fredrick, Maryland). Left channel discharge (facing upstream) averaged 0.716 cubic meters per second (range 0.417 to 1.45), and right channel discharge averaged 0.890 cubic meters per second (range 0.398 to 1.93 cms) during experimentation. At the confluence of the channels, the left channel width was 6.6 m, and the right channel width was 6.9 m. Riffle habitat dominated both channels. Water temperature during the first trial, second trial, and third trial each night averaged 19.9° C (SD = 2.9), 19.1° C (SD = 2.1), and 18.6° C (SD = 2.0), respectively.

Test odorants were applied to each channel 45 m upstream of the confluence of the channels (Figure 1-4). Peristaltic pumps were used to administer methanol solutions of a test odorant to one channel and a methanol vehicle solution to the other channel, which was of equal volume to the test odorant solution. Odorant application side was switched each trial. Given this design, experimental females selected which channel

Figure 1-4. Stream segment of the Ocqueoc River, MI, used to determine preference responses of pre-ovulatory females to putative migratory and mating pheromones. 45 m upstream of the channel confluence, one channel was baited with control vehicle, and the other was baited with a synthesized pheromone mixture. Odorants were switched each trial. Females tagged with passive integrated transponders (PIT tags) were released 250 m downstream of the odorant release point. Movements into each channel were determined with a cross-channel PIT antenna arrayed in each channel 2 m upstream of their confluence. Movements within 0.5 m of each odorant application location were determined with 1 m^2 PIT antennas (Odor$_R$, Odor$_L$). Figure presented in color.



to enter 45 m downstream of the odorant sources.

Females were internally tagged with passive integrative transponders (PIT tags, 23 mm, Oregon RFID, Portland, OR) by making a small incision in their abdominal cavity with a scalpel, placing the PIT tag inside, and applying Vetbond tissue adhesive (3M, St. Paul, MN) to seal the wound. Females were tagged at least 26 h prior to experimentation. Twenty females were released in each trial. PIT tagged females were acclimated in the experimental stream for 24 h prior to experimentation in 1 m^3 cages constructed of wood and plastic mesh. Acclimation cages were placed 250 m downstream of the odorant release locations. Females were pre-exposed to the test article 1 h prior to release, and their movement and distribution in the stream were monitored for 2 h after release. 2 m upstream of the channel confluence, PIT tag detectors in the right and left channels ascertained female channel selection. A 1 m^2 PIT tag detector was placed around each odorant release location to determine whether females entered within 0.5 m of the odorant release location (Figure 1-4).

Experimental treatments and trials.

The upstream movement and distribution of female sea lampreys were evaluated when control vehicle was applied to both channels (negative control); when larval sea lamprey extract (positive control) was applied to one channel and control vehicle was applied to the other channel; and when a mixture of putative synthesized pheromones was applied to one channel, and control vehicle was applied to the other. The mixtures of synthesized pheromones tested were: 1) PADS 10^{-12} M (Molar), PSDS 5×10^{-13} M, PZS 5 x 10^{-13} M, 3kPZS 5 x 10^{-13} M, 2) PADS 10^{-12} M, PSDS 5 x 10^{-13} M, PZS 5 x 10^{-13} M, and 3) 3kPZS 5 x 10^{-13} M. All concentrations reported are based on complete mixing with stream discharge from both channels. During positive control trials, larval extract was applied to one channel to reach an in-stream PADS concentration of 10^{-12} M (Table 1-4). Application concentrations of putative pheromone components were based on their relative concentration in larval extract (Table 1-4) and on ratios in published literature (Fine & Sorensen, 2008; Sorensen et al., 2005). Experiments were conducted at night because migratory sea lampreys are nocturnal (Applegate, 1950).

Given the brief sea lamprey migratory period, up to three trials were conducted per night to increase the possible number of treatments and replicates, while reducing the probability that the maturational status of the batch of experimental animals would change during the study. During the first trial per night, odorants were started 1 h prior to sunset (typically around 20:10), females were released at sunset, and odorant application and data recording was stopped 2 h after sunset. During the first trial per night, female response to larval extract (positive control), control vehicle (negative control), and a mixture of synthesized PADS, PSDS, PZS, 3kPZS were evaluated. Trials were conducted randomly from 27-May-2008 to 8-June 2008. During the second trial per night, odorants were started at 23:30 and ended at 02:30. Female responses to larval extract, control vehicle, and a mixture of synthesized PADS, PSDS, PZS were evaluated. Trials were conducted randomly from 31-May-2008 to 12-June 2008. During the third trial per night, odorants were started at 02:40 and ended at 05:40. Female responses to larval extract, control vehicle, and 3kPZS alone were evaluated. Trials were conducted randomly from 2-June-2008 to 12-June 2008.

Data analysis.

Differences in three response variables were evaluated among treatments: 1) the proportion of females that moved upstream into the bifurcated section, 2) the proportion of females that entered the baited channel, and 3) the proportion of females that entered within 0.5 m of the odorant in the baited channel. The numbers of females that moved upstream into the bifurcated stream segment were numerated, summed, and expressed as proportions according to treatment, where the denominator was the total number of females released. The numbers of females that entered the baited channel and control channel were numerated, summed, and expressed as proportions according to treatment, where the denominator was the total number of females that entered a channel. The numbers of females that entered within 0.5 m of each odorant source were numerated, summed, and expressed as proportions according to treatment, where the denominator was the total number of females that entered a channel. Experimental data were combined and analyzed in three ways: 1) within an experimental time period, differences in response variables among treatments were evaluated (i.e. first trial per night), 2) within all larval extract trials, differences in response variables at different time periods were evaluated, and 3) all experimental data were combined and differences in response variables among treatments were evaluated.

Fixed effect generalized linear models with a binomial distribution (logistic regression) were used to evaluate summarized data. For all combined data and data summarized within individual experimental time periods, variability in a response variable was explained by pheromone treatment. For data from combined larval extract trials, variability in a response variable was explained by the time of night the trial was conducted (1st, 2nd, or 3rd trial per night). During trials when pheromone was applied, the proportion of females entering the baited channel and proportion of females entering within 0.5 m of the odorant in the baited channel was statistically compared to negative control trials by assigning a "pheromone" channel. The right channel was always the "pheromone" channel during the first control trial within each group of analyzed data. The assigned "pheromone" channel was switched during each control trial.

Each response variable in each data set was also fit to a nested mixed effect generalized linear model with a binomial distribution, where variability in a response variable was explained by the fixed effect of pheromone treatment and the random effect of trial date. Each mixed effect model was compared to its nested fixed effect model using a likelihood ratio test to determine whether the mixed effect model significantly improved model fit. If the likelihood test yielded a p-value less than 0.05, then the mixed effect model improved fit and was used for statistical purposes instead of the fixed effect model. All generalized linear models used for data analysis showed no evidence of overdispersion or nonlinearities. Statistical results reported are from two-tailed tests.

RESULTS

Maturity state of females.

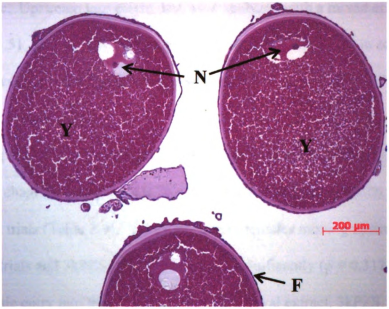
Oocytes of experimental animals were several days from ovulation. A nucleus was not visible in 84% and 80% of oocytes sampled on 3-June-2008 and on 12-June-2008, respectively. The lack of a nucleus indicates that oocytes were many days from being fully developed. Columnar follicular cells were not present in any of the oocytes evaluated, confirming that none were in late stages of emergence.

Responses of pre-ovulatory females to putative pheromones.

Results according to trial time. During the first trial per night, the proportion of females moving upstream into the bifurcated stream segment did not vary among treatments (Table 2-4). A higher proportion of females entered the treated channel during larval extract and PADS, PSDS, PZS, 3kPZS (mixture) trials than the randomly selected "treatment" channel during control trials (Table 3-4 and herein). The proportion of females entering the baited channel during larval extract and PADS, PSDS, PZS, 3kPZS trials did not vary significantly ($p = 0.400$, $df = 197$, z-stat = -0.84). A higher proportion of females entered within 0.5 m of the larval extract source and the PADS, PSDS, PZS, 3kPZS source than control odorant sources. Larval extract directed a higher proportion of females to within 0.5 m of the release location than did PADS, PSDS, PZS, 3kPZS ($p < 0.001$, $df = 197$, z-stat = -4.18). Upstream movement data were analyzed with a mixed effect model (Likelihood ratio test; $p = 0.009$, $df = 1$, $X^2 = 6.94$). Channel preference and entries within 0.5 m of the odorant source were evaluated with fixed effect models (Likelihood ratio tests; $p = 0.098$, $df = 1$, $X^2 = 2.78$; $p > 0.5$, $df = 1$, $X^2 = 0.01$;

Figure 2-4. Oocytes of a pre-ovulatory female taken from the batch of lampreys used for field experiments testing putative migratory and mating pheromones. Oocytes displayed were immature and did not have a nucleus or columnar follicular cells. About 80% of oocytes examined were in this stage of maturity. Y = yolk. Figure presented in color.

Figure 3-4. Oocytes of a pre-ovulatory female taken from the batch of lampreys used for field experiments testing putative migratory and mating pheromones. Oocytes displayed were immature, but were in later stages of development than oocytes in Figure 3-3 as they had a nucleus. No columnar follicular cells were present. About 20% of oocytes examined were in this stage of maturity. N = nucleus, Y = yolk, F = follicular cells. Figure presented in color.



respectively).

During the second trial per night, the proportion of females moving upstream into the bifurcated stream segment did not differ among treatments (Table 2-4). A higher proportion of females entered the baited channel during larval extract trials than the proportion entering the baited channel during PADS, PSDS, PZS trials and control trials (Table 2-4 and herein). A higher proportion of females entered within 0.5 m of the larval extract source than those that entered within 0.5 m of sources of PADS, PSDS, PZS and control vehicle. Upstream movement data were analyzed with a mixed effect model ($p < 0.001$, $X^2 = 51.51$, df = 1). Channel preference and entries within 0.5 m of the odorant source were evaluated with fixed effect models ($p > 0.50$, df = 1, $X^2 = -0.01$; $p > 0.50$, df = 1, $X^2 = -0.01$; respectfully).

During the third trial per night, the proportion of females moving upstream into the bifurcated channels was greater during larval extract trials and 3kPZS trials than during control trials (Table 2-4). The proportion of females moving upstream during larval extract trials and 3kPZS trials did not differ significantly ($p = 0.312$, df = 1, z-value = 1.01). Female entry into the channel treated with larval extract, 3kPZS, and control vehicle did not differ significantly (Table 3-4 and herein). A higher proportion of females entered within 0.5 m of a source of larval extract than a source of control odorant. Female entry within 0.5 m of a source of 3kPZS did not differ significantly from larval extract and control methanol treatments (3kPZS vs. Extract: $p = 0.070$, df = 1, z-value = -1.81). Upstream movement, channel preference, and entries within 0.5 m of the odorant source were evaluated with fixed effect models ($p = 0.403$, df = 1, $X^2 = 0.70$; $p > 0.50$, df = 1, $X^2 = 0.38$; $p > 0.50$, df = 1, $X^2 = 0.31$; respectively).

Table 2-4. The number of pre-ovulatory female sea lampreys that were released (n) and the proportion that moved upstream into the bifurcated stream segment when an odorant was applied to one channel and control vehicle was applied to the other, or when control vehicle was applied to both channels (Control Vehicle). Statistical results presented are separated into data collected during the first, second, and third trials per night. A p-value less that 0.05 indicates a significant difference when compared to control vehicle trials. Treatments with the same letter within each group did not differ significantly.

