ALTERING THE MIGRATORY ROUTES OF SEA LAMPREY THROUGH THE APPLICATION OF SEMIOCHEMICALS

By

Gregory Joseph Byford

A THESIS

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

Fisheries and Wildlife - Master of Science

2016

ABSTRACT

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The sea lamprey (*Petromyzon marinus)* is a highly destructive invasive species within the Laurentian Great Lakes, but, is a legally protected species within its native range in the North Atlantic Ocean. Sea lamprey use multiple chemical cues either emitted or released from multiple life stages to perceive its environment and assess opportunities and risks while selecting suitable habitat during their terminal reproductive migration. This makes the sea lamprey a great model organism to test how conflicting signals of opportunities and risk are used by aquatic organisms during reproductive migrations. Here, we report two field tests designed to better understand how a cue derived from the carcasses of adult sea lamprey and the odor released by burrowing larvae influence the behavioral decisions made by migratory-phase sea lamprey once they have entered a riverine environment and a laboratory test to see if sea lamprey are repulsed by the extract of another imperiled anadromous lamprey. In our field experiments we demonstrated that when both odors are present lamprey behavior is contextually dependent upon the lateral distribution of odors as they approach the odor sources. In our laboratory experiment, we demonstrated that sea lamprey are repulsed by an extract derived from Pacific lamprey (*Entosphenus tridentatus*). In whole, this information may be of use for managers developing novel strategies such as olfactory-mediated behavioral manipulation to achieve either control or conservation.

To my mother for all her support and care packages,
To my father for showing me what it means to work hard,
To my sister for all her assistance throughout the years

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. C. Michael Wagner, and the members of my graduate committee Dr. Mike Jones and Dr. Jim Miller for their support and assistance. I also thank all personnel of the United States Geological Survey at the Hammond Bay Biological Station, Millersburg, Michigan, and the United States Fish and Wildlife Service at Marquette Biological Station, Marquette, Michigan, for their assistance in animal capture, housing, maintenances and technical support during projects. I give special thanks to my field technicians and colleagues for their hard work, dedication and patience in regards to these projects: Amanda Pomranke, Carrie Kozel, Luke Baker, Seth Gibson, Erica Adams, Fawn Fisher, Jameson Farinosi, Nick Snowgold, Sean Soucy (USFWS), Skye Fissette, Dr. Trevor Meckley, Dr. John Hume and Dr. Tom Luhring.

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INTRODUCTION TO THESIS

Organisms seek information about their current and future surroundings to pursue fitness enhancing opportunities while avoiding perceived risks. Information can be obtained on one or more sensory modalities (e.g. visual, olfactory, and tactile) through personal experience or by observing the success or failure of others (i.e. social information) (Dall et al. 2005). In aquatic environments, chemical assessment of risks and opportunities is especially important because low light transmission and a heterogeneous habitat make other sensory modalities less reliable (Chivers and Smith 1998; Ferrari et al. 2010). Furthermore, the aqueous medium allows molecules containing information to persist within the environment while being transmitted over great distances (Wisenden and Chivers 2006; Ferrari et al. 2010). By this means information is easily accessible to individuals which encounter odor plumes and have the capacity to perceive them. This has resulted in the reliance on chemical information to inform behavioral decisions across a range of spatial and temporal scales in aquatic organisms (Lima and Dill 1990). However it can also create situations where multiple odor plumes overlap and contain opposing information (i.e. risks and opportunities).

In behavioral ecology, the tradeoff between avoiding risks and gaining fitness enhancing resources (food, habitat and mates) has been well studied (Lima and Dill 1990 and references within; Kats & Dill 1998). However, fewer studies have used migratory species to gain insight on how migrating species operate this tradeoff (Bentley et al. 2014). When movements across habitats are required to attain suitable sites for resource acquisition or reproduction the exposure to predators is believed to increase because of exposure to and unfamiliarity of new habitats (Yoder et al. 2004; Rittenhouse et al. 2009; Forrester et al. 2015, Pepino et al. 2015). For example, when a fish migrates from a lake or ocean into a river they become spatially more confined and are exposed to new sources of predation including mammals and shore birds. However, the unidirectional flow broadcasts information in such a way that it can be interpreted for migrants traveling upstream. Cues used to aggregate migrants (migratory pheromones) are typically broadcasted over larger spatial and temporal scales than predation cues (alarm cues) that operate only when predation has recently occurred. This creates a situation where these cues operate on different temporal and spatial scales but may be present together. How ought an animal perceive and behave when exposed to mixed information of opportunities and risks?

The sea lamprey relies heavily on its olfactory system to guide its migration from the lake or ocean into a suitable river in search for spawning habitat and mates (Applegate 1950, Manion and Hansen 1980; Binder and McDonald 2007; Vrieze et al. 2010; 2011). Sea lamprey do not migrate to a natal stream (Bergstedet and Seelye 1995; Waldman et al. 2008) like many other migratory fishes (Ogura & Ishida 1995). Instead, stream selection is based on the odor released passively by resident larvae which confirms past reproductive success and suitable larval habitat (Teeter 1980; Wagner et al. 2009). Much effort has been expended to elucidate several active components of larval odor (Sorenson and Vrieze 2003; Sorenson et al. 2005) as well as studying this attractant as a potential alternative management strategy for the invasive population in the Laurentian Great Lakes (Wagner et al. 2006; Meckley et al. 2012).

More recently, it has been discovered that sea lamprey are repulsed by the odor of dead conspecifics suggesting that risk information may be another critical olfactory cue used to accomplish their ultimate goals (Wagner et al. 2011, Bals and Wagner 2012). Bals and Wagner (2012) described various facets of the sea lamprey alarm system in a laboratory setting. In summary, they found that the alarm cue was present in all tissues of adult lamprey, is present in larval sea lamprey, and in a heterospecific lamprey, the silver lamprey (*Ichthyomyzon unicuspis*). Previously alarm substances were believed to be contained within epidermal club cells, which may still be true, however in sea lamprey the alarm substance is also contained elsewhere throughout the body and remains active after a 96 hour decay. Furthermore, Bals and Wagner (2012) demonstrated the alarm response attenuates in females once they are mature. Female attenuation of response to alarm cues when ovulating has been observed in other fish (Lastein et al. 2008). The full ecological function of the sea lamprey alarm cue has yet to be determined. This is in part because the cue can be a result of three situations: 1) dead larvae, 2) dead migrants, or 3) dead spawners. In each of these scenarios the cue carries a different ecological meaning for migrating lamprey. The chemical communication system of sea lamprey produces a unique opportunity to study how a long-term opportunistic cue (larval odor) and a short-term risk information (alarm cue) are used during their riverine migration.

The goal of the first chapter of this thesis was to gain a basic understanding of how the sea lamprey respond to unavoidable risk (bank-to-bank alarm cue application) when a natural opportunistic odor source is present (resident larvae). Using a modified approach from previously published work, we used experimental protocols for odor-mediated behavioral manipulation studies in a natural river (Wagner et al. 2009, Meckley et al. 2012) to examine whether sea lamprey respond to the odor of dead conspecifics in a threat-sensitive manner as they migrate upstream. The second chapter further examines the migratory response of sea lamprey in a riverine setting free of background odor sources. This field study aimed to better understand how the odor of dead conspecifics and the odor of resident larvae together may modulate their behavior while approaching critical decision making points along their migration such as a stream bifurcation. The results give us a better understanding as to how lamprey use long-term opportunistic and short-term risk information during their riverine migration. The third chapter used a previously published assay based on odor-mediated change of space use (Wagner et al. 2011, Bals and Wagner 2012) to examine whether the odor of a more distantly related and imperiled lamprey species (Pacific lamprey; *Entosphenus tridentatus*) repels sea lamprey. The results shed light on use of sea lamprey as a surrogate to test for repulsive odors of other imperiled lampreys which may have the potential to be deployed for conservation management.

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CHAPTER 1: THREAT-INSENSITIVE BEHAVIOR IN A MIGRATING FISH

ABSTRACT

In migrating animals, movement between habitats is critical, but also increases the probability of encountering predators. Habitat selection is generally guided by information related to habitat quality, but information about recent predation events can modify that choice. In aquatic environments, chemically mediated assessment of information is one strategy many organisms use in order to balance the tradeoff of habitat selection and predation risk. It has been demonstrated that multiple aquatic organisms assess risk information and modulate antipredator behaviors according to the degree of threat posed by predators (threat-sensitive predator avoidance hypothesis). Most studies testing the threat-sensitive predator avoidance hypothesis were conducted in a laboratory with non-migratory fishes. Less is known whether migratory fish exhibit threat-sensitive behaviors in a natural setting. We investigated whether sea lamprey (*Petromyzon marinus*), exhibit threat-sensitive behaviors to a putative conspecific alarm cue during their terminal reproductive migration. Using a two-channel design in a small river we activated the entire water column with four concentrations of a putative conspecific alarm cue (0 $\mu l\ L^{-1}$, 0.1 $\mu l\ L^{-1}$, 0.2 $\mu l\ L^{-1}$ and 1$\mu l\ L^{-1}$) and observed the behavior of migratory sea lamprey. We saw no evidence which strongly suggests that sea lamprey exhibit threat-sensitive behavior during their upstream riverine migration. During the sea lamprey's terminal reproductive migration, risk information may restrict their route of migration but may not affect their propensity to migrate upstream.

INTRODUCTION

Predation is a significant and pervasive selection force which shapes the behavioral decisions of all animals including movement (Lima & Dill 1990; Chivers and Smith 1998; Kats & Dill 1998). Movement between habitats is a crucial step for migratory animals which creates a behavioral trade-off when the movements needed to obtain suitable sites for reproduction also places them in unfamiliar environment and increases their risk of predation (Lucas and Baras 2001; Yoder et al. 2004; Rittenhouse et al. 2009; Forrester et al. 2015; Pepino et al. 2015). In aquatic environments, risk information often comes in the form of chemical social cues (alarm cues) that can be used to identify potential upstream threats (Lima & Dill 1990; Chivers & Smith 1998; Kats & Dill 1998; Ferrari et al. 2010).