Treatment- 1st Trial	**Trials**	**n**	**% Upstream**	**p-value (df, z-stat)**
Control Vehicle	3	60	67%	NA, A
Larval Extract	4	80	85%	*0.098 (258, 1.66) A*
PADS, PSDS, PZS, 3kPZS	6	120	75%	*0.442 (258, 0.77) A*
Treatment - 2nd Trial				
Control Vehicle	2	40	38%	NA, A
Larval Extract	2	40	65%	*0.424 (238, 0.80) A*
PADS, PSDS, PZS	8	160	42%	0.897 (238, 0.13) A
Treatment - 3rd Trial				
Control Vehicle	2	40	25%	NA, A
Larval Extract	2	40	50%	0.023 (199, 2.27) B
3kPZS	6	120	59%	< 0.001 (199, 3.58) B

Table 3-4. Number of pre-ovulatory female sea lampreys moving upstream into the bifurcated stream segment (n), percent females entering the pheromone-baited and control channels, and percent females entering within 0.5 m of the odorant release location (pheromone, control). Statistical results presented are separated into data collected during the first, second, and third trials per night. A p-value less that 0.05 indicates a significant difference from control vehicle trials. Treatments with the same letter within each group did not differ significantly.

Treatment- 1st Trial	**n**	**Pheromone Chan.**	**Control Chan.**	**p-value (df, z-stat)**	**Pheromone**	**Control**	**p-value (df, z-stat)**
Control Vehicle	40	40%	60%	NA, *A*	8%	8%	NA, *A*
Larval Extract	68	75%	25%	*<0.001 (195, 3.74), B*	62%	1%	*<0.001* (195, 4.46), *C*
PADS, PSDS, PZS, 3kPZS	90	69%	31%	*<0.001* (195, 3.28), *B*	28%	3%	*0.009* (195, 2.61), *B*
Treatment - 2nd Trial							
Control Vehicle	15	36%	64%	NA, A	20%	13%	*NA, A*
Larval Extract	26	62%	31%	*0.012* (105, 2.52), *B*	62%	0%	*0.015* (105, 2.44), *B*
PADS, PSDS, PZS	67	43%	57%	0.242 (105, 1.17), *A*	16%	12%	0.739 (105, -0.33), *A*
Treatment - 3rd Trial							
Control Vehicle	10	50%	50%	NA, *A*	0%	0%	NA, *A*
Larval Extract	20	80%	20%	0.101 (98, 1.64), *A*	55%	5%	*0.036* (98, 2.09), *B*
3kPZS	71	68%	32%	0.280 (98, 1.08), *A*	32%	6%	0.178 (98, 1.35), *AB*

Responses to larval extract throughout the night. The proportion of females swimming upstream into the bifurcated stream segment was significantly greater during the first trial per night than the third trial per night (Table 4-4). The proportion of upstream migrantes entering the channel baited with larval extract did not vary significantly depending on whether extract was applied during the first, second, or third trial per night (Table 5-4 and herein). The proportion of females entering within 0.5 m of the larval extract source did not differ significantly among trials conducted during the three time periods. Upstream movement data were analyzed with a mixed effect model ($p < 0.001$, df = 1, $X^2 = 13.24$). Channel preference and entries within 0.5 m of the odorant source were analyzed with fixed effect models ($p = 0.113$, df = 1, $X^2 = 2.51$; $p > 0.50$, df = 1, $X^2 < 0.01$; respectively).

Combined data analysis from all trials. When data from all trials were combined, the proportion of females moving upstream into the bifurcated stream segment during larval extract and 3kPZS trials was significantly greater than the proportion of females moving upstream during control vehicle or PADS, PSDS, PZS trials (Table 4-4; larval extract vs. PADS, PSDS, PZS: $p = 0.003$, df = 697, z-stat = -2.93; 3kPZS vs. PADS, PSDS, PZS = $p = 0.001$, df = 697, z-stat = -3.20). The proportion of females moving upstream did not differ between larval extract trials and PADS, PSDS, PZS, 3kPZS trials ($p = 0.194$, df = 697, z-stat = -1.30), and did not differ between larval extract trials and 3kPZS trials ($p = 0.100$, df = 697, z-stat = 1.645). Combined upstream movement data were evaluated with a mixed effect generalized linear model, where treatment was a fixed effect and trial date and time were random effects. Trial date was included in the model because likelihood ratio tests showed that trial date was an important random effect in models describing upstream movement within an individual time period. Treatment time was included in the model because it was an important fixed effect influencing upstream movement when all larval extract trials were compared.

Larval extract, a mixture of synthesized PADS, PSDS, PZS, 3kPZS, and 3kPZS alone elicited significant preference responses for the baited channel (Table 5-4 and herein). A mixture of PADS, PSDS, PZS did not elicit a significant preference for the baited channel. The proportion of females entering the channel baited with larval extract, PADS, PSDS, PZS, 3kPZS, and 3kPZS alone did not differ significantly. A higher proportion of females entered within 0.5 m of the larval extract source, the mixture of PADS, PSDS, PZS, 3kPZS, and the 3kPZS odorant source than sources of control odorant or a mixture of PADS, PSDS, PZS (PADS, PSDS, PZS vs. Extract: $p < 0.001$, df = 406, z-stat = 5.39; PADS, PSDS, PZS vs. PADS, PSDS, PZS, 3kPZS: $p = 0.10$, df = 406, z-stat = 1.66; PADS, PSDS, PZS vs. 3kPZS: $p = 0.0321$, df = 406, z-stat = 2.14). Larval extract lured significantly more females to within 0.5 m of the pheromone release location than did a mixture of PADS, PSDS, PZS, 3kPZS or 3kPZS alone ($p < 0.001$, df = 406, z-stat = -4.56; $p < 0.001$, df = 406, z-stat = -3.66; respectively). Data were analyzed with a fixed effect generalized linear model where variability in channel preference and entry within 0.5 m of the odorant source was explained by treatment. Trial date was not included in the model because likelihood ratio tests within individual time periods showed that trial date was an unimportant random effect describing channel preference and movement within 0.5 m of an odorant source. Treatment time was not included in the model because it did not influence channel preference or movement within 0.5 m of the odorant source when all larval extract trials were compared.

Table 4-4. The number of pre-ovulatory female sea lampreys that were released (n) and the proportion that moved upstream into the bifurcated stream segment when an odorant was applied to one channel and control vehicle was applied to the other, or when control vehicle was applied to both channels. Statistical results presented are separated into the data collected during larval extract trials at different times of night and combined data from all trials. A p-value less that 0.05 indicates a significant difference when compared to the first larval extract trial per night (Positive Controls) or control trials (All Trials). Treatments with the same letter within each group did not differ significantly.

Positive Controls	Trials	n	% Upstream	p-value (df, z-stat)
Larval Extract 1st	4	80	85%	NA, A
Larval Extract 2nd	2	40	65%	0.155 (158, -1.42) AB
Larval Extract 3rd	2	40	50%	<0.001 (158, -3.87) B
Treatment - All Trials				
Control Vehicle	8	160	41%	NA, A
Larval Extract	8	160	71%	*0.009 (697, 2.61) BC*
PADS, PSDS, PZS, 3kPZS	6	120	75%	*0.481 (697, 0.705) AC*
PADS, PSDS, PZS	8	160	42%	0.365 (697, -0.906) A
3kPZS	6	120	59%	*<0.001 (697, 3.84) B*

Table 5-4. Number of pre-ovulatory female sea lampreys moving upstream into the bifurcated stream segment (n), percent females entering the pheromone-baited and control channels, and percent females entering within 0.5 m of the odorant release location (pheromone, control). Statistical results presented are separated into data collected during larval extract trials at different times of night (Positive Control) and combined data from all trials (All Trials). A p-value less that 0.05 indicates a significant difference from the first larval extract trial per night or control trials. Treatments with the same letter within each group did not differ significantly.

Positive Controls	**n**	**Pheromone Chan.**	**Control Chan.**	**p-value (df, z-stat)**	**Pheromone**	**Control**	**p-value (df, z-stat)**
Larval Extract 1st	68	75%	25%	NA, *A*	62%	1%	NA, *A*
Larval Extract 2nd	26	62%	31%	0.572 (111, -0.57), *A*	62%	0%	0.984 (111, -0.02), *A*
Larval Extract 3rd	20	80%	20%	0.645 (111, 0.46), *A*	55%	5%	0.588 (111, -0.54), A
Treatment - All Trials							
Control Vehicle	65	42%	58%	NA, *A*	9%	11%	NA, *A*
Larval Extract	114	75%	25%	*<0.001 (402, 4.28), B*	60%	2%	*<0.001 (402, 5.78), C*
PADS, PSDS, PZS, 3kPZS	90	69%	31%	*0.001 (402, 3.35), B*	28%	3%	*0.007 (402, 2.72), B*
PADS, PSDS, PZS	67	43%	57%	0.839 (402, 0.20), A	16%	12%	0.223 (402, 1.22), A
3kPZS	71	68%	32%	*0.003 (402, 3.02), B*	32%	6%	*0.002 (402, 3.11), B*

DISCUSSION

Upstream movement of pre-ovulatory females into the bifurcated stream segment was influenced by the time of night females were released. During larval extract trials females were more likely to move upstream during the first trial per night. This result is consistent with a study that showed that migratory lampreys are most active during the early portion of the night (Binder & McDonald, 2008). The proportion of females moving upstream during a trial also varied from night to night given the same odorant treatment. Variation in the proportion of females moving upstream on different trial dates and times is likely related to temperature or other stream conditions (Binder & McDonald, 2008; Applegate, 1950). In the analysis of all upstream movement data, a mixed effect model with random effects of trial date and time allowed for the simultaneous evaluation of all odorant treatments by accounting for variation in the data attributed to trial time and date.

Pre-ovulatory females displayed similar preference responses to migratory pheromones regardless of the trial time. Females that moved upstream into the bifurcated stream segment showed similar preference responses to larval extract throughout the night. Date of the trial did not influence female entry into the baited channel or entry within 0.5 m of any odorant treatment because the random effect of trial date in mixed effect models did not significantly improve model fit. Given that preference responses of females did not change throughout the night or on different trial dates, all experimental data describing entry into the baited channel and entry within 0.5 m of an odorant source were combined and analyzed with a fixed effect model.

A benefit of conducting three trials per night was that changes in female maturity and responsiveness to pheromone treatments over the experimental period were reduced because only 16 days were required to conduct the study rather than 35 days needed if only one trial per night were conducted. Histological analyses of gonads showed that females used in the study were several days from ovulation and the developmental state of the gonads did not change substantially over the duration of the study. Conducting three trials per night did generate the potential confounding effect that behavioral responses of females to odorants may vary through the night. Statistical analyses showed that female preference responses to larval extract did not vary through the night and that behavioral responses to all odorant treatments did not vary among trials. Therefore, the discussion below will focus on analyses of response variables of all combined data.

Synthesized 3kPZS influenced the large-scale movement patterns of pre-ovulatory females in a natural stream. 3kPZS alone at 5×10^{-13} M lured more females into the baited channel than the control vehicle. The ability of 3kPZS to lure females into the baited channel of the river did not differ from that of extracted migratory pheromone. 3kPZS also influenced the fine-scale movement patterns of pre-ovulatory females in streams. 3kPZS lured more females within 0.5 m of the odorant release point than the control vehicle. The ability of 3kPZS to lure females to the exact point of release was less than that of larval extract. Larval extract lured about 60% of females that moved upstream within 0.5 m of the odorant application, while 3kPZS only lured about 30% of females to within 0.5 m. Surprisingly, the mixture of putative migratory pheromones, PADS, PSDS, PZS (Sorensen et al., 2005), did not lure females into the baited channel or to within 0.5 m of the odorant release point, unless the mixture was spiked with 3kPZS.

Data that demonstrate attraction of pre-ovulatory females to 3kPZS are highly unexpected given the current understanding of sea lamprey chemical communication. In field conditions, pre-ovulatory females have not shown a preference response to 3kPZS (Siefkes et al., 2005). In a two-choice maze, pre-ovulatory females have not shown a preference response to spermiated male washings which contain 3kPZS (Siefkes et al., 2005). The discrepancies in results are likely attributed to the fact that the current study evaluated behavioral responses at night when pre-ovulatory females migrate, whereas previous studies were conducted during the day.

Whether pre-spermiated males show preference responses to 3kPZS remains unknown. In a two-choice maze, pre-spermiated males were not attracted to washings from spermiated males (Siefkes et al., 2005). However, these experiments were also conducted during the day when pre-spermiated males are inactive. If 3kPZS is a major component of the migratory pheromone, pre-spermiated males will likely be attracted to 3kPZS because larval odorants attract both sexes in a two-choice maze (Vrieze & Sorensen, 2001; Bjerselius et al., 2000). Spermiated male washings may also be attractive to sexually immature adult sea lampreys. Immature lampreys may show strong preference responses to spermiated male washings because they contain 3kPZS and they act as priming cues, which increase maturational rates (Chung-Davidson et al., in prep). Furthermore, spermiated male washings may be an honest indicator of suitable spawning habitat, the ultimate destination for migrating lampreys. Future experiments should directly compare in-stream responses of immature male and female sea lampreys to larval and spermiated male washings at night.

It was equally unexpected that putative migratory pheromone components PADS, PSDS, PZS did not induce preference responses in pre-ovulatory females in a natural stream. The chemical purity and stereo-configuration of synthesized PADS, PSDS, PZS have been confirmed by an outside party and match that published in Sorensen et al. 2005 (S-S. Yun, personal communication). It now seems plausible that PADS, PSDS, PZS may not function in streams, but at river mouths, where they could act as settlement cues that retain females until conditions are suitable to enter the stream and migrate (Applegate, 1950). Alternatively, behavioral responses of females to PADS, PSDS, PZS may be an artifact of the laboratory two-choice maze employed by Sorensen et al. 2005.

Results show that 3kPZS may induce upstream movement in pre-ovulatory females. In the combined analysis of all data, more females moved upstream to the bifurcated stream segment when 3kPZS was applied than when control vehicle or a mixture of PADS, PSDS, PZS was applied. Because 3kPZS is released by both spermiated males and larvae, upstream movement upon detection of 3kPZS would bring females closer to larval and spawning habitat. High 3kPZS concentrations may indicate the presences of spermiated males on spawning grounds and may speed sexual maturation (Chung-Davidson et al., in prep). Furthermore, although this difference was not statically significant (Table 4-4, $p = 0.10$), 3kPZS alone induced a larger proportion of females to move upstream than larval extract or a mixture of PADS, PSDS, PZS, 3kPZS. These data support the hypothesis that PADS, PSDS, or PZS may serve as settlement cues for sexually immature adult sea lampreys by slowing upstream movement. The above hypothesis is intriguing, but also very speculative. Additional field experiments are needed to determine the function of PADS, PSDS, and PZS.

Pre-ovulatory and ovulated females respond similarly to synthesized 3kPZS. First, 3kPZS induced directed upstream movement in pre-ovulatory and ovulated females. Previous experiments in the same study stream documented that when 3kPZS was applied at 10^{-12} M, about 90% of ovulated females moving upstream into the bifurcated segment swam up the baited channel and within 0.5 m of the 3kPZS source (Chapter 3). In the present study, nearly 70% of pre-ovulatory females that swam upstream entered the 3kPZS-baited channel and 30% entered within 0.5 m of the release point. The precision by which ovulated females locate the source of 3kPZS is higher than pre-ovulatory females. This makes sense biologically because ovulated females would need to locate the exact position of a spermiated male on a nest, whereas pre-ovulatory females only need to locate suitable habitats within streams while migrating. Ovulated females track plumes of 3kPZS by moving directly upstream when the pheromone is detected, as well as moving sidestream and downstream when the plume is lost, likely using odor-conditioned rheotaxis (Chapter 3). Pre-ovulatory females may also use odor-conditioned rheotaxis because they navigate similar hydrodynamic environments as ovulated females. More research should be conducted to understand how pre-ovulatory females track pheromone plumes of 3kPZS.

It is intriguing to consider how 3kPZS may function as the main component of both the migratory and mating pheromone. Pre-ovulatory females were equally likely to enter channels baited with 3kPZS and larval extract, but fewer females located the exact source of 3kPZS. Similarly, ovulated females are lured to the exact released point of 3kPZS, but spermiated male washings retain ovulated females at the release point longer than 3kPZS (Chapter 3) and induce reproductive behaviors (Chapter 5). Larval and spermiated male sea lamprey likely release additional pheromone components that induce behaviors specific to the migratory pheromone or mating pheromone, whereas 3kPZS could be a pheromone component released by both life stages to simply induce directed upstream movement.

Given data from the present study and past studies, I now postulate that 3kPZS is an aggregational pheromone that induces directed upstream movement in pre-spermiated males, pre-ovulatory, and ovulated females to amass them near spawning grounds and aggregate spawning pairs on nests for reproduction. Following this line of logic, it is possible that the migratory pheromone is defined by the minor components that influence stream settlement (Moore & Schleen, 1980) and fine scale in-stream preference responses. PADS, PSDS, or PZS are likely not the components that direct fine scale upstream movement, but may function as settlement cues for lampreys at river mouths. The mating pheromone may be defined by minor components that induce near-source attraction, retention on the nest, and mating behaviors (Chapters 3 and 5). Additional mating pheromone components are currently being characterized and identified (Chapter 5).

The discovery that 3kPZS may be an aggregational pheromone has major implications for how sea lamprey populations in the Great Lakes may be managed with pheromones (Twohey et al., 2003), and accentuates the potential utility of 3kPZS. Based on previously published data, it appeared that two separate pheromones, consisting of separate components, could enhance sea lamprey management. The migratory pheromone could be used early in the season to direct immature migratory lampreys into preferred streams, stream channels, or baited traps. The capture of additional males would enhance the sterile male release program, and removal of fertile females would reduce reproductive potential. The mating pheromone could escalate final attempts to reduce the number of viable eggs by removing ovulated females immediately prior to spawning. Given the proposed revision to the sea lamprey pheromone paradigm, it appears that 3kPZS alone induces the sought after behavioral responses of both the migratory and mating pheromone; directed upstream movement. 3kPZS could be used to modify the behavior and distribution of females during the entire migratory period, in addition to the spawning period. Applications of 3kPZS may actually be most effective during the migratory period when competition from spermiated males will be nonexistent. Applying only 3kPZS for pheromone-based control is a distinct advantage to applying multiple components because a single compound is cheaper, easier to register with regulatory agencies, and simple to apply.

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CHAPTER 5

BEHAVIORAL RESPONSES OF OVULATED FEMALE SEA LAMPREYS TO 3-KETO ALLOCHOLIC ACID AND PHEROMONE EXTRACTS

ABSTRACT

Spermiated male sea lampreys (*Petromyzon marinus*) release 3-keto petromyzonol sulfate (3kPZS), which induces robust upstream movement in ovulated females, but fails to retain them on nests or induce spawning behaviors with the potency of the natural male pheromone. The purpose of this study was to search for mating pheromone components that retain females on nests. The first objective was to test the in-stream responses of ovulated females to the putative pheromone component 3-keto allocholic acid (3kACA), which was hypothesized to increase the potency of 3kPZS by functioning to retain females on nests and induce spawning behaviors. 3kACA alone did not induce preference responses in females, and mixing 3kACA with 3kPZS did not increase the potency of 3kPZS to attract or retain females on a nest. The second objective was to determine if extracts of spermiated male washings from XAD7HP and C18 resins contain all behaviorally active mating pheromone components. Extraction products were tested in a new in-stream bioassay, which allowed the display of mating behaviors and the simultaneous comparison of two odorants. XAD7HP resin captured and eluted all behaviorally active pheromone components in spermiated male washings, but C18 solid phase extraction did not. The third objective was to determine the release location of unidentified pheromones by screening spermiated male washings from the head and tail region for behavioral activity using the in-stream bioassay. All behaviorally active pheromones were present in spermiated male head washings. Future efforts to identify pheromone components should use XAD extraction techniques, analysis of head

washings, and the in-stream bioassay to isolate compounds that elicit behaviors in natural streams.

INTRODUCTION

Chemical communication in sea lamprey (*Petromyzon marinus*) is critical to the successful completion of their multi-phase life history in a single spawning event. Filter feeding larval sea lampreys, prior to transformation into parasites of large fishes, live in natal streams (Applegate, 1950). Larvae excrete migratory pheromones that direct the migration of adult lampreys from expansive ocean or lake environments to suitable habitats within streams (Sorensen & Vrieze, 2003). Fully mature males build nests in riverine riffle habitats and secrete a mating pheromone that is highly attractive to ovulated females, luring them to spawning nests (Li, 2005). Interference with this chemical communication system may offer unique and environmentally sound ways of managing the sea lamprey (Twohey et al., 2003), a destructive invader of the Laurentian Great Lakes (Smith & Tibbles, 1980). For practical widespread use of a pheromone in pest management, the chemical structure of the pheromone must be identified and synthesized. Historically, sea lamprey pheromones have been identified through extraction of compounds from lamprey holding water, separation of extraction products (fractionation), testing of fractions for electro-olfactogram (EOG) potency, testing of EOG potent fractions in a two-choice maze, and identification of compounds which elicit responses in a two-choice maze (Sorensen et al., 2005; Li et al., 2002).

All putative sea lamprey pheromones identified to date are bile acids. The sea lamprey olfactory organ is highly discriminatory to a wide variety of bile acids and is especially sensitive to sulfated bile acids (Sorensen et al., 2005; Siefkes & Li, 2004; Li & Sorensen, 1997; Li et al., 1995). Three sulfated bile acids, petromyzonamine disulfate

(PADS), petromyzosterol disulfate (PSDS), and petromyzonol sulfate (PZS) (Sorensen et al., 2005) and one non-sulfated bile acid, allocholic acid (ACA) (Bjerselius et al., 2000), have been hypothesized to function as migratory pheromone components released by larvae to direct adults upstream. Interestingly, two putative mating pheromone components released by spermiated males, 3-ketopetromyzonol sulfate (3kPZS) and 3-keto allocholic acid (3kACA), are nearly identical in structure to putative migratory pheromone components and are only differentiated by their 3-keto functional group (Yun et al., 2003). Most recently, four unusual unsaturated bile acids with 4-ene configurations have been identified from washings of spermiated males and have been suspected to function as mating pheromone components (Yun et al., in prep).

Taken together, 11 putative bile acid sea lamprey pheromones have been chemically identified, but only one has elicited behavioral responses in natural streams when tested (3kPZS). All putative pheromones were extracted from holding water of larvae or mature male lampreys and most were potent olfactory stimulants and attractants in a two-choice maze. Historically, results of two-choice maze experiments have rarely been verified in natural streams. More recently, however, researchers have utilized field environments in attempts to confirm the function of putative pheromones because synthesized compounds and permits have become available. Results of field experiments testing putative pheromone components have often not corroborated results obtained in laboratory two-choice mazes. The most striking example is that a mixture of putative migratory pheromone components, PADS, PSDS, and PZS, do not elicit preference responses in migratory females in natural streams (Chapter 4, Wagner et al. unpublished data), whereas they elicit responses in a two-choice maze (Sorensen et al., 2005).

Similarly, synthesized 3kPZS and spermiated male washings (SMW) induced indistinguishable behavioral responses in ovulated females in a two-choice maze (Li et al., 2002), but in a natural stream synthesized 3kPZS did not retain ovulated females on nests with the potency of spermiated male washings (Chapter 3). The overarching purpose of this study was to search for mating pheromone components that retain females on nests and induce spawning behaviors.