It has been demonstrated that chemical alarm responses can either be elicited at a point which the stimulus is of sufficient concentration to elicit a response (i.e. threshold response, Stabell and Lwin 1997), or individuals may modulate antipredator behaviors according to the degree of threat posed by predators through risk assessment (i.e. threat-sensitive predator avoidance hypothesis, Helfman 1989). Dupuch et al. (2004) demonstrated the northern redbelly dace (*Phoxinus eos*) exhibited more intense fleeing responses when exposed to greater concentrations of a damage-released alarm cue followed by increased freezing behavior closer to substrate and away from the alarm cue injection point. Jachner and Rydz (2002) similarly demonstrated that roach (*Rutilus rutilus*) increased the time spent hiding in a refuge when exposed to greater concentration of a damage-released alarm cue and spent less time foraging. However, multiple experiments demonstrated that other fish species (e.g. fathhead minnow (*Pimephales promelas*), juvenile convict cichlids (*Archocentrus nigrofasciatus*), rainbow trout (*Oncorhynchus mykiss*), and pumpkinseed *(Lepomis gibbosus*)) do not react in a threat-sensitive manner (Lawrence & Smith 1989; Brown et al. 2001; Mirza & Chivers 2003; Marcus & Brown 2003; Roh et al. 2004). Migratory fish are not trading off foraging opportunities and avoiding predation as many migratory fish cease feeding during their migration (Applegate 1950, Groot & Margolis 1991). To increase fitness, migratory fish are trading off reproductive opportunities and the risk of predation. Many migratory fishes are semelparous (i.e. only have one spawning event), therefore, they cannot delay reproduction or their fitness may be decreased or fully relinquished (Groot & Margolis 1991; Cook et al. 2014). To our knowledge threat-sensitive behavior has not been demonstrated in any semelparous migratory animals, but is important to test because it could have major fitness consequences if the behavior delays reproductive components such as securing habitat and finding a mate. In light of this, responding to risk information in a threat-sensitive fashion seems maladaptive for semelparous migrants. However, these cues have limited time-spans to be of use to individual migrants (Wisenden et al. 2009), therefore, they ought to act upon the information before it is lost.

As migrants move upstream, threat-sensitive responses would help migrants avoid the source (i.e. a predation event) if a lateral gradient of the cue is perceivable, but may delay upstream movement if a lateral gradient is not perceivable. If migrants stopped and waited for the cue to be absent when a lateral gradient is not present, they may never fully utilize the valuable information of the predator’s location upstream making their route more dangerous when this information is gone. Being able to distinguish between different levels of predation threats (hungry or satiated) and exhibiting flexible responses to predators can help lower the costs of anti-predator behaviors (Licht 1989).

In the Laurentian Great Lakes, sea lamprey (*Petromyzon marinus*), cease feeding in lacustrine environments and begin a terminal reproductive migration into riverine environments

(Applegate 1950). Sea lamprey are highly selective when choosing rivers for spawning and only spawn in approximately 10% of streams within the Great Lakes basin (Morman et al. 1980). The sea lamprey’s semelparous migration is guided by the odor of stream-resident larvae which confirms habitat suitable for rearing of larvae is present and past reproductive success has occurred (Teeter 1980; Bjerselius et al. 2000; Vrieze et al. 2010; 2011). More recently it has been demonstrated that sea lamprey possess an alarm signaling system which may modulate its behavior during its terminal reproductive migration within riverine environments (Wagner et al. 2011; Bals and Wagner 2012). It has been demonstrated that the putative conspecific alarm cue can restrict migrants into areas free of the cue when the cue is applied to achieve a dilution of 0.2 $\mu l\ L^{-1}$ or greater (Bals, 2012). Bals (2012), did not observe any threat-sensitive behavior when applying putative alarm cue to channel with an opposing channel free of the risk cue, but this may be because their experiment captured the behavior of migrants in close proximity to a simulated predator attack where the gradient of the risk cue could be detected and avoided. Further downstream of a predator’s attack, the putative alarm cue will activate the entire water column and the lateral gradient of risk information is not available to inform more precisely the location of the information source. How do sea lamprey tradeoff reproductive opportunities and risk information when risk is unavoidable and at varying levels? If migrants do exhibit threat-sensitive suppression of upstream movement, the application of a putative alarm cue could help aggregate spawning activity lower in riverine systems. Hence, the use of a putative conspecific alarm cue may be able to contribute to reaching the strategic goals of the Great Lakes Fishery Commission (GLFC) including reducing use of the lampricides, while increasing the effectiveness and efficiency of sea lamprey control methods (GLFC 2001; 2011).

Here, we report a field experiment which investigated whether migratory sea lamprey exhibit threat-sensitive behavioral responses to different in stream concentrations of a putative alarm cue when the entire river channel is fully activated (no lateral differences). We hypothesized that migrating sea lamprey will exhibit threat-sensitive suppression of upstream movement to a putative conspecific alarm cue when the odor activates the entire water column. We predicted: (1) that migrants will suppress upstream movements in direct proportion to the amount of risk as indicated by a putative conspecific alarm cue; (2) that the rate of upstream movement by migrants will be lowest with high risk and will be highest with no risk; and (3) that migrants will no exhibit lateral preferences when the lateral gradient of risk information is equal.

METHODS

Approach

We tested our hypotheses in a small river (9.5 m width), where we created two channels of equal width (4.75 m) to observe whether sea lamprey exhibit threat-sensitive behaviors during upstream migration. To ensure that migratory lamprey exhibited previously demonstrated avoidance behavior to the putative conspecific alarm cue we activated one of the channel widths with 1 µl L^{-1} and predicted that migrating lamprey would avoid the activated channel and migrate up the channel free of risk information, previously demonstrated by Bals (2012). To test for threat-sensitive behavior, we ran one 3 h long trial each night where the entire water column was fully activated with one of four dilutions with a putative conspecific alarm cue (0 µl L^{-1}, 0.1 µl L^{-1}, 0.2 µl L^{-1} and 1 µl L^{-1}) and observed lamprey movements.

Study site

All trials were conducted between May 28 and July 7, 2014 in the Ocqueoc River, a tributary to Lake Huron in Northern Michigan, USA. The experimental reach (45°24'37.52" N, 84° 2'54.05" W) was located upstream of a barrier used to limit the distribution of the invasive sea lamprey population in Lake Huron. Hence, we were only permitted to release female sea lamprey. However, there are no differences in the response of migratory-phase male and female lampreys to the putative alarm cue before maturation (Bals & Wagner 2012). A 6.7 m section of the stream was divided into two equal sized channels which were chemically homogenous from each other (see Fig. 1.1). Movements around the structure were monitored by using four fixed passive integrated transponder (PIT) antennas (two in each separate channel) and a four channel multiplexer (Oregon RFID, Portland, Oregon, USA). Stream temperatures ranged from 14° – 22°

C. Discharge was estimated daily with a flow meter (Hach H950, Frederick, MD, USA) using the velocity-area method (Gore 2006) and ranged from 1.10 – 2.16 m^3s^{-1} during the experiment.

Test subjects

We obtained actively migrating female sea lampreys from the U.S. Fish and Wildlife service as part of the annual sea lamprey control program during May – June 2014. These individuals had entered riverine environments and had begun their migration before being caught in traps at two tributaries of Lake Huron (the Cheboygan and Ocqueoc Rivers) or one tributary of Lake Michigan (Carp Lake River). Following capture, they were transported to the Hammond Bay Biological Station (HBBS, Millersburg, Michigan, USA), separated by sex using external characteristics, per Siefkes et al. (2003), and placed into 1000 L holding tanks which receive a continuous flow of fresh water from Lake Huron (100% exchange every 2 h). All sea lamprey were held at the station for a minimum of 48 h prior to experimental use. Each individual test subject was physically examined for signs of physical damage and monitored to ensure normal behavior. Lampreys released into the stream were not recovered. Uses of fishes and experimental procedures were approved by the Michigan State University Institutional Animal Care and Use Committee via permit # AUF 01/14-007-00.

PIT-tag implantation procedure

Prior to use in a trial, we internally tagged individual migratory-phase female sea lampreys with a 32 mm PIT tag (Oregon RFID, Portland, OR, USA) by making a 3 mm lateral incision along the low abdomen below the first dorsal fin. Each individual was examined for ovaries before the tag was inserted. After insertion, the incision was sealed with tissue adhesive (VetBond^{TM} tissue adhesive, 3M Company). Animals then recovered from the procedure in a 200L holding tank receiving a continuous flow of water for 24 h prior to use in a trial.

Alarm cue collection

The odor that putatively contains an alarm cue was extracted from freshly killed adult male and female sea lamprey following procedures detailed of Bals & Wagner (2012). To extract the cue we used a pair of 2.08 m Sohxlet apparatus (Ace Glass Inc., Vineland, NJ) each equipped with water-cooled Allihn condensers and 12 L solvent reservoirs heated with hemispherical mantles to 75° – 80° C. Carcasses (N=9/batch) were extracted with a 50:50 solution of 200 proof ethyl alcohol and deionized water for three cycles. Extract was then roto-evaporated (Buchi Rotoevaporator R-220, New Castle, DE) at 40° C under a vacuum to remove ethanol, producing ~5.2 L of alarm cue and stored at -20° C until use. Before use in experiments, alarm cue was slowly brought to melting temperature prior to being mixed into its carboy for that night’s trial.

Half-stream trial procedure

Twelve hours prior (approximately 10:00) to the beginning of trials, 80 test subjects were stocked into two holding cages (40 in each) to acclimate to stream conditions. Each trial lasted 2 hours, we began pumping the stimulus at 21:00 every night and released test subjects at 21:10. We began pumping one of two treatments, either alarm cue or a solvent control, one meter upstream of the experimental arena (see Fig. 1-1) at a rate of 40 ml min^{-1} using laboratory-grade peristaltic pumps (MasterFlex model 7533-20) to activate half of the stream width to the desired concentration (1 µl L^{-1}). Four replicates were completed for each treatment. The channel receiving the stimulus was alternated across replicates within each treatment (each treatment received 2 trials with the stimulus applied in the right and left channels). Test subjects were released from the cages and lamprey movements across the PIT antennas were recorded to a single multiplexer. After the trial was completed at 23:00, we measured the residual amount of either alarm cue or a solvent control left from the trial to ensure that we had pumped a sufficient amount to reach our desired instream dilution, then we remixed the proper amounts again and reset the pump rate if needed. A second trial was then run, following the same procedures previously stated from 23:30 until 1:30. Following both trials, we downloaded the data onto a PDA and transferred it to a computer for analysis.

Full-stream trial procedure

Twelve hours prior (approximately 10:00) to the beginning of trials, 40 test subjects were stocked into two holding cages (20 in each cage) to acclimate to stream conditions. Each trial lasted 3 hours, we began pumping the stimulus approximately 120 meters upstream of the PIT array at 22:00 and released test subjects at 22:30. We introduced our stimulus (see Fig 1.1) at a rate of 40 ml min^{-1} using laboratory-grade peristaltic pumps (MasterFlex model 7533-20) which either pumped alarm cue or solvent control to activate the full stream width to the desired concentration. Four replicates were completed per dilution. The channel receiving the alarm cue was alternated across replicates within each treatment (each treatment received an equal number of trials 2 with the stimulus applied on the right and left side). Test subjects were released from the cages and lamprey movements across the PIT antennas were recorded to a single multiplexer. Following each trial, we downloaded the data onto a PDA and transferred it to a computer for analysis.