A mating pheromone released by spermiated male sea lampreys can be expected to contain multiple components because females must display multiple behaviors to successfully spawn; for instance, migration to the spawning grounds, landing on a nest, and mating (Chapter 1). In insects minor pheromone components are often slight chemical modifications of the major component (Howse et al., 1998), and it is possible that this theme is conserved in vertebrate pheromones. Spermiated male sea lampreys have been shown to release several compounds that are slight modifications of 3kPZS. For example, four unusual 4-ene derivatives of 3kPZS were isolated from spermiated male washings (Yun et al., in preparation). 4-ene bile acids were not tested for EOG potency or for preference responses in a two-choice maze, but were identified based on evidence that they resembled 3kPZS. Field trials showed that synthesized 4-ene bile acids did not induce strong preference responses in ovulated females and did not retain them on spawning nests (Yun et al., in preparation).

3kACA, a novel bile acid, has also been isolated from spermiated male washings (Yun et al., 2003). 3kACA resembles 3kPZS because it has the same 3-keto functional group as 3kPZS, but lacks the sulfate. 3kACA is a potent olfactory odorant in female sea lampreys and is distinguishable from 3kPZS as it binds to separate olfactory receptors

(Siefkes & Li, 2004). 3kACA is a primary pheromone suspect and has been hypothesized to function alone or to enhance the potency of 3kPZS (Yun et al., 2003), but has not been tested in stream environments. Therefore, the first objective of this study was to determine whether 3kACA alone, or when mixed with 3kPZS, modifies ovulated female behavior in streams. Experiments were specifically designed to determine whether 3kACA influences one or more of the multiple behaviors females exhibit to successfully mate. It was tested whether 3kACA directs long distance upstream movement of ovulated females, influences spawning nest selection, influences retention on a nest, or increases the likelihood females will exhibit mating behaviors on a spawning nest.

Recent field studies have revealed a clear necessity to develop more effective methods to identify pheromones that modify lamprey behavior in natural environments. Two methods have been primarily used to extract pheromones from lamprey holding water. One procedure utilizes Amberlite XAD7HP resin (XAD, Sigma-Aldrich, St. Louis, MO) packed in glass columns. XAD extraction procedures were developed to extract large quantities of migratory pheromones from larval holding water (Fine et al., 2006). A second procedure utilizes C18 solid phase extraction (sep-pak, Waters Corporation, Milford, MA). During the identification of 3kPZS, C18 solid phase extraction was used to extract and concentrate 3kPZS from spermiated male washings (Li et al., 2002). Therefore, a potentially practical method to extract and concentrate large quantities of male pheromone for chemical identification would be to employ both techniques in succession. Large quantities of male pheromone could be extracted from SMW using XAD resin (Fine et al., 2006). The XAD extract could then be concentrated using C18 sep-paks, thereby preparing the sample for analytical separation techniques. Therefore, the second objective in this study was to determine whether XAD7HP and C18 solid phase extraction techniques effectively capture and elute all behaviorally active mating pheromone components.

A bioassay must be used throughout the extraction and fractionation process to determine whether behaviorally active components are still present in the extract sample or fraction (Yambe et al., 2006). If the bioassay limits the display of pheromone-induced behaviors, conclusions could be uninformative or erroneous. Accordingly, the second objective this study was accomplished by developing an in-stream bioassay that allows direct comparison of two odors and the display of pheromone-induced behaviors, such as retention on a nest, rock movements, and tail fans. A new bioassay was necessary because traditional two-choice maze methods do not allow the display of mating behaviors and currently have a 9% success rate in determining whether a compound modifies sea lamprey behaviors in natural streams (11 compounds identified, 1 elicits in-stream behaviors).

Identification of the release location of pheromones can eliminate many potential compounds as pheromones. For example, if all behaviorally active compounds are released through the head region, then compounds released through the urine could be eliminated as possible pheromones. 3kPZS is released in the head region likely through glandular cells in the gills (Siefkes et al., 2003), but it is unknown where pheromone components that retain females on nests are released. Therefore, the third objective of this study was to use the in-stream bioassay to determine whether pheromones that retain females on nests are excreted through the head region or tail region.

METHODS

General methods.

Experimental animals. Use of sea lampreys was approved under Michigan State University Institutional Animal Use and Care Committee permit 05/06-066-00. Sea lampreys were captured in mechanical traps from Great Lakes tributaries from May through July during 2005, 2006, and 2008 by the United States Fish and Wildlife Service, Marquette, MI. Males and females were manually separated. Males were identified by their dorsal ridge and females were identified by their soft abdomen (Vladykov, 1949). Females were further classified as pre-ovulatory or ovulated. Ovulated females expressed eggs upon manual pressure to the abdomen, whereas pre-ovulatory females did not. Pre-ovulatory females were stored at the United States Geological Survey Hammond Bay Biological Station, Millersburg, MI, in 1000 L flow-through tanks fed with Lake Huron water at ambient temperatures ranging from 4 to 18 °C. Groups of pre-ovulatory females were moved to cages (~ 1 m^3) located in Lake Huron tributaries to allow them to mature in stream environments. Pre-ovulatory females typically ovulated within seven to 10 days of being placed in a cage. Ovulated females were removed from the stream and taken back to the biological station for tagging prior to experimentation.

Ovulated females were used in all experiments described in this study. All ovulated females were fitted with two 10 cm colored polyethylene streamer tags (Hallprint, Adelaide, Australia) to increase in-stream visibility for visual observation. In 70 m 3kACA trapping experiments, ovulated females were also fitted with external radio telemetry tags (Model 393, Advanced Telemetry System, Isanti, Minnesota, USA), and females were tracked in streams with directional radio antenna and receiver (Lotek

Engineering Incorporated, Newmarket, Ontario, Canada). In all other experiments, ovulated females were externally tagged with 23 mm glass encapsulated external passive integrated transponders (PIT tags, Oregon RFID, Portland, Oregon, USA). Movements of PIT tagged females in the stream were monitored using PIT tag antennas connected to a multiplexer (Oregon RFID, Portland, Oregon, USA). Female tagging occurred at least 12 h prior to experimentation. Tagged females were place in an acclimation cage (1 m^3) in the experimental stream at least 8 h prior to experimentation. All experiments were conducted during daylight hours. Females were released in groups numbering three to 11 animals depending on the individual experiment. Each sea lamprey released in an experiment was considered an individual sample because previous research showed that ovulated females move independently of each other when released in groups (Siefkes et al., 2005). Trial date was evaluated as a random effect in all statistical analyses to determine whether females released on a given date behave differently than females released on another dates. The random effect of trial date never explained sufficient variation in the data to merit its inclusion in any statistical model (as determined by likelihood ratio tests). This supports the assumption that an individual female can be considered an individual sample.

Experimental stream. Experiments were conducted in the Upper Ocqueoc River, MI, USA. The Upper Ocqueoc River was historically infested with sea lampreys (Applegate, 1950), but a barrier built several km downstream currently prevents sea lamprey infestation. Accordingly, it was assumed that no natural pheromones from wild sea lamprey were present. The experimental stream segment was located on a historic spawning riffle above Ocqueoc Falls characterized by shallow fast flow with gravel and bolder substrate (Applegate, 1950). During experimentation, stream discharge ranged from 0.70 cubic meters per second (cms) to 1.34 cms depending on recent precipitation events. Discharge was estimated at least once weekly or after significant precipitation using a Marsh-McBirney Flow-Mate flow meter (Marsh-McBirney Incorporated, Fredrick, Maryland, (McMahon et al., 1996). Most experimental designs in this study used a bifurcated section of stream at the study site, where each channel had similar discharge, water velocity, and substrate (Siefkes et al., 2005). In some experimental designs the two channels were considered the arms of a natural two-choice maze, where female preference for an odorant was determined by baiting channels with different odorants and observing female distribution in the two channels (Johnson et al., 2005).

Synthesized odorants. Application of 3kPZS, 3kACA, and related bioactive components to the experimental stream was approved by the Michigan Department of Environmental Quality and United States Environmental Protection Agency through experimental user permit 75437-EUP-2. 3kPZS and 3kACA were synthesized by Bridge Organics (Vicksburg, Michigan, USA). Purity of synthesized compounds was greater than 95% as determined by mass spectrometry, nuclear magnetic resonance, high pressure liquid chromatography, and thin-layer chromatography. Peristaltic pumps were used to administer methanol solutions of a test odorant to the stream at specific molar (M) concentrations (Chapter 3). Reported molar concentrations are the final in-stream concentration of the test article when fully mixed with stream discharge, not the stock solution applied to the stream.

Natural odorants. Some experiments required the use of natural male mating pheromone collected from spermiated males. Natural odorants were collected by placing spermiated males in a known volume of water for a known amount of time. The water in which spermiated males had been held was termed spermiated male washings (SMW). Washings prepared for experimental use were aliquoted into 1 L bottles and stored at temperatures below -20° C until experimentation. Washings were analyzed for 3kPZS concentration using the liquid chromatography/mass spectrometry method described in Chapter 3. Given known concentrations of 3kPZS in SMW, release rates of SMW were standardized according to 3kPZS concentration. Washings were applied to the stream by thawing the needed volume of SMW, mixing it with river water, and pumping the water/washings solution into the stream.

Objective 1: 3kACA experimental methods.

General approach. The objective of this subset of experiments was to determine if 3kACA functions in streams to modify the migratory or spawning behavior of ovulated female sea lampreys. Specifically, it was evaluated whether 3kACA alone or when added to 3kPZS (mixture) influences: 1) long distance upstream movement to a spawning ground, 2) the ability of females to locate a nest on a spawning ground, and 3) female behaviors in a spawning nest.

Does 3kACA direct long distance upstream movement? The experimental goal was to test whether 3kACA alone or mixtures of 3kPZS and 3kACA influence female movement patterns over 70 m or 650 m stream distances and increase trap capture efficiency. A sea lamprey trap was set in each channel of the bifurcated stream with specifications according to those described in Chapter 3. In the first experiment, females were released 70 m downstream of baited traps, and the capture rates of females was observed: 1) when both traps were baited with control vehicle (methanol control vehicle), 2) when one trap was randomly baited with 3kACA at 10^{-11}, 10^{-12}, 10^{-13}, or 10^{-14} M and the other trap was baited with control vehicle, and 3) when one trap was randomly baited with a mixture of 3kPZS and 3kACA at a 1:0.1 ratio with 3kPZS equal to 10^{-11}, 10^{-12}, 10^{-13}, or 10^{-14} M and the other trap was baited with control vehicle. Trials were conducted randomly without replacement from 2-July-2005 to 30-July-2005, and odorant application started between 08:15 and 11:45. Groups of four to six females were pre-exposed to the odorants for 30 min prior to release. Upon release, females were tracked for 2 h and capture rates in traps were recorded. Statistical differences in capture rates among traps baited with different test articles were evaluated with generalized linear models assuming a binomial data distribution (logistic regression). All logistic regression models used in this study showed no evidence of overdispersion.

In a second experiment, the goal was to determine whether adding 3kACA to 3kPZS increased the proportion of females moving upstream over 650 m and the proportion of females captured in traps. Upstream movement and the capture rate of females over a 650 m distance were observed: 1) when both traps were baited with control vehicle, 2) when one trap was baited with 3kPZS 10^{-12} M and the other trap baited with control vehicle, and 3) when one trap was baited with a mixture of 3kPZS 10^{-12} M + 3kACA 10^{-13} M and the other trap was baited with control vehicle. Trials were conducted randomly without replacement from 31-May-2006 to 9-June-2006, and odorant application for each trial started between 08:00 and 09:00. Groups of nine to 11 females were pre-exposed to the odorants for 1 h prior to release. During one control trial only four females were released because of scarcity. Upon release, females were tracked for 8 h and the number of females that moved upstream into the trapping array, the number captured in traps, and the time it took to capture the lampreys were recorded. Significant differences in the proportion of females moving upstream and the proportion captured in traps baited with different odorant treatments were evaluated with logistic regression models, where the probability of upstream movement or capture was explained by odorant treatment. Differences in the time to capture were evaluated with a general linear model, where time to capture was explained by odorant treatment.