Data analysis

To demonstrate that a putative alarm cue was present in the sohxlet extracted odor (half-channel experiment) we examined the channel selection, defined as the proportion of individual lamprey choosing the channel activated with either a solvent control or a putative alarm cue determined by first passage through the PIT array. Channel selection was analyzed with a three-way analysis of variance (ANOVA) test with stimulus odor (factor), water temperature (continuous), and stream discharge (continuous) as fixed effects in the model.

To ascertain whether migratory sea lamprey exhibited threat-sensitive responses to increasing full stream concentrations of a putative alarm cue, we examined: 1) the rate of migration, defined as the proportion of sea lampreys moving upstream and detected at the PIT array; 2) time of arrival, estimated as the time from opening the release cage to first detection at the PIT array for each individual which moved upstream; and 3) channel selection, defined as the proportion choosing the right channel as determined by first passage through the right side of the PIT array (Both channels are activated equally, confirmed via dye testing, so analyzing one channel will allow us to observe potential side bias). Migration rate and channel selection (response variables) were analyzed with separate three-way ANOVA tests with alarm cue concentration (factor), water temperature (continuous), and stream discharge (continuous) as fixed effects in the model. Prior to analysis the proportion data were arcsine (square-root) transformed and tested for normality via Shapiro-Wilk's test. The transformed values for migration rate (W = 0.97, P = 0.86) and channel selection (W = 0.92, P = 0.18) met the normality assumption for ANOVA. Time of arrival for all individuals were highly left-skewed and failed normality tests after applying standard transformations (e.g. log) as well as Box-Cox transformation suggestions (Box & Cox 1964). Consequently, differences in swim-up time across alarm cue dilutions were examined with a non-parametric Kruskal-Wallis equality-of-populations rank test, using the 'kwallis2' command which also provides multiple comparison testing (Caci 1999). All statistical analyses were performed with STATA ver. 14.1 (StataCorp LP).

RESULTS

Our ANOVA model ($F_{3,7}$ = 11.16, P = 0.02) suggests that the addition of the alarm cue to one half of the channel had a strong effect on which side of the channel that lampreys chose when swimming upstream ($F_{1,7}$ = 18.05, P = 0.01). Neither stream discharge ($F_{1,7}$ = 0.2, P = 0.67) nor river temperature ($F_{1,7}$ = 0.00, P = 0.99) had effects on which channel migrating lampreys actively chose. This ensured that our stimulus odor still contained a putative alarm cue after our sohxlet extracted material was roto-evaporated.

The proportion of individuals which were detected swimming upstream during the 3 h observation period was consistently high and per treatment are as follows: 0 µl L^{-1} = 83.1%, 0.1 µl L^{-1} = 88.7%, 0.2 µl L^{-1} = 88.7%, and 1 µl L^{-1} = 85.3% (see Fig. 1.2). Activating the entire stream width with a putative alarm cue had no effect on the proportion of lamprey swimming upstream (ANOVA, overall model $F_{5,15}$ = 1.06, P = 0.43; alarm cue concentration effect $F_{3,15}$ = 0.49, P = 0.69). Similarly, neither river temperature ($F_{1,15}$ = 0.95, P = 0.35) nor stream discharge ($F_{1,15}$ = 0.01, P = 0.92) effected the proportion of lamprey actively migrating upstream.

The time between being released until first detection varied greatly between individuals. The range and average time of all individuals per treatment is as follows: 0 µl L^{-1}, range = 266 – 7033 s, average = 1605 s; 0.1 µl L^{-1}, range = 242 – 5118 s, average = 1707 s; 0.2 µl L^{-1}, range = 277 – 8649 s, average = 1806 s; and 1 µl L^{-1}, range = 152 – 8565 s, average = 2262 s (see Fig. 1.3 and Table 1.1). Differences did exist between alarm cue dilution treatments in regards to the time from release to arrival (Kruskal-Wallis, χ^2 = 15.97, df = 3, P = 0.001). However, the only difference between treatments was between the control (0 µl L^{-1}) and the highest alarm cue concentration (1 µl L^{-1}; obs. diff. = 76.98, crit. diff. = 51.74).

Our model (ANOVA, $F_{5,\,15} = 0.39$, $P = 0.84$) suggests that neither alarm cue concentration ($F_{3,\,15} = 0.49$, $P = 0.69$), stream discharge ($F_{1,\,15} = 1.31$, $P = 0.27$), or river temperature ($F_{1,\,15} = 0.95$, $P = 0.35$) did not influence the channel which migrants chose when swimming upstream past the PIT array.

DISCUSSION

As predicted, when a putative conspecific alarm cue was applied to one stream channel, migrants consistently avoided the activated channel and swam up the odor free channel. This result further supports that sea lamprey may restrict their migratory routes in presence of a putative alarm cue (Bals, 2012).

However, our hypothesis that migrating sea lamprey exhibit threat-sensitive responses to a putative conspecific alarm cue when activating the entire water column was not supported by our results. There were no statistical differences in the proportion of individuals actively swimming upstream during our three hour trial across the four alarm cue concentrations. We observed that approximately the same proportion of migrants swam upstream across all alarm cue concentrations used. During their reproductive migration, sea lamprey may tolerate risk information when a lateral gradient is not perceivable, and only once a lateral gradient of risk is apparent do lamprey modulate their behavior to avoid the source of risk. It has been demonstrated that foraging fish will tolerate more risk when their needs are greater or the energetic rewards are higher (Smith 1981; Abrahams & Dill 1989; Brown & Smith 1996).

We did not observe any threat-sensitive responses by migrants in the average time of their arrival at the PIT array either. However, there was a significant difference in the average time from release to first detection between the control and 1 $\mu l\ L^{-1}$ trials. No other significant differences were observed between any other treatments in regards to time of arrival. Several biological phenomena could explain why we saw a difference in migrant's time of arrival at the highest concentration and not at others. As previously mentioned in the introduction, responses are usually either elicited once at a threshold concentration, or the magnitude of the response can be graded based on the level of threat perceived by the current concentration. If the response was based on a threshold the two lowest concentrations (0.1 and 0.2 µl L^{-1}) may not have be able to elicit a response. However, Bals (2012) was able to direct > 90% of migrants away from the alarm cue activated channel with a concentration of 0.2 µl L^{-1}, so it is unlikely that this concentration was unperceivable in our experiment. This observed difference could also be explained by a change in behavior when exposed to the putative conspecific alarm cue at our highest concentration. Sea lamprey may have reduced their upstream movement or activity which is a common antipredator response (Chivers & Smith; Kats & Dill 1998, Ferrari et al. 2010). However, another possible explanation for this observation may be an artifact of our experimental set up. Our experiment was designed to simulate a predation event where lamprey were suddenly exposed to the alarm cue and released shortly after. Being held as a group in a cage where subjects were unable to seek refuge may effect individual responses when they are released from the cage. Visual observations of animals exhibiting fright responses at the dilutions of 0.2 and 1 µl L^{-1} were common, where a number of individuals were observed porpoising and moving downstream (G.J. Byford, *pers. observation*). It is likely this difference in time of arrival may be an experimental artifact, however, there is no clear observable time lag at the beginning of the survival curves which indicates some migrants were still swimming upstream rather quickly.

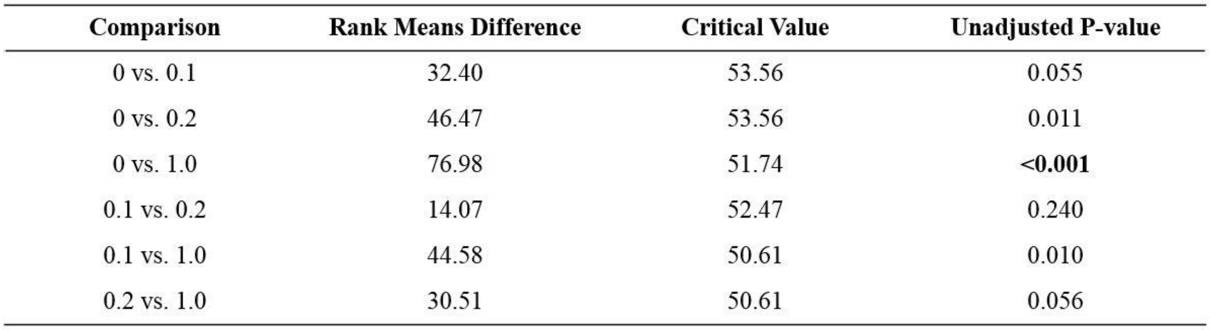
There was great variation between the times it took individuals to be detected from whey they were released within all treatments. Some individuals swam up quickly, while others swam up very late in trials. This variation in time of arrival may be explained by individual personality differences of migrating sea lamprey. Some aquatic organisms have been demonstrated to consistently exhibit behaviors towards managing risk and vary individually on a scale from shy to bold (Coleman & Wilson 1998; Brown et al. 2009; Brown et al. 2014). One study has tested for consistent individual differences in sea lamprey and demonstrated individual repeatable differences in their general activity, latency to leave a refuge and response to a predator cue (McLean, 2014). Personality differences may cause some individuals to be bolder and move upstream quicker when risk information is present and other shy individuals may take longer to move upstream when risk information is present or not at all.

However, these results must also be discussed considering a post-hoc discovery that upstream of our experimental section in the Ocqueoc River a small population of sea lamprey larval was discovered during annual electro-fishing surveys (Aaron Jubar, USFWS, *pers. comm.*). They reported catching 25 of 35 sea lamprey larvae at seven of eight sampling locations above our study site and 10 more larvae downstream of our site. The survey catches implies that 9,405 larvae were present above the barrier of which 7,076 were > 100 mm in length (Aaron Jubar, USFWS, *pers. comm.*). The proportion of individuals greater than > 100 mm suggests that the larval infestation occurred at least 2 years ago meaning the odor emitted by larvae has been present in the system for that time. We do not have water sample data to quantify the molar concentrations of the known larval bile acids, but much work has been done to establish the sea lamprey's perception at pico-molar concentrations (Vrieze et al. 2010; Meckley et al. 2012).