Does 3kACA influence short range preference for spawning nests? The experimental goal was to test whether 3kACA alone, or when mixed with 3kPZS, increased female preference for a spawning nest. Female preference and retention on spawning nests baited with different ratios of 3kPZS and 3kACA were directly compared. Four spawning nests with specifications according to Applegate 1950 were constructed perpendicular to stream flow and spaced approximately 1 m apart in the Ocqueoc River (Figure 1-5). Four specific ratios of 3kPZS and 3kACA were randomly applied to each nest: 1) 3kPZS 10^{-12} M alone (1:0), 2) 3kPZS 10^{-12} M + 3kACA 10^{-12} M (1:1), 3) 3kPZS 10^{-12} M + 3kACA 10^{-13} M (1:0.1), and 4) 3kACA 10^{-12} M (0:1). The 3kPZS:3kACA ratio in SMW has been reported at about 1:0.05 (Yun et al., 2003), but is highly variable (S-S Yun, personal communication). Application of each ratio to a spawning nest was randomized by placing the names of each ratio in a hat. The first ratio drawn out of the hat was applied to the furthest left nest looking upstream. The second ratio drawn was applied to the second furthest left nest looking upstream. This random drawing continued for the third and forth ratios. Trials were conducted from 13-June-2006 to 28-June-2006, and odorant application for each trial started between 08:00 and

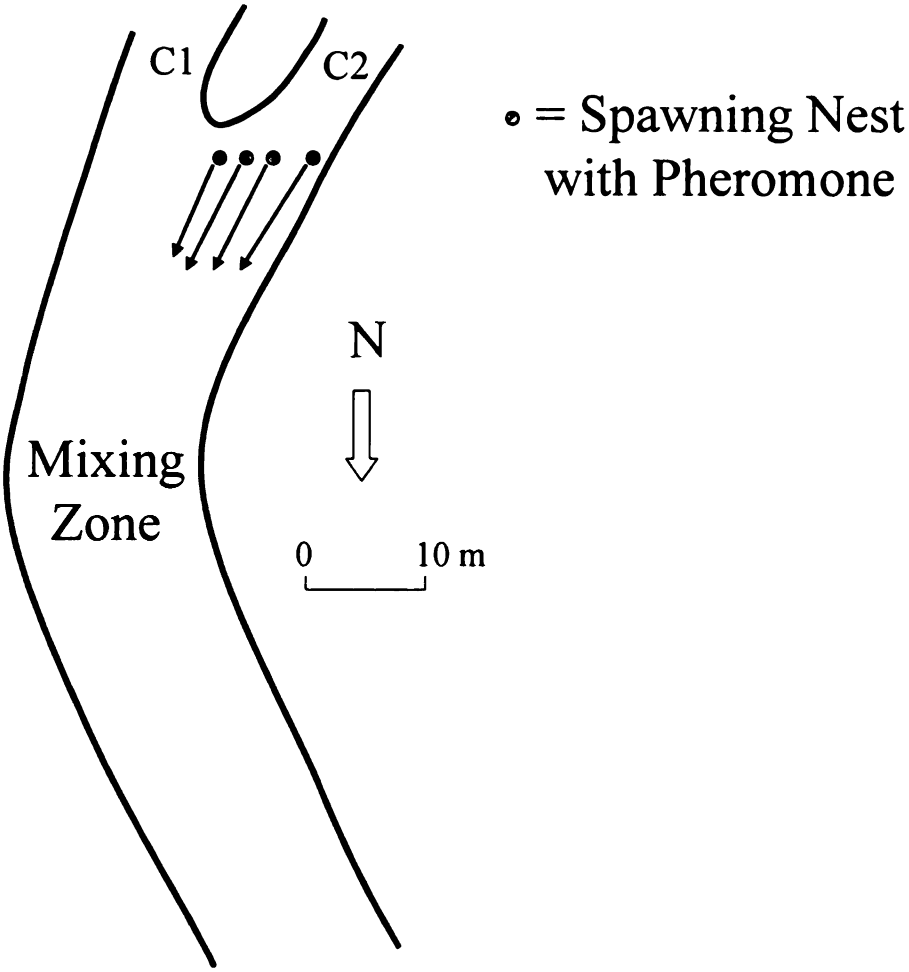

Figure 1-5. Ocqueoc River segment used to test female preference and retention to spawning nests baited with different ratios of 3kPZS and 3kACA. Four spawning nests were constructed 1 m apart. Nests were randomly baited with a ratio of 3kPZS:3kACA at 1:0, 1:1, 1:0.1, and 0:1. Ovulated females were released 250 m downstream of pheromone-baited nests.

13:00. Females were released 250 m downstream of the odorants. Groups of three to six females were pre-exposed to the odorants for 30 min prior to release. Upon release females were tracked for 2 h and the first nest visited by each female was recorded as was retention at that nest. The distribution of females visiting the odorant treatment was evaluated with a logistic regression model where entry into a nest was explained by pheromone treatment. Difference in retention time at the first odorant treatment visited was evaluated with a general linear model where retention at the nest was described by the pheromone treatment.

Does 3kACA influence female spawning behaviors in a nest? The experimental goal was to test whether 3kACA increased the number of mating behaviors females exhibit in spawning nests. Five confined stream channels 1.2 m wide and 5.0 m long were constructed across the Ocqueoc River channel using plywood to separate flow in each channel and sandbags to prevent water from mixing between channels (Figure 2-5). Block nets were placed at the upstream and downstream ends of each channel to prevent females from switching channels. Stream channels had similar physical characteristics, such as depth, water velocity, and substrate. In each channel, average water depth ranged from 0.20 m to 0.25 m, average water velocity ranged from 0.20 m/sec to 0.28 m/sec, and the substrate was gravel. The confinement of water in each channel was confirmed with rhodamine dye tests (Turner Designs, Rhodamine WT, Sunnyvale, CA, USA), where dye was applied to one channel, and water was sampled in neighboring channels and analyzed with a luminescence spectrometer (Perkin Elmer LSS55, Downers Grove, IL, USA). 1 m downstream of the upstream blocknet in each channel, a sea lamprey nest was constructed according to specifications in Applegate 1950. The nest in each channel

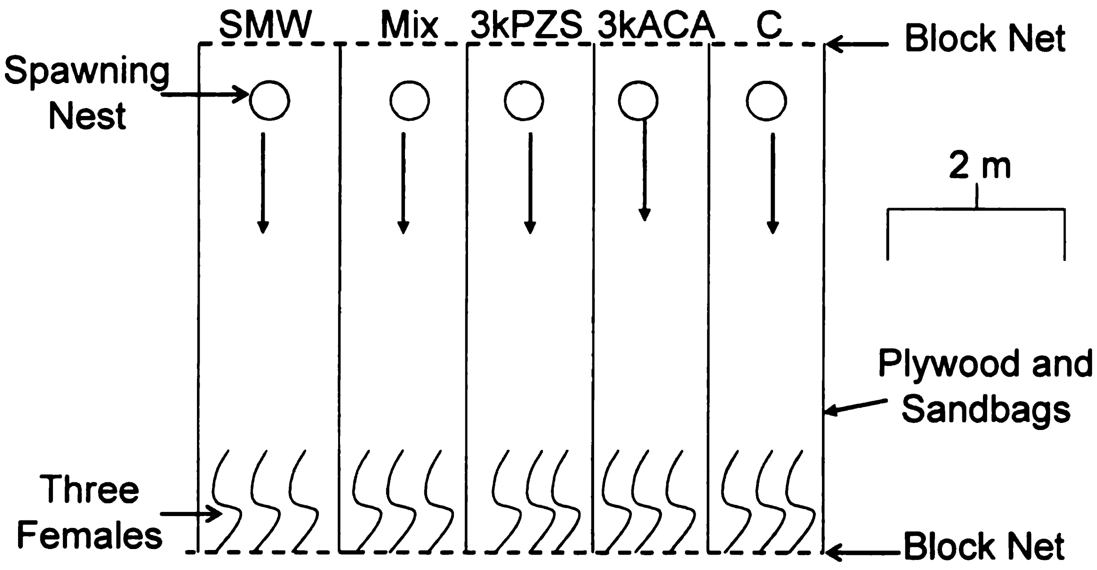

Figure 2-5. Experimental design used to determine if 3kACA induced spawning behaviors. Five nearly identical stream channels were created using plywood and sandbags to divide the flow of the Ocqueoc River, MI. Test articles (SMW = spermiated male washings, Mix = 3kPZS 10^{-12} M + 3kACA 10^{-13} M, C = control vehicle) were randomly applied to a spawning nest at the upstream end of each channel. Three ovulated females were released in each channel. Females were retained in each channel by block nets. Arrows within each channel indicate direction of water flow.

received one of the following odorants during a trial: 1) SMW with a 3kPZS concentration of 10^{-12} M, 2) 3kPZS 10^{-12} M + 3kACA 10^{-13} M, 3) 3kPZS 10^{-12} M, 4) 3kACA 10^{-12} M, and 5) control vehicle. SMW were prepared by placing 20 spermiated males in 110 L of deionized water for 24 h. A different odorant was randomly applied to each nest during a trial. Application of odorants to spawning nests was randomized as in the 3kACA spawning nest experiment described above. Trials were conducted from 20-July-2006 to 24-July-2006, and odorant application for each trial started between 08:00 and 10:00. Three females were released in each channel. Females were held in acclimation cages at the downstream end of each channel prior to experimentation. Females were pre-exposed to the odorants for 30 min prior to release. Upon release, female entries into nests were recorded for 4 h. Spawning behaviors, namely rock movements and tail fans, were recorded while females were in the nest by three naïve observers on a deck built above the stream channels. A rock movement was defined as when a female used its oral disk to pick up a rock in the nest and move it. A tail fan was defined as when a female rapidly moved its tail, thereby moving silt out of the spawning nest. Variation in the number of females that visited nests baited with different test articles were evaluated with a logistic regression model, where entry into a nest was explained by odorant treatment. Variation in the frequency of rock movements and tail fans in nests baited with different odorants were evaluated with general linear models, where the number of spawning behaviors exhibited was explained by odorant treatment.

Objectives 2 and 3: Pheromone extraction, head and tail washings, and field bioassay methods.

Extraction methods. Spermiated male washings were produced by placing 20 spermiated male sea lampreys in 200 L of deionized water for 36 h. 12 L of the 200 L of deionized water evaporated during the 36 h washing. 88 L of SMW were aliquoted into 1 L bottles for use as bioassay positive controls. The remaining 100 L of SMW were pre-filtered with a 75 micron inline Hytrex filter (Osmonics, Minnetonka, MN) and passed through 1 kg of XAD resin packed in a glass column (50 mm x 1200 mm, Sothern Analytical) at 0.5 L/min (Fine et al., 2006). Water that passed through the XAD resin was collected (XAD waste water). 25 L of XAD waste water was aliquoted into 1 L bottles for bioassay use. The remaining 75 L of XAD waste water was used in another experiment. XAD resin was eluted with 3.3 L of methanol. 3.2 L of XAD extract (methanol solution) was collected for bioassay use. Test articles for use in the bioassay were frozen at -80° C simultaneously.