Our negative results for threat-sensitive responses in migrating sea lamprey when a small larval population is present helps to improve our understanding of how lamprey use multiple olfactory cues during their migration and how they could serve utility in alternative control strategies. We demonstrated that currently we do not possess a repellant which suppresses upstream movement in rivers we would expect a lamprey to choose for spawning (one where past reproductive success has occurred). However, we were still able to restrict the migratory route of > 95% of lamprey (135 of 141) which swam upstream when the putative conspecific alarm cue was applied to only one channel, which is similar to the > 99% avoidance (122 of 123) achieved when the odor of larvae was not present (Bals 2012). The difference in the magnitude of avoidance with and without the odor of larvae present may be of interest to managers who want to achieve strong and consistent responses throughout the migratory season. This odor may be used at stream bifurcations or tributaries to aggregate spawners, but if the response to the putative alarm cue is not as strong when both channels also are emitting the odor of larvae, aggregating spawners may not be as successful. Due to the fecundity (egg production) of female sea lamprey (Applegate 1950), only a few individuals need to spawn successfully in an area in order to produce several thousand larvae. This continues the call and need for control strategies which are consistent and highly effective. Olfactory behavioral manipulation shows promise for the sea lamprey control program, however, more rigorous testing is needed before it can be used to achieve highly consistent and effective results which are cost effective.

APPENDIX

TABLES



Comparison	Rank Means Difference	Critical Value	Unadjusted P-value
0 vs. 0.1	32.40	53.56	0.055
0 vs. 0.2	46.47	53.56	0.011
0 vs. 1.0	76.98	51.74	**<0.001**
0.1 vs. 0.2	14.07	52.47	0.240
0.1 vs. 1.0	44.58	50.61	0.010
0.2 vs. 1.0	30.51	50.61	0.056

Table 1.1: Results of the post-hoc Rank-Means (RM) test of the times of arrival at the PIT antennas during our three hour observation period. Comparison refers to the alarm cue concentrations being compared; Rank Means Difference is the reported RM difference; Critical Value is the critical RM value for that comparison. The unadjusted P-value is reported. P-values in bold indicate significantly different rank-means after adjustment for multiple comparisons.

FIGURES

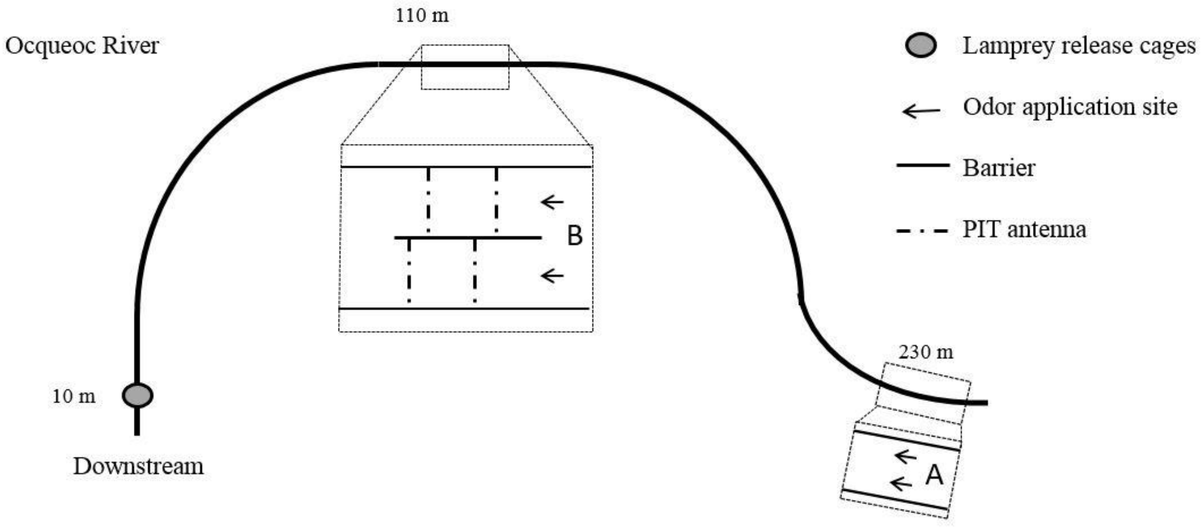


Figure 1.1: Diagram of the Ocqueoc River field site. Sea lampreys were held in cages approximately 100 m downstream of the experimental section. Movements were monitored with paired Passive Integrated Transponder (PIT) antennas as they swam upstream past the odor application site. For the full-stream width experiment (A), odors were introduced 120 m above the most upstream section of the barrier to activate the entire water column. For the half-channel experiment (B), odors were introduced 1 m above the most upstream section of the barrier in the middle of the respective channel to activate only one channel. All distances displayed are distances from the most downstream point of the site.

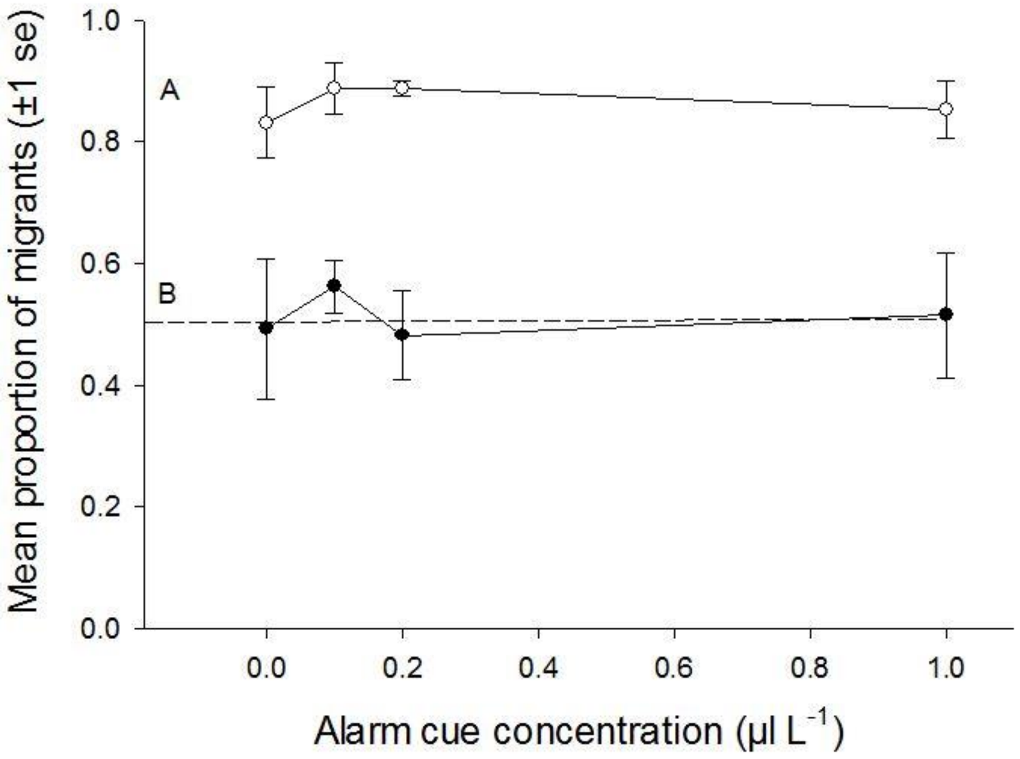


Figure 1.2: Mean proportion (± 1 se) of released female lampreys observed moving upstream in the Ocqueoc River when exposed to the alarm cue at various dilutions during the full-stream experiment. (A) Mean proportion that swam upstream and were detected at the PIT array. (B) The proportion of upstream swimming lampreys that were detected in the right channel (dashed line indicates null expectation of equal proportion on either side of the channel). N=4 for each concentration.

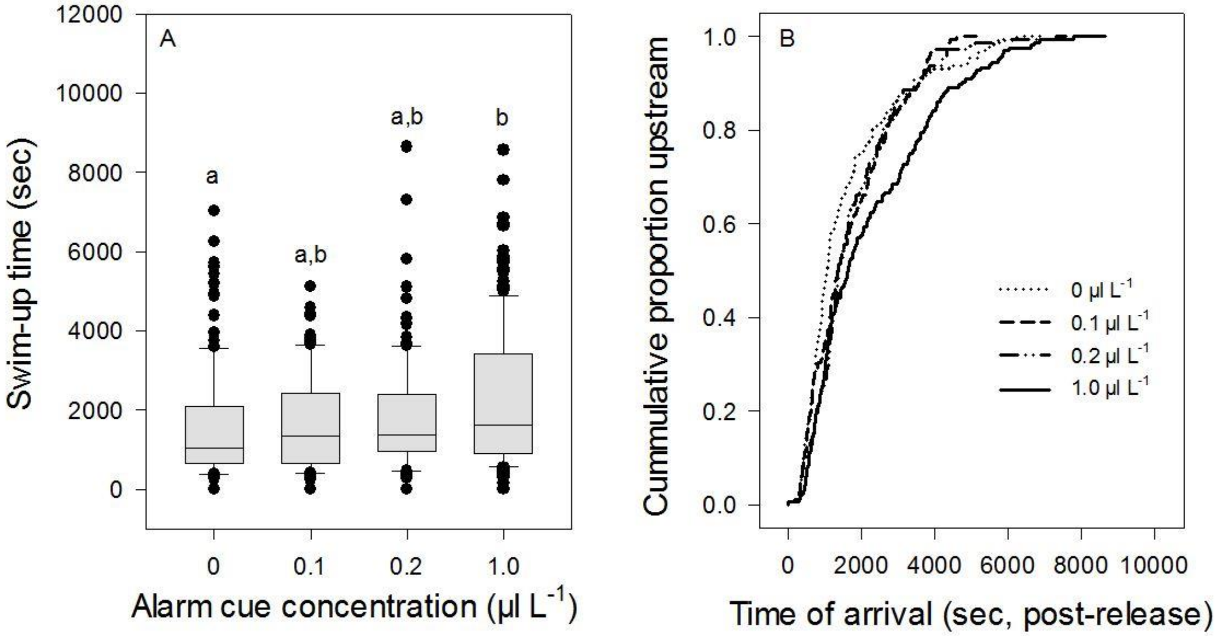


Figure 1.3: (A) Box-plot of the individual swim-up times (elapsed time of arrival at the PIT antennas) during the field experiment for each alarm cue concentration (0 µl L^{-1} = solvent control). Different letters indicate statistically different time per post-hoc pairwise Kruskal-Wallis test performed on rank data. (B) Cumulative proportion of animals detected at the PIT antennas as a function of time since release for each alarm cue concentration. Only lampreys that swam up to the PIT antennas are included.

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CHAPTER 2: APPARENT RISK OF PREDATION ALTERS MIGRATORY SEA LAMPREY RESPONSE TO A HABITAT-SELECTION CUE

ABSTRACT

Migratory animals are confronted with a challenge when movements to secure suitable habitat for reproduction also increase their vulnerability to predation. In aquatic environments, chemically mediated assessment of information (risks and opportunities) has been one strategy organisms use to inform decision-making when confronted with these contrasting cues. The sea lamprey uses the odor released passively by stream resident larvae to select rivers where past reproductive success has occurred. As migrating sea lamprey enter riverine environments they become spatially confined, increasing their vulnerability to shoreline predators and avoid the odor of dead conspecifics consistent with antipredator behavior. Using a two-channel design in a small river we sought to create avoidable and unavoidable risk scenarios which migrating lamprey may encounter once in a riverine environment. In the presence of larval odor, more migrants actively swam upstream and did so more quickly. Also, migrants were still attracted to the larval odor channel even when concurrently present with risk. Fewer migrants actively swam upstream and did so slower when risk information went from avoidable to unavoidable. It appears that when encountering both cues, sea lamprey will continue to migrate upstream and appear to be able to select routes which are associated with less risk.

INTRODUCTION

Movement between foraging and reproductive habitats is a common feature of the life cycles of many animals (Lucas and Baras 2001; Milner-Gulland et al. 2011). In particular, migrating animals frequently must move through unfamiliar space to secure suitable sites for reproduction, increasing the risk of encountering new predators (Yoder et al. 2004, Rittenhouse et al. 2009, Forrester et al. 2015, Pepino et al. 2015). Information about habitat quality (for reproduction or rearing of offspring) generally guides habitat selection (Doving and Stabell 2003; Doving et al. 2006), but local information about recent or past predation can modify that choice and the route taken during migration (Kotler et al. 1991; Heithaus & Dill 2002; Olsson et al. 2002; Creel et al. 2005).

Choosing high-quality habitat influences an individual's immediate success as well as its lifetime fitness (Franklin et al. 2000). In order to maximize fitness, organisms must seek opportunities to gain resources (e.g. food, habitat, and mates) while combating risky situations, such as avoiding predation. The tradeoff between seeking opportunity and attending to risk is mediated by the reception of information across multiple sensory systems (Kim et al. 2009). However, in aquatic environments chemically-mediated communication is particularly important because the heterogeneous habitat with limited visibility and low light transmission can make visual information less reliable (Chivers & Smith 1998; Kats & Dill 1998; Ferrari et al. 2010). The aqueous medium is ideal for broadcasting molecules containing information regarding opportunities (e.g. mates, habitat) and risk (e.g. predation, sub-optimal habitat) over substantial distances (Wisenden and Chivers 2006; Wisenden 2008; Ferrari et al. 2010). Therefore, many aquatic organisms depend on chemical information to modulate behavioral decisions across spatial and temporal scales (Lima and Dill 1990). However, an individual's response to chemical information can differ based on their internal state (e.g. hunger level, Brown & Smith 1996; maturation state, Lastein et al. 2008; Bals and Wagner 2012), previous experiences (Ferrari & Chivers 2006), as well as personality differences (Brown et al. 2014).

The nature of broadcasting information in aquatic environments, suggests that animals frequently are faced with multiple sources of information which may be conflicting. The most common outcome to receiving multiple inputs is that one type of stimulus trumps the response to other stimuli and the animal will perform just one behavior (Hinde 1970). For example, when the hermit crab (*Clibanarius vittatus*) detects a predator odor and a food odor simultaneously, the food-searching behavior switches to performing antipredator defense behaviors (Hazlett 1996). A second potential outcome to multiple inputs may be a summation of responses related to the multiple inputs received (Hinde 1970). Animals may execute distinct behavior patterns simultaneously (Davis 1979), or synergistic behavior may occur when multiple inputs are perceived with similar valence making the decision more reliable (Beehler et al. 1993). Several studies have observed the relationship between foraging and predation risk and demonstrated that short-term predation risk negatively influences foraging rates (Milinski & Heller 1978; Mittelbach 1986; Mittelbach and Chesson 1987; Werner & Hall 1988; Kats and Dill 1998). However, it is less understood how aquatic organisms use multiple signals during a reproductive migration compared to foraging. The most well studied aquatic migrations is that of salmonids which are thought to rely on chemical information to locate suitable habitat (Doving and Stabell 2003; Doving et al. 2006), however, more recently sockeye salmon have been observed to move between spawning stream habitats and lake habitats to adapt to differences in predation risk (Bentley et al. 2014). It has also been demonstrated that migrating ungulates will alter their migratory routes, using different habitats to lower the risk of predation and do so within partial and full migration strategies (Fortin et al. 2005; Hebblewhite & Merrill 2006; 2007; 2009). How do aquatic organisms resolve the challenge of pursuing opportunities while avoiding the risk of predation during reproductive migrations?

The sea lamprey (*Petromyzon marinus*) uses olfaction during its terminal nocturnal migration to locate and choose suitable riverine habitat to reproduce (Applegate 1950; Teeter 1980; Binder and McDonald 2007; Vrieze et al. 2010; 2011). Sea lamprey have shown to be highly selective in choosing rivers for spawning and only spawn in approximately 10% of streams within the Laurentian Great Lakes basin (Morman et al. 1980) However, this semelparous migration does not end within the natal stream (Bergstedt and Seelye 1995; Waldman et al. 2008) like that of many anadromous fishes such as salmon (Ogura & Ishida 1995). It has been shown that both migratory strategies may rely on olfaction (Hasler et al. 1978; Teeter 1980; Vrieze et al. 2010; 2011) where both are likely experience similar conflicts between information about opportunities and risks. Migratory sea lamprey use the odor released passively by burrowing conspecific larvae to identify and select rivers where habitat is suitable for larval rearing and past reproductive success has occurred (Bjserlius et al. 2000, Vreize et al. 2010; 2011). Actively migrating lamprey are highly attracted to larval odor and tends to avoid habitats where the odor is absent (Wagner et al. 2006). However, sea lamprey do not modulate their response to greater larval odor concentrations (Wagner et al. 2009) and the response diminishes as individuals mature during the migratory season (Bjerselius et al. 2000, Meckley et al. 2012). More recently, it has been demonstrated that sea lamprey possess an alarm signaling system which may modulate the response to larval odor (Wagner et al. 2011; Bals and Wagner 2012). However, it is not fully understood how this alarm signaling system mediates lamprey behavior throughout their terminal riverine migration. When present throughout the water column from bank to bank, the alarm cue fails to block or delay lamprey as they migrate upstream when a small larval population is present (Byford Chapter 1). But, the alarm cue has been shown to restrict migrants into areas free of the cue, in both systems free of the odor of resident larvae and when the odor of resident larvae is present (Bals 2012; Byford Chapter 1). As indicated in the previous paragraph, sea lamprey will only choose to migrate within riverine systems where the odor of resident larvae is present. The odor of larvae attracts lampreys upstream, in part, to ensure that reproduction occurs upstream of suitable larval rearing habitat and the alarm cue may allow them to avoid areas of high predation along the way, or where unavoidable, modulate their movement paths and timing to minimize risk. When exposed to combinations of conflicting chemical cues where risk may be avoidable or unavoidable and a conspecific attractant may be present or absent, do migrating sea lamprey use the available information to modulate the timing of their movements and select the route which minimizes risk?

We postulated that migrating sea lamprey modulate their movements relative to the amount and distribution of conspecific attractants and repellants present. We predicted that: (1) that a conspecific alarm cue will decrease the upstream movement of migrating test subjects which will be greatest when only a conspecific attractant is present; (2) test subjects will be attracted to the channel emitting a conspecific attractant even when a conspecific alarm cue is present; and (3) that the rate of upstream movement will be faster when a conspecific attractant is present and will be slower when a conspecific alarm cue is present.

METHODS

Approach

We tested our hypotheses in a small river, where we created two chemically separate channels of equal width to observe the migratory behavior of sea lamprey to scenarios of conflicting cues. We ran two ninety minute trials each night using the same treatment, but alternating the channel in which the treatment was applied. The experimental treatments are as follows:

1. Larval odor vs. river water (positive control, opportunity). This treatment tests whether the animals were responding to the migratory cue (larval odor) by moving upstream in high numbers (Prediction 1.1) and choosing the side of the channel activated with the larval odor to ensure entry into the proper spawning stream (Prediction 2.1). Activating one side of the channel only simulates the presence of a single suitable tributary upstream.
2. Larval odor vs. alarm cue (opportunity + avoidable risk). This treatment tests whether the animals reduce upstream movement when the migratory cue (larval odor) is contrasted with avoidable risk compared to the positive control (Prediction 1.2). This treatment also tests whether more animals chose to actively migrate up the larval odor side when contrasted with alarm cue compared to the positive control (Prediction 2.2).
3. Larval odor + alarm cue vs. alarm cue (opportunity + unavoidable risk). This treatment tests whether more animals actively migrated when the entire water column was activated with risk and larval odor was present compared to the absence of larval odor (Prediction 1.3) and whether more animals chose to migrate up the larval odor channel compared to the positive control (Prediction 2.3).

4. Alarm cue vs. river water (Alarm cue control, avoidable risk), This treatment tests whether the animals were responding to a putative alarm cue by moving upstream less compared to treatment #1 (Prediction 1.4) and avoiding the channel activated with the putative alarm cue and choosing the risk free side (Prediction 2.4).
5. Alarm cue vs. alarm cue (negative control, unavoidable risk). This treatment tests whether the animals moved upstream less when risk activates the entire water column compared to when a lateral gradient is present (Treatment #4; avoidable) where migrants can escape the odor (Prediction 1.5) and whether migrants prefer migrating up one channel compared to the other when all information is equal between channels (Prediction 2.5).

In our analyses we tested three response variables; the proportion of lampreys moving upstream (Predictions 1.1, 1.2, 1.3, 1.4, and 1.5), the side chosen (Predictions 2.1, 2.2, 2.3, 2.4, and 2.5), and the time of arrival of those individuals who made the decision to swim upstream (Prediction 3).

Study site

All trials were conducted between June 6 – 23, 2015 in the Ocqueoc River (width = 9.8 m, mean discharge = 2.18 m^3s^{-1}), a tributary to Lake Huron in Northern Michigan, USA (45° 24’36.63°N, 84° 2’53.83°W) which historically had large regular runs of sea lamprey before a barrier was installed (Applegate 1950). Since, the study site was located upstream of a sea lamprey barrier, we were permitted to release only female lamprey to prevent larval infestation. There are no sex differences in behavioral responses to either cue during their migratory phase (Bjerselius et al.