1 L of XAD extract was re-extracted using 100 g of C18 resin packed in a sep-pak (two 50 g C18 Sep-Pak 35 CC cartridges connected in series). The liter of XAD extract was diluted into 9 L of deionized water to reduce the concentration of methanol to ensure extraction by C18 resin. Only 8.2 L of the 10.0 L of diluted XAD extract was re-extracted with C18 due to time constraints. The 8.2 L of water that passed through the C18 sep-pak was collected (C18 waste) and aliquoted into 1 L bottles for bioassay use. 91 ml of C18 extract was eluted from the sep-pak for bioassay use. If my objective had been to isolate and identify putative pheromone components, the 91 ml of C18 extract would have been freeze-dried and fractionated by HPLC. However, the objective of this study was to confirm that extraction techniques captured unknown pheromones. Hence, the 91 ml of C18 extract was diluted into 909 ml of methanol to reconstitute the original

1 L volume of XAD extract that was started with. Test articles collected from the C18 extraction were frozen at -80° C simultaneously.

The concentration of 3kPZS in: 1) SMW, 2) XAD extract, 3) XAD waste water, 4) C18 extract, and 5) C18 waste water was determined using the LC/MS method described in Chapter 3 (Table 1-5). 3kPZS from SMW was accounted through the XAD and C18 extraction procedures and showed that little 3kPZS degraded during the extractions and while frozen. 3kPZS accounting also showed that XAD resin captured 3kPZS, but some 3kPZS passed through the resin into the waste water (Table 2-5 and herein). C18 sep-pak extraction captured and eluted 3kPZS with very high efficiency.

Head and tail washings collection. Head and tail washings of spermiated males were collected using a modified version of the device constructed by Siefkes et al. 2003, where a dry chamber was added to separate the head and tail regions to ensure no water mixed between compartments. A spermiated male was placed in a plexiglass tank, which separated water exposed to the anterior section of the lamprey from the posterior section of the lamprey. 7 L of deionized water was put in each compartment and aeration was provided in both compartments. One male was placed in the washings tank for 1 h. After an hour the male was removed, and the head and tail washings were frozen at -20° C. Head and tail washings were collected on 20 males. When all washings were collected, head and tail washings were thawed, combined into a single batch according to their source (140 L each), and refrozen at -20° C. The concentration of 3kPZS in head and tail washings was determined using the LC/MS method. 3kPZS was only detected in head washings (Table 1-5), confirming the results of Siefkes et al. 2003.

Table 1-5. The volume, 3kPZS concentration, and total 3kPZS in test articles generated for bioassay use to determine if XAD and C18 resins capture unknown pheromone components, and whether unknown pheromone components are released through the head or tail region.

Test Article	Volume (L)	3kPZS (mg/L)	3kPZS for Bioassay (mg)
SMW	88.0	2.1	184.8
XAD Extract	2.2	17.9	39.4
XAD Waste	25.0	0.5	12.5
C18 Extract	1.0	25.0	25.0
C18 Waste	8.2	0.0	0.0
Head Wash	140.0	0.1	14.0
Tail Wash	140.0	0.0	0.0

Table 2-5. This table displays accounting of 3kPZS present in spermiated male washings (SMW) as it was extracted by XAD resin and C18 resin. SMW and XAD extract were the starting products of the XAD and C18 extraction, respectively. The remaining test articles were the end products of each extraction. For example, the first block can be understood as the following: the total volume of SMW that passed through XAD resin, the concentration of 3kPZS in the SMW, and the total amount of 3kPZS present in the SMW; the total volume of XAD extract collected, the concentration of 3kPZS in XAD extract, and the total amount of 3kPZS in XAD extract; the total volume of XAD waste water collected, the concentration of 3kPZS, and total amount of 3kPZS in XAD waste water. If 100% of 3kPZS was eluted from the XAD resin, the total amount of 3kPZS in SMW should equal the sum of the total amount of 3kPZS in the XAD extract and XAD waste.

Test Article	Volume (L)	3kPZS (mg/L)	Total 3kPZS (mg)
SMW	100	2.1	210
XAD Extract	3.2	17.9	57
XAD Waste	100	0.5	50
XAD Extract	8.2	1.8	15
C18 Extract	1.0	25	25
C18 Waste	8.2	0	0

Behavioral assay. Behavioral responses of ovulated females to extraction and washings products were evaluated in the Upper Ocqueoc River at the same bifurcated stream segment used for 3kACA experiments. In each stream channel, two sea lamprey nests were constructed 1.25 m apart. Two test articles were directly compared by applying a test article to each nest and allowing females to choose an odorant immediately downstream of the nests. Test articles switched nests each trial. Both channels of the river were used simultaneously to conduct independent experiments, thereby doubling the number of trials conducted. To insure that water was not mixing between channels, sandbags were used to fortified sections of the island. Females were held in a 1 m^3 acclimation cage located 45 m downstream of the baited nests. After a 30 min odorant introduction period, five to 10 PIT tagged ovulated females were released per trial for 2 h. Odorant introduction began between 07:30 and 12:00 in all trials. PIT tag antennas connected to a multiplexer determined which nest females entered and how long they were retained. The total number of rock movements and tail fans in each nest per trial were visually observed and recorded by naïve observers stationed between the nests. Significant differences in the first odorant visited were evaluated with logistic regression models, where the first nest visited was explained by pheromone treatment. Significant differences in retention, rock movements, and tail fans were evaluated with general linear models, where the behavior (i.e. rock movements) was explained by pheromone treatment. Nest choice and retention on a nest were evaluated at alpha = 0.05. Rock movements and tail fans were evaluated at alpha = 0.10 because females exhibited relatively few mating behaviors. Exceptions to this statistical framework are stated when individual comparisons are described below.

Direct comparisons of test articles. Ten pairs of test articles were directly compared using the in-stream bioassay. Odorant release rates were standardized by applying each test article to reach a final in-channel 3kPZS concentration of 5×10^{-13} M. For comparisons of SMW from the head and tail regions an equal volume of washings were applied to each nest. Direct comparisons of test articles were categorized into three groups: XAD extraction comparisons, C18 extraction comparisons, and head and tail washings comparisons. The first group of direct comparisons ($n = 4$) evaluated the following products of XAD extraction. 1) Spermiated male washings (collected prior to extraction, SMW) were compared to XAD extract of the same SMW in the right channel (determined by looking upstream) with trials being conducted from 1-July-2008 to 7-July-2008. If XAD resin extracted and eluted all behaviorally active pheromone components in SMW, female attraction, retention, and spawning behaviors in nests baited with XAD extract and SMW should be equal. 2) SMW were compared to XAD extract + XAD waste water in the left channel from 1-July-2008 to 7-July-2008. If XAD extraction captured, but failed to elute all behaviorally active pheromone components with the same experimental efficiency, female attraction, retention, and spawning behaviors in nests baited with SMW should be greater than nests baited with XAD extract + XAD waste. 3) XAD extract was compared to synthesized 3kPZS in the right channel from 8-July-2008 to 18-July-2008. If XAD captured and eluted 3kPZS plus additional unidentified pheromone components, female attraction, retention, and spawning behaviors in the nest baited with XAD extract would be greater than nests baited with synthesized 3kPZS. 4) Synthesized 3kPZS was compared to synthesized 3kPZS + XAD waste in the left channel from 8-July-2008 to 18-July-2008. If behaviorally active pheromone components passed through the XAD resin, female attraction, retention and spawning behaviors in the nest baited with 3kPZS + XAD waste would be greater than nests baited with synthesized 3kPZS. In this comparison, the logistic regression model, which evaluated data describing the first nest visited included the explanatory variables of pheromone treatment and nest location because females preferred to enter the right nest first regardless of pheromone treatment ($p = 0.002$, $df = 1$, $X^2 = 9.49$).

The second group of comparisons ($n = 4$) evaluated products of C18 extraction. 1) C18 extract (of XAD extract of SMW) was compared to SMW in the right channel from 20-July-2008 to 25-July-2008. If XAD and C18 resin extracted and eluted all behaviorally active pheromone components, female attraction, retention, and spawning behaviors in nests baited with C18 extract and SMW should be equal. 2) SMW were compared to C18 extract + C18 waste in the right channel from 26-July-2008 to 30-July-2008. If C18 resin captured, but failed to elute all behaviorally active pheromone components captured by XAD extraction with the same efficiency, female attraction, retention, and spawning behaviors in nests baited with SMW should be greater than nests baited with C18 extract + C18 waste. In this comparison, the logistic regression model, which evaluated data describing the first nest visited, included the explanatory variables of pheromone treatment and nest location because females preferred to enter the right nest first regardless of pheromone treatment ($p = 0.026$, $df = 1$, $X^2 = 4.94$). Also, the general linear model evaluating rock movements included the explanatory variables of pheromone treatment and nest location because females exhibited more rock movements in the right nest regardless of pheromone treatment ($p = 0.013$, $df = 9$, t-stat $= -3.11$). 3) C18 extract was compared to synthesized 3kPZS in the left channel from 20-July-2008 to

25-July-2008. If XAD and C18 resin captured and eluted 3kPZS plus additional unknown pheromone components, female attraction, retention, and spawning behaviors in the nest baited with C18 extract would be greater than nests baited with synthesized 3kPZS. In this comparison, the logistic regression model, which evaluated data describing first nest visited, included the explanatory variables of pheromone treatment and nest location because females preferred to enter the right nest first regardless of pheromone treatment ($p = 0.017$, $df = 1$, $X^2 = 5.73$). 4) Synthesized 3kPZS was compared to 3kPZS + C18 waste in the left channel from 26-July-2008 to 30-July-2008. If behaviorally active pheromone components were captured and eluted from XAD resin, but passed through the C18 resin, female attraction, retention, and spawning behaviors in the nest baited with 3kPZS + C18 waste would be greater than nests baited with synthesized 3kPZS.

The volume of waste water applied to a nest when mixed with XAD extract, C18 extract, or synthesized 3kPZS equaled the amount of SMW applied to the neighboring nest or the amount of SMW that would have needed to be extracted with XAD or C18 resin to yield the amount of synthesized 3kPZS applied to the neighboring nest. When XAD extract + XAD waste was applied to the stream (XAD waste contained 3kPZS), the amount of XAD extract applied was reduced, not the amount of XAD waste water, to achieve equal 3kPZS release rates in each nest.

The third group of comparisons (n = 2) evaluated head and tail washings from spermiated males. 1) Head washings were compared to tail washings in the right channel from 31-July-2008 to 5-Aug-2008. Nests baited with head washings may attract more females because 3kPZS was only present in head washings. Females visiting nests baited with tail washings may be retained longer and exhibit more spawning behaviors if unknown pheromone components are present in tail washings. 2) Head washings were compared to tail washings + synthesized 3kPZS in the left channel from 31-July-2008 to 7-Aug-2008. Tail washings were spiked with 3kPZS to ensure that some females would visit the tail washings nest. If unknown pheromone components were present in tail washings, female attraction, retention, and spawning behaviors in nests baited with 3kPZS + tail washings would be greater than nests baited with head washings.

RESULTS

3kACA experimental results.

3kACA does not direct upstream movement. 3kACA alone, when applied to traps at concentrations ranging from 10^{-11} to 10^{-14} M, did not induce females to swim upstream 70 m into traps. Capture rates of females in 3kACA-baited and control traps did not differ significantly and were less than 10% (Table 3-5 and herein). Capture rates of females in traps baited with a mixture of 3kPZS and 3kACA at a 1 to 0.1 ratio were high and significantly greater than traps baited with control vehicle. All females were captured in the pheromone-baited trap when 3kPZS was applied to the stream. Capture rates of traps baited with mixtures of 3kPZS and 3kACA at 10^{-11}, 10^{-12}, or 10^{-13} M did not differ significantly and averaged 64%. High capture rates of females in traps baited with 3kPZS and 3kACA were likely attributed to the presence of 3kPZS because control traps and traps baited with 3kACA did not capture significant proportions of females.