2000; Wagner et al. 2006; Bals and Wagner 2012). We divided the stream into two channels of equal width with a two twenty-two foot long impervious PVC barrier which keep each channel chemically separate (see Fig. 2.1). To ensure that the odor plumes from both channels were mixed from bank-to-bank at the release cages we ran three trials to observe Rhodamine WT concentrations (ppb) at the point of release. Using a Turner Designs DataBank logger equipped with a series Cyclops-7 Rhodamine WT probe we determined that the odor from each channel was bank-to-bank at the release cages ensuring that test subjects experienced both plumes upon release (see Fig. 2.1). We monitored lamprey movements with passive integrated transponder (PIT) tags and four fixed PIT antennas (Oregon RFID, Portland, OR, USA). Temperature in the stream ranged from 14° – 21° C and discharge ranged from 1.70 – 2.64 $m^3 s^{-1}$ during the experiment.

Experimental subjects

We obtained actively migrating female sea lampreys from the U.S. Fish and Wildlife service as part of the annual sea lamprey control program during May – June 2015. These individuals had entered riverine environments and had begun their migration before being caught in traps at two tributaries of Lake Huron (the Cheboygan and Ocqueoc Rivers) or one tributary of Lake Michigan (Manistique River). Following capture they were transported to the Hammond Bay Biological Station (HBBS, Millersburg, Michigan, USA), separated by sex using external characteristics, per Siefkes et al. (2003), and placed into 1000 L holding tanks which receive a continuous flow of fresh Lake Huron water (100% exchange every 2 h). All sea lamprey were held at HBBS for a minimum of 48 h prior to experimental use. Each individual test subject was physically examined for signs of physical damage and monitored to ensure normal behavior. Lampreys released into the stream were not recovered. All procedures for subject handling and experimentation were approved by the Michigan State University Institutional Animal Care and Use Committee (permit # AUF 01/14-007-00).

PIT-tag implantation procedure

Prior to use in a trial, we internally tagged migratory-phase female sea lampreys with a 23 mm PIT tags (Oregon RFID, Portland, OR, USA) by making a 3 mm lateral incision along the lower abdomen below the first dorsal fin. Each individual was examined for ovaries before the tag was inserted. After insertion, the incision was sealed with Vet-Bond tissue adhesive. Animals then recovered from the procedure in a 200 L holding tank receiving a continuous flow of water for 24 h prior to use in a trial.

Larval odor collection

We used extracted larval odor from three separate year batches (2009, 2013 and 2014).The larval odor extract was collected following the methods of extraction by Fine et al. (2006). Captive populations of larval sea lamprey (over 20,000) were held in flowing 500 L holding tanks at HBBS for collection of larval pheromone extracts in years 2009, 2013 and 2014. The tanks had sand substrate for refuge and larvae were fed yeast weekly. The larval-conditioned water passed through vertical columns containing 500 g of methanol-activated Amberlite XAD7HP resin (Sigma-Aldrich, St. Louis, Missouri, USA.) using peristaltic pumps (Cole-Parmer, Vernon Hills, Illinois, USA). Three to four columns were loaded for up to 24 h at a time. Each column was then eluted with 4 L of methanol and eluents were concentrated using a model R-210 roto-evaporator (Buchi Rotovapor, Flawil, Switzerland) and stored at -80° C. All larval extracts were fully thawed, pooled and thoroughly mixed before further analyses were conducted. Petromyzonamine disulfate (PADS), a component of larval extract with known chemical structure (Sorenson et al. 2005), was used as a benchmark compound when calculating the volume of extract to consistently apply to the stream. Prior to use, the concentration of PADS of each year (2009, 2013 and 2014) was determined using high performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) by Dr. Ke Li (Michigan State University). For reference, the PADS concentration was found to be 262 μg L^{-1} in the larval extract derived in the 2009 batch, 1275μg L^{-1} in the 2013 batch and 89.8μg L^{-1} in the 2014 batch. Larval odor was slowly brought to melting temperature prior to being mixed for use in experiments.

Alarm cue collection

The odor that putatively contains an alarm cue was extracted from freshly killed adult male and female sea lamprey following procedures detailed of Bals & Wagner (2012). To extract the cue we used a pair of 2.08 m Sohxlet apparatus (Ace Glass Inc., Vineland, NJ) each equipped with water-cooled Allihn condensers and 12 L solvent reservoirs heated with hemispherical mantles to 75° – 80° C. Carcasses (N=9/batch) were extracted with a 50:50 solution of 200 proof ethyl alcohol and deionized water for three cycles. Extract was then roto-evaporated (Buchi Rotoevaporator R-220, New Castle, DE) at 40° C under a vacuum to remove ethanol, producing

~5.2 L of alarm cue and stored at -20° C until use. Before use in experiments, alarm cue was slowly brought to melting temperature prior to being mixed into its carboy for that night's trial.

Trial procedure

Eleven hours prior (approximately 1100) to the start of the first trial PIT-tagged female lamprey were stocked into two metal holding cages at the downstream end of the study site. Two trials were run each night which consisted of the same treatment. We used the same treatment for both trials each night so that the animals in the second trial experienced the same downstream mix of odors as they experienced in the first trail. For the first two replicates of each treatment only 20 test subjects were used per replicate due to limited availability of experimental subjects. The following four replicates of each treatment used 30 test subjects per replicate, following the original study design. Discharge was estimated using the cross-section method (Gore, 1996) with a flow meter (Hach F950, Frederick, MD, USA) after the experimental subjects were stocked. We started our first trial at approximately 2200 h and applied one of the five odor treatments to our two-choice barrier. When the putative alarm cue was applied to be avoidable it was introduced into one of the two channels to reach an in-stream concentration of 0.2 $\mu l\ L^{-1}$ which was predicted to have a strong but not complete repulsion (Bals 2012). When the putative alarm cue was applied to be unavoidable it was introduced into both channels to reach an instream concentration of 0.2 $\mu l\ L^{-1}$. Larval odor was applied to one of the two channels to reach an in stream concentration of 5.0×10^{-13} μg/L PADS, a concentration shown to be highly attractive in the field (Wagner et al. 2006).

Data analysis

We tested Prediction #1 (1.1, 1.2, 1.3, 1.4 and 1.5), that a conspecific alarm cue affects a migrant's upstream movement, with a generalized linear mixed-model (GLMM) for binomial responses using a logit link function with treatment (factor), time (factor), water temperature (continuous), stream discharge (continuous) as fixed effects and Julian date as a random effect in the model. Time is defined as whether test subjects were released in the first or the second trial of the night, and Julian date is defined as the day in which test subjects were placed in acclimation cages for that night's trials. To test for overall differences in upstream movement between treatments, we ran a Fisher's protected least-significant difference (LSD; $\alpha = 0.05$) to test all pairwise comparisons.

We tested Prediction #2 (2.1, 2.2, 2.3, 2.4 and 2.5), that test subjects will always be attracted to the channel emitting a conspecific attractant even when a conspecific alarm cue is present, using individual Z-tests testing if the distribution of migrants between channels differed from a 50:50 distribution. We also analyzed the two treatments (Treatment 2: alarm cue vs. river water & Treatment 3: alarm cue vs. alarm cue) which did not included larval odor to test whether migrants were avoiding the alarm cue when given a route with no risk information and whether our field site had a side bias when both channels were emitting the same information.

We tested Prediction #3, that test subjects will arrive sooner at the PIT antennas after being released when larval odor is present and individuals will arrive later when only alarm cue is present, using a Kruskal-Wallis rank sum test. We used a non-parametric analyses for this data because its distribution was heavily left-skewed, and was unable to meet the normality assumption for parametric testing using common transformations (e.g. log or square root transformation) as well as using Box-Cox transformation (Box & Cox 1964). Consequently, differences in swim-up time across alarm cue dilutions were examined with a non-parametric Kruskal-Wallis equality-of-populations rank test, using the ‘kwallis2’ command which also provides multiple comparison testing (Caci 1999). All statistical analyses were performed in STATA ver 14.1 (StataCorp LP).

RESULTS

Our GLMM suggests that treatment (z = -6.54, P < 0.001), and river temperature (z = -2.60, P = 0.009) significantly influence individual decisions to swim upstream, but time (z = -1.60, P = 0.11) and stream discharge (z = 0.77, P = 0.44) do not. Post-hoc pairwise comparison suggest that the following comparisons of treatments are significant: 1 vs. 3 (P = 0.001), 1 vs. 4 (Prediction 1.4; P < 0.001), 1 vs. 5 (P < 0.001), 2 vs. 3 (P = 0.004), 2 vs. 4 (P < 0.001), 2 vs. 5 (P < 0.001), 3 vs. 5 (Prediction 1.3; P = 0.004). Treatment #1 (Larval odor control) had the highest proportion of upstream migrants (130 out of 158 released; Figure 2.3) satisfying prediction 1.1. However, prediction 1.2 was not met, treatment 1 was not found to have statistically more upstream migrants than in treatment 2 (P = 0.49). Also, prediction 1.5 was not met (4 vs. 5; P = 0.21), we did not see a difference in upstream movement whether a lateral gradient was present or not at the PIT antennas when only a putative alarm cue was introduced.

As predicted, in all treatments (1, 2, & 3) where larval odor was present, the odor was always attractive even when being concurrently present with alarm cue (Prediction 2.1, Treatment 1: Larval odor vs. river water, z = 5.156, P < 0.001; 4: Larval odor vs. Alarm cue, z = 31.969; P < 0.001; and 5: Larval odor + Alarm cue vs. Alarm cue z = 3.375, P < 0.001, Fig. 2.4). When larval odor was not present and migrants were given the choice between alarm cue and a risk free channel, lamprey actively avoided the channel (Prediction 2.4, Treatment 4: alarm cue vs. river water, z = 18.759, P < 0.001). When migrants were presented with the same amount of alarm cue from both channels (all is equal situation), there distribution did not differ from a 50:50 distribution (Prediction 2.5 Treatment 5: Alarm cue vs. Alarm cue, z = -1.255, P = 0.21) suggesting that there were no side biases created by our two channel design.

The time between being released until first detection varied greatly between individuals (see Fig 2.3). Our Kruskal-Wallis test ($\chi^2 = 112.24$, df = 4, $P < 0.001$) suggests that differences do exist between treatments in regard to individuals time of arrival at the PIT antennas. Pair-wise comparisons suggest that the following contrasts treatments were significantly different: 1 and 2, 1 and 3, 1 and 4, 1 and 5, as well as 2 and 5 (see Table 2.3 and Figure 2.5). These significant comparisons suggest that the addition of a putative alarm cue decrease the swim up times of individuals meeting Prediction 3.

DISCUSSION

As predicted, in single-odor scenarios where spatial information was given (larval odor on one side or alarm cue on one side), the animals consistently sought the larval odor or avoided the alarm cue. These results support behaviors demonstrated in other experiments using migratory sea lamprey which allows us to assume our experiment represents the animal's decision-making in its migratory phase (Wagner et al. 2006; Meckley et al. 2012; Bals 2012).

Our hypothesis, that migrating sea lamprey will modulate their movements while migrating in riverine environments relative to the presence/absence and lateral distribution of conspecific attractants and repellants, was supported by multiple results. However, the application of a putative alarm cue only reduced the upstream movement of migrants in the presence of the migratory cue (larval odor) when the alarm odor fully activated the entire water column (unavoidable) and did not reduce upstream movement when a lateral gradient (avoidable) was presented. Furthermore, the application of a putative alarm cue only increased the number of migrants choosing the larval odor side when the alarm odor was applied to one channel (avoidable) and decreased the amount of migrants choosing the larval odor side when applied to both channels (unavoidable). These results suggest that unavoidable risk may have a larger impact on migrant's decision-making than does avoidable risk which may be explained by threat-sensitive responses to differences in the amount of alarm cue used between Treatments 4 & 5 and/or that the animals may change refuge seeking behavior in response to different odor landscapes.

In Treatment 2 (opportunity and avoidable risk), we activated one stream channel to 0.2 μl L^{-1} compared to Treatment 3 opportunity and unavoidable risk) where both channels were activated with 0.2 μl L^{-1}. It has been demonstrated that other aquatic organisms match their antipredator behaviors proportionally to the degree of threat posed by predators through risk assessment (Helfman 1989; Dupuch et al. 2004; Jachner & Rydz 2002), however, this is not true for all aquatic organisms (Lawrence & Smith 1989; Brown et al. 2001; Mirza & Chivers 2003; Roh et al. 2004). In the first chapter of this thesis, we tested for threat-sensitive responses in upstream movement when the entire water column was activated with a putative conspecific alarm cue. We saw no differences in the total proportion of individuals actively migrating upstream between the four dilutions of alarm cue (0, 0.1, 0.2, and 1 μl L^{-1}), and only saw a difference in the average time of arrival between the control (0 μl L^{-1}) and our highest alarm cue application (1 μl L^{-1}). These results suggest that sea lamprey are not exhibiting threat-sensitive responses to varying levels of risk information when it fully activates the entire water column. Furthermore, the use of these risk cues are limited by time (Wisenden et al. 2009), so waiting for the cue to be absent when a lateral gradient is not perceivable would not allow the migrant to utilize information about the predators location for avoidance once a lateral gradient is perceivable. Though the animal's response may not be proportional to the degree of threat posed, the sea lamprey has shown that it can perceive a lateral gradient of risk and avoid the side where the source of risk information is being emitted (Bals 2012; Byford Chapter 1).

We did not expect that fewer migrants would choose the channel emitting the migratory cue when risk is unavoidable compared to our positive control. We expected that proportion of migrants to be the same or higher than our positive control. As previously mentioned, it has been demonstrated how sea lamprey perceive and avoid risk with a lateral gradient (Bals 2012; Byford Chapter 1), however, it has not been finely measured how migrants move within a river when the entire water column is activated with risk. Our Treatment 5 (unavoidable risk), demonstrated that in this scenario that the distribution of lamprey within the stream is not statistically different from a 50:50 distribution. When the risk activates the entire water column lamprey may try to use deeper migratory routes in the middle of rivers where they are less susceptible to mammalian shoreline predators. Though larval odor appeared less attractive when risk was unavoidable, the distribution of migrants was significantly different from a 50:50 distribution showing it was still attractive and lamprey tolerated risk to ensure it did not miss the opportunistic signal. Previously, it has been shown that fish will tolerate more risk when their needs are greater, such as foraging when starving (Smith 1981; Abrahams & Dill 1989; Brown & Smith 1996).

In the treatments where the only odorant present was the putative conspecific alarm cue (Treatment 4 avoidable risk; and 5 unavoidable risk) we observed some individuals still actively swimming upstream relatively quickly, however, their appeared to be a delay for all migrants. In Treatment 5 we did not observe our first migrant until 498 seconds after being released and in Treatment 4 approximately 10% (7 or 67) were detected swimming upstream before 500 seconds. This delay is an interesting behavior, which may be a true response or may be an artifact of our experimental design. Our experimental design simulates a predator's attack upstream, where lamprey have been caged for several hours and are suddenly exposed to the putative conspecific alarm cue. When they are released shortly after exposure, visual observations of animals exhibiting fright responses as they move out of the cage are frequent as well as animals moving downstream (G.J. Byford, *pers. observation*). This experimental artifact is likely what is causing the delay in observing individuals migrate upstream, and may explain why the cumulative proportion curves of the alarm cue only treatments do not asymptote as quickly than the other treatments. Additional migrants may have been detected if we ran longer trials, however, based on the slopes of the curves it was not likely to be many.

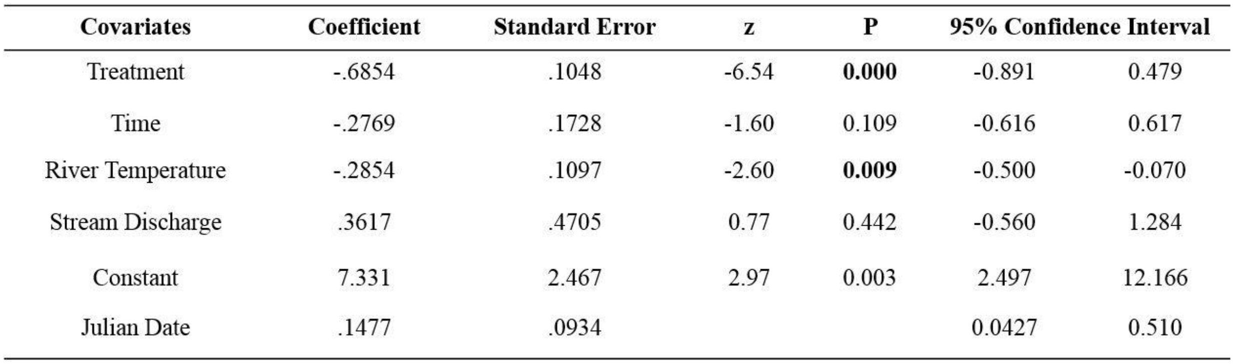
There was great variation between individual's decisions to go upstream or not and the time it took them to be detected from when they were released within all treatments. One explanation for the individual variance of these responses may be explained by differences in the individual personalities of migrating sea lamprey. It has been demonstrated that aquatic organisms vary individually and consistently in how they exhibit behavioral tactics towards managing risk and do so on a scale from shyness to boldness (Coleman & Wilson 1998; Brown et al. 2009; Brown et al. 2014). This would explain why some individuals, with bolder personalities, still chose to swim upstream in scenarios where only alarm cue (risk information) was present, and that more individuals chose to swim upstream when the migratory cue was present because the scenario was perceived less risky by most personalities. Only one study tested for consistent individual differences in sea lamprey and demonstrated that there are individual differences in their general activity, latency to leave a refuge and response to a predator cue (McLean 2014). So, it may be a possible explanation which needs to be further explored to fully understand how sea lamprey use conflicting signals during their terminal reproductive migration.

Understanding how sea lamprey use public sources of opportunity and risk information throughout their terminal reproductive migration is important for managers who are trying to meet objectives of either control or conservation. The putative conspecific alarm substance and the migratory cue, larval odor, both strongly modulated the channel choice of individuals. Furthermore, when both odors are applied in opposite channel (Treatment 2, opportunity & avoidable risk), in a "push-pull strategy" only 1 migrant out of 116 individuals detected moving upstream chose the side activated with risk. The use of olfactory cues within integrated pest management strategies for many insects has been widely studied and has been used with success

(Miller & Cowles 1990; Agelopoulos et al. 1999; Cook et al. 2007), where insects are diverted from valuable crops while being attracted to less desirable crops. A push-pull strategy was using a partial sex pheromone, 3-keto-petromyzonol sulfate, and a putative conspecific alarm cue was applied at a barrier intergraded trap to test whether its use could improve trap catches and efficacy (Hume et al. 2015). Hume et al. (2015) demonstrated that sea lamprey could locate traps twice as quickly using the push-pull strategy or by simply just applying the putative conspecific alarm cue, however, they did not find any differences in trap catches. A push-pull strategy may also be useful in rivers where lamprey spawn that do not have dams. Here, a push-pull manipulation could be used to try to aggregate spawners into the lowermost tributary(s) of the river system to reduce the total area which must be treated by lampricides. Push-pull manipulation using olfactory cues sounds promising and may one day be of use within the sea lamprey control program, yet many uncertainties still exist. First, a long-term push-pull experiment must take place which observes its success over an entire migratory season. As lamprey mature, the response to larval odor attenuates (Bjerselius et al. 2000) and migrants also may habituate to the putative conspecific alarm cue (Bals 2012) which present challenges for creating consistent responses for effective control. Secondly, before push-pull strategies are implemented rigorous testing of different attractants and repellants throughout the entire migratory season is needed. Since lampreys attenuate and use different olfactory cues as they mature, likely more signals will need to be used for the most effective push-pull control. Olfactory behavioral manipulation will not be a silver bullet for managers, however, will likely serve utility within programs for control and conservation.