Adding 3kACA to 3kPZS did not increase the likelihood that females would swim upstream and enter the pheromone-baited trap over a 650 m stream distance. Traps baited with 3kPZS alone and a mixture of 3kPZS and 3kACA did not differ significantly in capture rate and lured about 38% of females upstream and 33% of females into traps (Table 4-5 and herein). The time taken for females to enter traps baited with 3kPZS and a mixture of 3kPZS and 3kACA did not differ significantly and averaged 207 min (SD = 110 min). 3kPZS likely induced directed upstream movement because addition of 3kACA did not increase the proportion of females moving upstream. During control trials only 3% of females moved upstream, and no females were captured.

Table 3-5. Number of ovulated females captured when both traps were baited with control vehicle (Control), when one trap was baited with control vehicle and the other trap was baited with 3kACA, and when one trap was baited with control vehicle and the other was baited with a mixture of 3kPZS (molar, M) and 3kACA at a 1 to 0.1 ratio. All females were captured in the pheromone-baited trap when 3kPZS was applied to the stream. Data were evaluated with logistic regression. Statistics reported on the horizontal plane are contrasts between control treatments and mix treatments (example Control vs. Mix 10^{-11}). Statistics on the vertical plane report the overall significance of the model. Treatments that share a letter are not significantly different ($\alpha = 0.05$).

Trap Bait (M)	n	Captured (n)	Trap Bait (M)	n	Captured (n)	p-value (df, X^2)
Control	24	2 A	Control	24	2 A	NA
3kACA 10^{-11}	24	2 A	Mix 10^{-11}	24	15 B	0.001 (1,11.70)
3kACA 10^{-12}	25	0 A	Mix 10^{-12}	24	18 B	<0.001 (1,15.93)
3kACA 10^{-13}	19	0 A	Mix 10^{-13}	19	10 B	0.004 (1,8.28)
3kACA 10^{-14}	12	1 A	Mix 10^{-14}	25	0 A	0.966 (1,0.00)
	X^2	4.94		**X^2**	43.87	
	df	4		**df**	4	
	p-value	0.293		**p-value**	< 0.001	

Table 4-5. Number of ovulated females that moved upstream 650 m and were captured when both traps were baited with control vehicle (Control), when one trap was baited with control vehicle and the other trap was baited with 3kPZS, when one trap was baited with control vehicle and the other was baited with at mixture of 3kPZS (M) and 3kACA at a 1 to 0.1 ratio. Time (min) indicates the average time for captured females to swim upstream and enter the baited trap. All females were captured in the pheromone-baited trap. Upstream movement and capture data were evaluated with logistic regression. Time to capture was evaluated with general linear model. Treatments that share a letter are not significantly different ($\alpha = 0.05$).

Treatment (M)	N	Upstream (n)	Captured (n)	Time (min)
Control	33	1 A	0 A	NA
3kPZS 10^{-12}	40	13 B	12 B	195 A
Mix 10^{-12}	41	16 B	15 B	217 A
	X^2	19.59	22.09	**F-Stat**=0.30
	Df	2	2	**NDF/DDF**=1/28
	p-value	<0.001	<0.001	**p-value**=0.586

3kACA does not influence female preference for spawning nests. Only nests baited with 3kPZS alone and mixtures of 3kPZS and 3kACA were visited by ovulated females. Nests baited with 3kACA (0:1) did not attract ovulated females (Table 5-5 and herein). The proportion of females attracted to nests baited with a 1:0, 1:1, and 1:0.1 ratio of 3kPZS to 3kACA did not differ significantly. Retention on the first nest visited did not differ significantly among the three nests baited with 3kPZS and averaged 407 sec (SD = 534 sec). 3kPZS likely induced movement to nests and retention in nests because the addition of 3kACA to 3kPZS did not increase nest attractiveness or retention.

3kACA does not influence female spawning behaviors in a nest. Spermiated male washings, a mixture of 3kPZS and 3kACA, and 3kPZS alone lured about 50% of females into the baited nest, which was significantly greater than nests baited with 3kACA or control vehicle (Table 6-5 and herein). 3kACA alone did not lure females into nests. Females in nests baited with SMW exhibited a total of 118 rock movements and 34 tail fans, which was significantly greater than all other test article treatments (p-value < 0.05 for all comparisons). When 3kPZS, 3kPZS + 3kACA, 3kACA, and control vehicle were applied to a nest, females averaged three rock movements and zero tail fans.

In-stream bioassay results.

XAD extraction comparisons. In-stream bioassay comparisons show that XAD resin captured and eluted all behaviorally active pheromone components in SMW. Equal proportions of females visited nests baited with XAD extract and SMW. Females on nests baited with XAD and SMW showed equal numbers of rock movements and tail fans (Tables 7-5 and 8-5 and herein). Further, XAD extract, when compared to synthesized

Table 5-5. The first pheromone treatment visited by ovulated females when presented with four nests baited with different pheromone treatments: 3kPZS alone, 3kPZS and 3kACA mixed at a 1 to 0.1 ratio, 3kPZS and 3kACA mixed at a 1 to1 ratio, and 3kACA. The average time females spent on the first nest visited (Retention). The first compound listed in the treatment column was applied to reach an in-stream concentration of 10^{-12} M. Nest visit data were evaluated with logistic regression. Retention time was evaluated with a general linear model. Treatments that share a letter are not significantly different ($\alpha = 0.05$).

Treatment	**Visit (n)**	**Retention (sec)**
3kPZS	17 A	448 A
3kPZS(1)+3kACA(0.1)	16 A	415 A
3kPZS(1)+3kACA(1)	14 A	347 A
3kACA	0 B	NA
$\mathbf{X^2}$	32.39	**F-Stat** = 0.138
df	3	**NDF/DDF** = 2/44
p-value	<0.001	**p-value** = 0.872

Table 6-5. Number of ovulated females that were released (n) in channels baited with an odorant treatment and the number of females that moved upstream into the baited nest. "Rock" and "fan" indicate the total number of rock movements and tail fans exhibited by females in the baited nest. Nest entry data evaluated with logistic regression and number of rock movements and tail fans evaluated with general linear models. Treatments that share a letter were not significantly different ($\alpha = 0.05$). SMW and 3kPZS applied to reach a 3kPZS concentration of 10^{-12} M. 3kACA applied to reach a concentration of 10^{-12} M. Mix = 3kPZS + 3kACA at a 1 to 1.0 ratio.

Treatment	**n**	**Nest (n)**		**Rock**	**Fan**
SMW	15	9 A		118 A	34 A
3kPZS	15	9 A		5 B	0 B
Mix	15	6 A		5 B	0 B
3kACA	15	0 B		0 B	0 B
Control	15	3 B		2 B	1 B
	X^2	22.43	**F-Stat**	2.18	2.047
	df	4	**NDF/DDF**	4/20	4/20
	p-value	<0.001	**p-value**	0.108	0.126

3kPZS, attracted more females, retained them longer, and elicited more rock movements and tail fans. Collectively, these results demonstrate that 1) XAD extract contains pheromone components in addition to 3kPZS, and 2) that XAD extract likely contains all behaviorally active mating pheromone components found in SMW.

The efficiency of XAD resin to extract and elute 3kPZS was low. XAD resin captured and eluted about 25% of the 3kPZS present in the 100 L of SMW that was passed through it. About 25% of the 3kPZS passed directly through the resin and into the waste water and about 50% of 3kPZS was not eluted from the resin or was lost elsewhere in the extraction process (Table 2-5). An unknown quantity of unidentified pheromone components also passed through the XAD resin. Synthesized 3kPZS spiked with XAD waste water was more attractive, retained females longer, and induced more spawning behaviors than 3kPZS alone (Tables 7-5 and 8-5 and herein). Also, when SMW and XAD extract were directly compared, SMW retained females significantly longer than XAD extract. Unidentified pheromone components are likely eluted from the XAD resin because comparisons of SMW and XAD extract plus XAD waste resulted in equal behavioral response from females.

C18 extraction comparisons. In-stream bioassay comparisons showed that C18 resin captured and eluted 3kPZS with high efficiency (Table 2-5), but unidentified pheromone components were not present in the C18 extract in sufficient quantities to elicit a behavioral response. Nests baited with SMW attracted more females, retained females longer, and induced more rock movements and tail fans than C18 extract of the XAD extract (Tables 7-5 and 8-5 and herein). C18 extract did not retain females longer or induce more spawning behaviors when compared to synthesized 3kPZS. These data demonstrate that C18 extract likely only contained 3kPZS at behaviorally active quantities. Even when C18 waste water was added to C18 extract, SMW were more attractive and induced more spawning behaviors, demonstrating that unidentified pheromone components did not pass through the C18 resin. Supporting this conclusion are comparisons of 3kPZS and 3kPZS spiked with C18 waste water, where each test article equally attracted and retained females, but neither odorant induced mating behaviors.

Comparisons of head and tail washings. All behaviorally active mating pheromone components were excreted through the head region of spermiated males. When head and tail washings were directly compared, females never visited the nest baited with tail washings (Tables 7-5 and 8-5). Females in nests baited with head washings were retained and exhibited mating behaviors at levels comparable to females in nests baited with whole SMW. Females showed equal attraction for nests baited with head washings and tail washings spiked with 3kPZS, but head washings retained females significantly longer and induced more mating behaviors than tail washings spiked with 3kPZS.

Table 7-5. Distribution of ovulated females when presented with two nests baited with different products of XAD and C18 extraction. "n" is the total number of females released. "A(n)" and "B(n)" are the number of females that entered nest A or B first. "A" represents the first odorant listed in the direct comparison column and "B" represents the second odorant listed in the direct comparison column. Retention lists the average time spent within 0.5 m of the first nest visited. Distribution data were evaluated with logistic regression and retention data were evaluated with a general linear model. NA= statistics test not applicable. SMW = spermiated male washings, XAD = XAD extract of spermiated male washings, waste = water that passed through an extraction resin, C18 = C18 extract of XAD extract of spermiated male washings, Head = head washings, and Tail = tail washings.

Treatment	**Distribution**				**Stats**	**Retention**		**Stats**
Direct Comparison	Trials	n	A (n)	B (n)	p-value (df, X^2)	A (sec)	B (sec)	p-value (NDF/DDF,F-Stat)
SMW *vs.* XAD	6	46	10	12	0.664 (1,-0.19)	1508	796	***0.042 (1/20,4.69)***
SMW *vs.* XAD+waste	6	51	10	17	0.179 (1,-1.80)	1017	1265	0.534 (1/18,-0.40)
XAD *vs.* 3kPZS	9	69	24	3	***>0.001 (1,18.34)***	645	175	0.185 (1/25,1.86)
3kPZS+waste *vs.* 3kPZS	8	67	27	15	***0.019 (1,5.53)***	1252	88	***>0.001 (1/40,21.06)***
SMW *vs.* C18	6	48	20	10	0.071 (1,3.27)	1698	367	***0.003 (1/23,11,44)***
SMW *vs.*C18+waste	6	54	25	14	***0.037 (1,4.36)***	1030	316	***0.002 (1/31,11.91)***
C18 *vs.* 3kPZS	6	49	16	8	***0.050 (1,3.80)***	749	81	0.052 (1/15, 4.44)
3kPZS+waste *vs.* 3kPZS	6	54	10	12	0.893 (1,-0.02)	62	238	0.081 (1/18,-3.43)
Head *vs.* Tail	4	38	15	0	***>0.001 (1,20.19)***	1444	NA	NA
Head *vs.* Tail+3kPZS	8	69	14	15	0.837 (1,-0.04)	926	207	***0.002 (1/26,11.31)***

Table 8-5. Total number (n) of rock movements and tail fans ovulated females exhibited in two nests baited with different products of XAD and C18 extraction. "A" represents the first odorant listed in the direct comparison column and "B" represents the second odorant listed in the direct comparison column. Data were evaluated with a general linear model. NA = stats test not applicable. SMW = spermiated male washings, XAD = XAD extract of spermiated male washings, waste = water that passed through an extraction technique, C18 = C18 extract of XAD extract of spermiated male washings, Head = head washings, and Tail = tail washings.

Treatment	**Rock**		**Stats**	**Fan**		**Stats**
Direct Comparison	A (n)	B (n)	p-value (NDF/DDF,F-Stat)	(n)	(n)	p-value (NDF/DDF,F-Stat)
SMW *vs.* XAD	50	42	0.853 (1/10,0.04)	31	25	0.805 (1/10,0.6)
SMW *vs.* XAD+waste	44	64	0.669 (1/10,-0.19)	33	39	0.868 (1/10,-0.03)
XAD *vs.* 3kPZS	27	0	***0.084 (1/16,3.47)***	15	0	***0.094 (1/16,3.23)***
3kPZS+waste *vs.* 3kPZS	78	0	***0.050 (1/14,4.61)***	15	0	0.108 (1/14,2.94)
SMW *vs.* C18	91	4	***0.055 (2/9,4.07)***	39	0	***0.025 (2/9,5.68)***
SMW *vs.* C18+waste	95	11	***0.009 (2/9,8.26)***	59	3	0.141 (1/10,2.56)
C18 *vs.* 3kPZS	14	0	0.111 (1/10,3.06)	3	0	0.341 (1/10,1.00)
3kPZS+waste *vs.* 3kPZS	0	1	NA	0	0	NA
Head *vs.* Tail	85	0	NA	29	0	NA
Head *vs.* Tail+3kPZS	61	8	0.127 (1/14,2.63)	19	0	NA

DISCUSSION

3kACA alone, when applied over a thousand-fold range of concentrations did not influence the behavior of ovulated females in streams during the day. 3kACA did not induce females to move upstream over 70 m or 650 m distances. When 3kACA was baited into a trap, the capture rate of ovulated females did not increase. When 3kACA was applied to a nest it did not attract, retain, or induce ovulated female mating behaviors.

Behavioral responses of ovulated females to mixtures of 3kPZS and 3kACA did not differ significantly from that of 3kPZS alone. Over a 70 m distance, traps baited with a mixture of 3kPZS and 3kACA captured 64% of females, which was higher than the 46% female capture rate in traps baited with 3kPZS alone observed in a previous study (Chapter 3). However, during 650 m trapping experiments, the proportion of females swimming upstream during 3kPZS and 3kPZS + 3kACA treatments did not differ, and the number captured in traps baited with the two test articles did not differ. Additionally, in nesting experiments, a nest baited with 3kPZS alone lured and retained females as well as mixtures of 3kPZS and 3kACA at 1:1 ratios and 1:0.1 ratios. Also, a mixture of 3kACA and 3kPZS did not induce more rock movements or tail fans than nests baited with 3kPZS. All field results support the conclusion that 3kACA does not induce behavioral responses in ovulated females. Field results also do not support the hypothesis that minor components of the sea lamprey pheromone may resemble the major pheromone component, as is common in insects (Howse et al., 1998). However, 3kACA may function as a priming pheromone. Recently 3kACA has been shown to slow the maturation of pre-spermiated males by offsetting the priming response elicited by 3kPZS (Chung-Davison et al., in prep).

Spawning channel experiments demonstrated that SMW contains pheromone components in addition to 3kPZS and 3kACA that induce reproductive behaviors in nests. To date, six putative mating pheromone components have been identified using two-choice mazes and only 3kPZS has elicited behavioral responses in natural streams. The lack of success in identifying biologically relevant pheromones highlights the need to reevaluate pheromone extraction techniques and shift bioassays from the lab to the field. The field bioassay developed in this study functioned well to distinguish differences in the behavioral responses of females to two test articles. The evaluation of four response variables at once in the bioassay improved experimental efficiency. The simultaneous evaluation of two test articles can yield clear conclusions on whether female responses to test articles differed. Comparing two odorants at the same time is statistically more powerful than comparing two groups of females that were exposed to different odorants at different times because variability is reduced in the behavioral responses of females and in stream conditions. In most comparisons the bioassay was unbiased; females showed equal preference for the two nests used in the bioassay and were equally likely to be retained and display mating behaviors in each nest. Occasionally, females would prefer the right nest in each channel regardless of the pheromone treatment applied. Right nest biases were accounted for by including the nest effect in the statistical model; however, this reduced experimental power due to a loss of a degree of freedom.

XAD7HP resin effectively captured and eluted male mating pheromone components that induced rock movements and tail fans. XAD extract can be confidently probed for unidentified pheromone components because this study confirmed they were present in the extract. Although XAD resin extracted all pheromone components, it did so with low efficiency. In Fine et al. 2006, 3kPZS was extracted with XAD7HP with 81% efficiency. The XAD7HP extraction conducted in this study only yielded 25% of the 3kPZS present in the SMW, although the same equipment was used. Analysis of water that passed through the XAD resin showed that 25% of 3kPZS in the SMW was present in the waste water. Bioassay comparisons determined that unidentified pheromone components were also in the waste water at high enough quantities to elicit behavioral responses. Pheromone components may have passed into the waste water due to poor packing of XAD resin in the glass column. Poor packing would result in channelization of water through the column reducing resin surface area and the time pheromones were exposed to the resin. Using more XAD resin and slower flow rates would likely improve extraction efficiency. 50% of 3kPZS was likely retained on the resin and not eluted when washed with 100% methanol because it was not present in the extract or waste water. Rinsing the resin with more than 3 L of methanol may improve elution efficiency. Alternatively, stronger solvents could be used to increase XAD elution efficiency.

C18 sep-paks extracted and eluted 3kPZS from XAD extract with high efficiency, but did not contain behaviorally active quantities of pheromone components that induce retention and mating behaviors in a nest. Surprisingly, the field bioassay showed that C18 resin likely captured unidentified pheromone components that induced mating behaviors, but did not release them when the resin was rinsed with 100% methanol. Li et al. 2002 likely had excellent success isolating and purifying 3kPZS from SMW because they employed C18 sep-paks. Ironically, attempts to identify additional pheromone components were hampered by employing C18 sep-paks because they were not present in the extract. A main conclusion of this study is that C18 resin should not be used to concentrate XAD extract of spermiated male washings when searching for unidentified pheromone components.

Bioassay comparisons of head and tail washing demonstrated that all behaviorally active pheromone components were excreted through the head region. These results confirmed the findings of Siefkes et al. 2003; 3kPZS is excreted from the head region, likely through gill glandular cells. Spermiated males may also release unidentified pheromone components across the gills using glandular cells. Because all pheromone components are released through the head, compounds released in the urine or sperm can be eliminated as pheromone components that induce mating behaviors. If this method of elimination was used initially, 3kACA could have been disregarded as a putative pheromone component without extensive in-stream testing or synthesis because 3kACA is present in tail washings (S-S Yun, personal communication).

The in-stream bioassay and XAD extraction of spermiated male washings will be vital to the identification of additional pheromone components. XAD extract should not be concentrated with C18 resin as my experiments have shown that C18 resin does not elute some behaviorally active compounds. Large quantities of SMW have already been extracted with XAD resin. Future researchers could fractionate XAD extract by re-extracting XAD extract with XAD resin and eluting with a gradient of methanol. XAD fractions could be tested in the in-stream bioassay for behavioral activity. Especially critical would be two comparisons: 1) 3kPZS + XAD fraction vs. 3kPZS and 2) SMW vs.

3kPZS + fraction. If the XAD fraction contained an unidentified pheromone component, nests baited with 3kPZS + XAD fraction would have higher female retention and mating behaviors than nests baited with 3kPZS. If all unidentified pheromone components were present in the fraction, retention and mating behaviors in the 3kPZS + XAD fraction nest would not differ significantly from nests baited with SMW. Testing XAD fractions in the in-stream bioassay could quickly reveal which fraction(s) contain compounds that elicit behaviors in natural environments. Approximately 12 fractions could be tested over a 70 day field season using two channels.

Once an active XAD fraction is identified, compounds present in the fraction could be isolated and identified using analytical chemistry techniques. Additional potential pheromone components could also be eliminated by comparing compounds present in the active fraction from head washings to compounds present in the active fraction from tail washings. Large quantities of head and tail washings have already been collected and extracted with XAD resin. Compounds present in the active fraction of head washings, but not present in the active fraction of tail washings would be likely pheromone candidates. In addition, XAD extract of spermiated male head washings and non-spermiated males head washings could be contrasted to eliminate additional compounds. EOG could be used to determine the olfactory potency of component candidates in the active fraction. Highly potent olfactory stimulants unique to spermiated male head washings could be isolated, identified, synthesized, and tested in the bioassay for final confirmation of pheromonal function.

In conclusion, this study demonstrated that 3kACA did not function in streams as a mating pheromone component to induce ovulated female preference responses, retention on a nest, or mating behaviors. Given that 3kACA did not elicit a behavioral response, a new in-stream bioassay was developed, and XAD and C18 extraction techniques were evaluated. XAD extract of SMW contained unidentified pheromone components, but C18 extract did not. All pheromone components are excreted by males through the head region. Future efforts to identify pheromone components should utilize XAD extraction techniques, analyses of head and tail washings, and the in-stream bioassay to systematically eliminate compounds as potential pheromones and isolate compounds that elicit behaviors in natural streams. Newly identified pheromone components could be valuable tools for managing sea lampreys in the Great Lakes (Twohey et al., 2003).

CONCLUSION TO DISSERTATION

Throughout this dissertation, experiments conducted in nature helped to reveal the ecological significance of putative sea lamprey migratory and mating pheromone components to determine their possible use in sea lamprey management. 3kPZS, the supposed mating pheromone, was shown to induce upstream movement in ovulated females and was able to direct them into baited traps and away from natural pheromone signals (Chapter 3). Interestingly, behavioral observations showed that 3kPZS only induced a migratory response in ovulated females and did not retain females on nests or induce spawning behaviors with the potency of the natural mating pheromone released by males. In Chapter 4, the responses of pre-ovulatory females to 3kPZS were re-evaluated in a natural stream at night because 1) 3kPZS elicited a migratory response in ovulated females, 2) previous tests of pre-ovulatory female responses to 3kPZS were potentially flawed, and 3) putative migratory pheromone components, PADS, PSDS, PZS, identified in laboratory mazes, have not elicited behavioral responses in pre-ovulatory females. Surprisingly, only 3kPZS, the putative "mating" pheromone, and not PADS, PSDS, PZS (putative migratory pheromone components) directed upstream movement in pre-ovulatory females, and the response elicited by 3kPZS did not vary from that induced by natural larval pheromone over long distances (Chapter 4). No longer does it appear that 3kPZS is a specific mating pheromone, but rather a general aggregational pheromone that directs upstream movement of adult lampreys regardless of sexual maturity status. Hence, 3kPZS could be used to modify the behavior and distribution of females during the entire migratory period, in addition to the spawning period. Synthesized 3kPZS must yet be tested in management contexts containing wild lampreys to confirm its utility for sea lamprey control.

In Chapter 5, I began investigating mating pheromone components that retain females on nests. 3kACA was hypothesized to function as a mating pheromone component that retains females on nests and induces mating behaviors because of previous laboratory experiments. Unexpectedly, 3kACA failed to elicit any behavioral response in ovulated females in natural streams. Therefore, an extraction technique was developed to concentrate unidentified mating pheromone components from mature male washings. A new in-stream bioassay was developed to allow the display of mating behaviors on nests. Future use of the field bioassay will enable additional mating pheromone components to be identified and characterized in stream environments. Throughout the dissertation, it was emphasized that researchers should utilize natural stream environments whenever possible to identify and characterize sea lamprey pheromone components, because that is the environment where pheromone components have ecological significances.

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