APPENDIX

TABLES

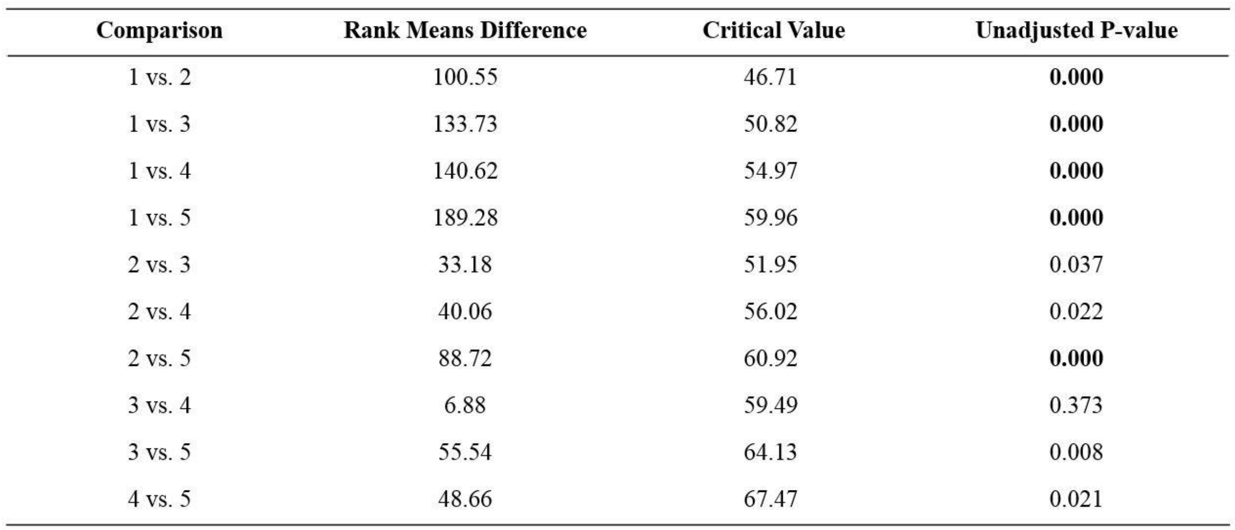


Covariates	Coefficient	Standard Error	z	P	95% Confidence Interval	
Treatment	-.6854	.1048	-6.54	**0.000**	-0.891	0.479
Time	-.2769	.1728	-1.60	0.109	-0.616	0.617
River Temperature	-.2854	.1097	-2.60	**0.009**	-0.500	-0.070
Stream Discharge	.3617	.4705	0.77	0.442	-0.560	1.284
Constant	7.331	2.467	2.97	0.003	2.497	12.166
Julian Date	.1477	.0934			0.0427	0.510

Table 2.1: The results from the generalized linear mixed-model (GLMM) for binomial responses using a logit link function used to observe how upstream movement was influenced by treatment (factor), time (factor), river temperature (continuous), and stream discharge (continuous) as fixed factors as well as Julian date as a random effect. Bold p-values are significant.

Treatment	z-score	p-value
1. Larval odor vs. river water	5.156	< 0.001
2. Larval odor vs. Alarm cue	31.969	< 0.001
3. Larval odor + Alarm cue vs. Alarm cue	3.375	< 0.001
4. Alarm cue vs. river water	18.7598	< 0.001
5. Alarm cue vs. Alarm cue	-1.255	0.21

Table 2.2: The results of the Z-tests which tested whether each treatments distribution of migrants was statistically different from a 50:50 distribution. The distribution of migrants of all treatments (1, 2, 3, and 4) except treatment 5 (alarm cue vs. alarm cue) were statistically different from a 50:50 distribution.



Comparison	Rank Means Difference	Critical Value	Unadjusted P-value
1 vs. 2	100.55	46.71	**0.000**
1 vs. 3	133.73	50.82	**0.000**
1 vs. 4	140.62	54.97	**0.000**
1 vs. 5	189.28	59.96	**0.000**
2 vs. 3	33.18	51.95	0.037
2 vs. 4	40.06	56.02	0.022
2 vs. 5	88.72	60.92	**0.000**
3 vs. 4	6.88	59.49	0.373
3 vs. 5	55.54	64.13	0.008
4 vs. 5	48.66	67.47	0.021

Table 2.3: Table 1. Results of the post-hoc Rank-Means (RM) test of the times of arrival at the PIT antennas during our ninety minute observation period. Comparison refers to the alarm cue concentrations being compared; Rank Means Difference is the reported RM difference; Critical Value is the critical RM value for that comparison. The unadjusted P-value is reported. P-values in bold indicate significantly different rank-means after adjustment for multiple comparisons.

FIGURES

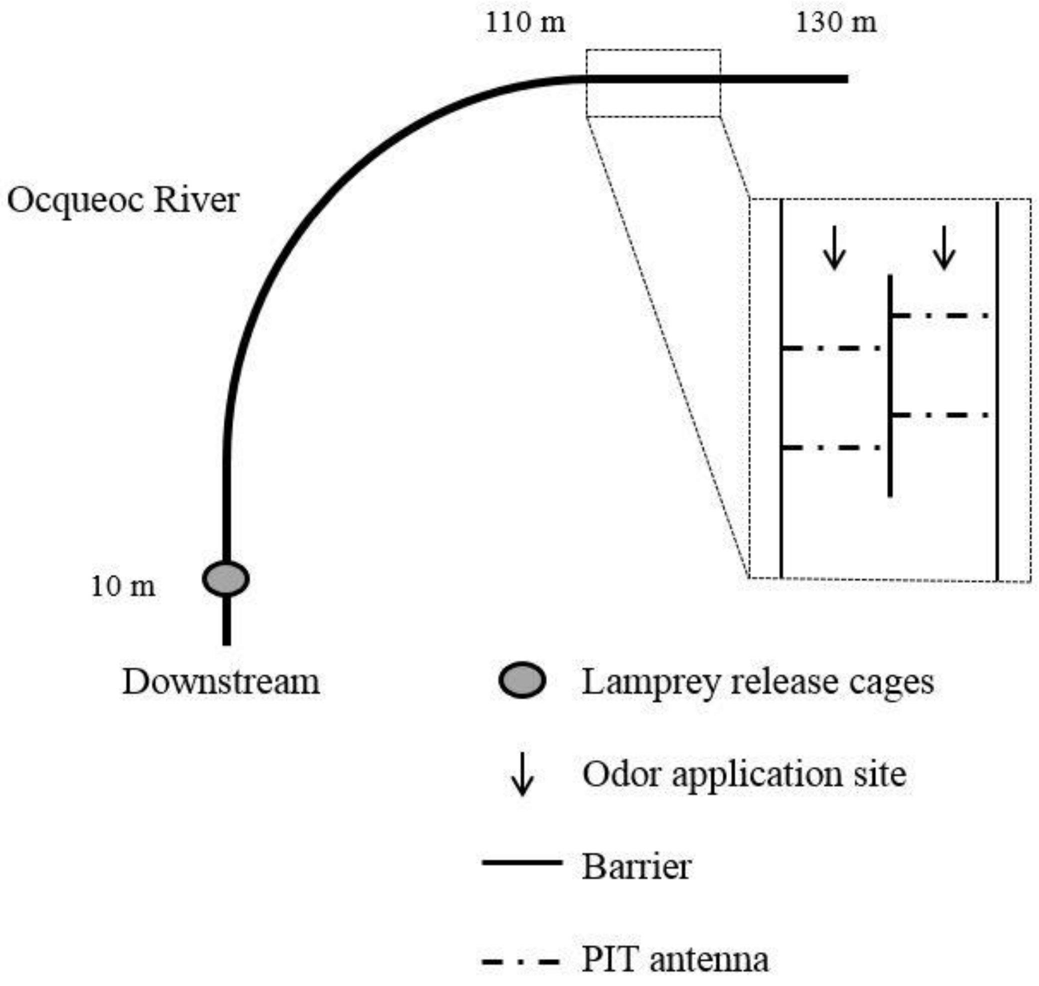


Figure 2.1: Diagram of the Ocqueoc River field site. Distances are displayed as distance upstream from the most downstream point of the site. Test subjects were acclimated and released in cages roughly 100 m downstream of the passive integrated transponder (PIT) antennas within the experimental arena. Movements were monitored as they passed the alarm cue application site(s) 1 meter upstream of the end of the mid-stream barrier. Stimulus odors were applied in the middle of each separate channel

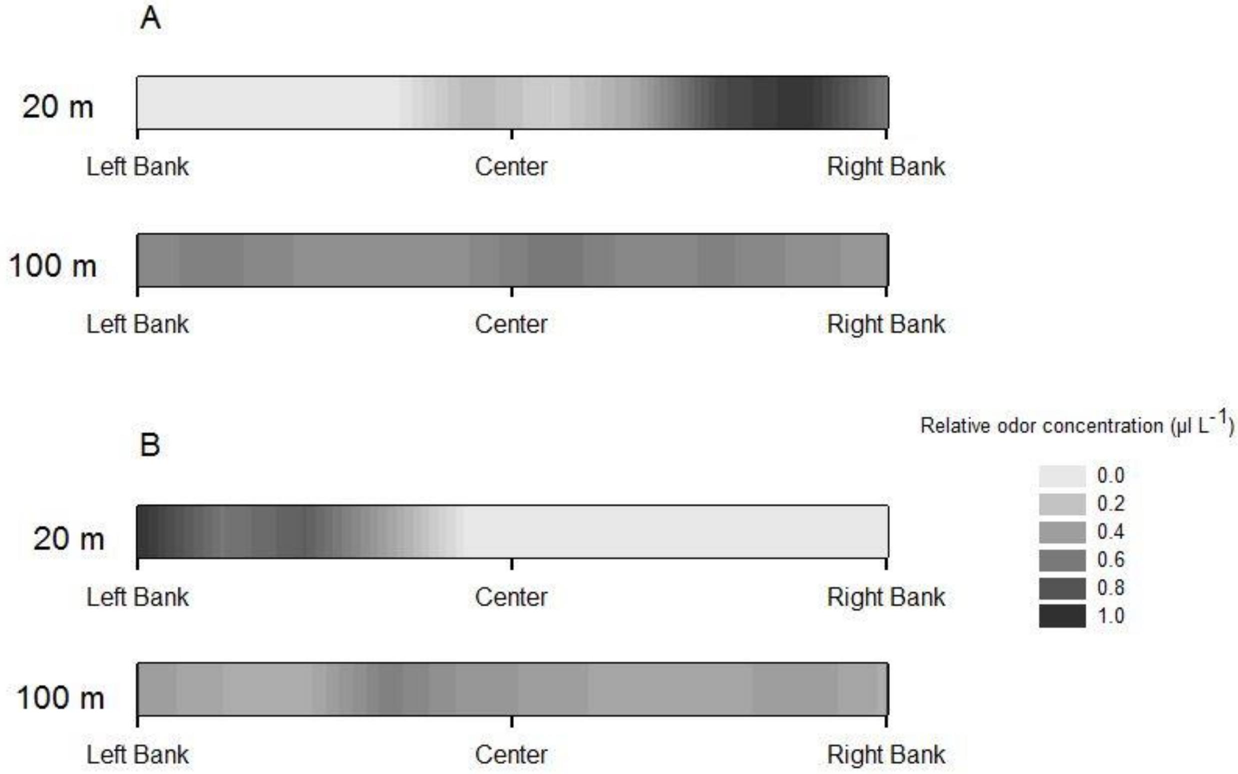


Figure 2.2: A lateral depiction of the relative concentration of rhodamine dye recorded from left bank to right bank facing upstream during dye testing. Shown are transects taken at 20 meters downstream of our odor application sites and 100 meters downstream at the release cages. Rhodamine dye was pumped and recorded separately from both the right (A) and left channels (B). This data shows that the odors were fully and relatively evenly mixed at the release cages and that there was a clear odor gradient as migrants approached the simulated bifurcation.

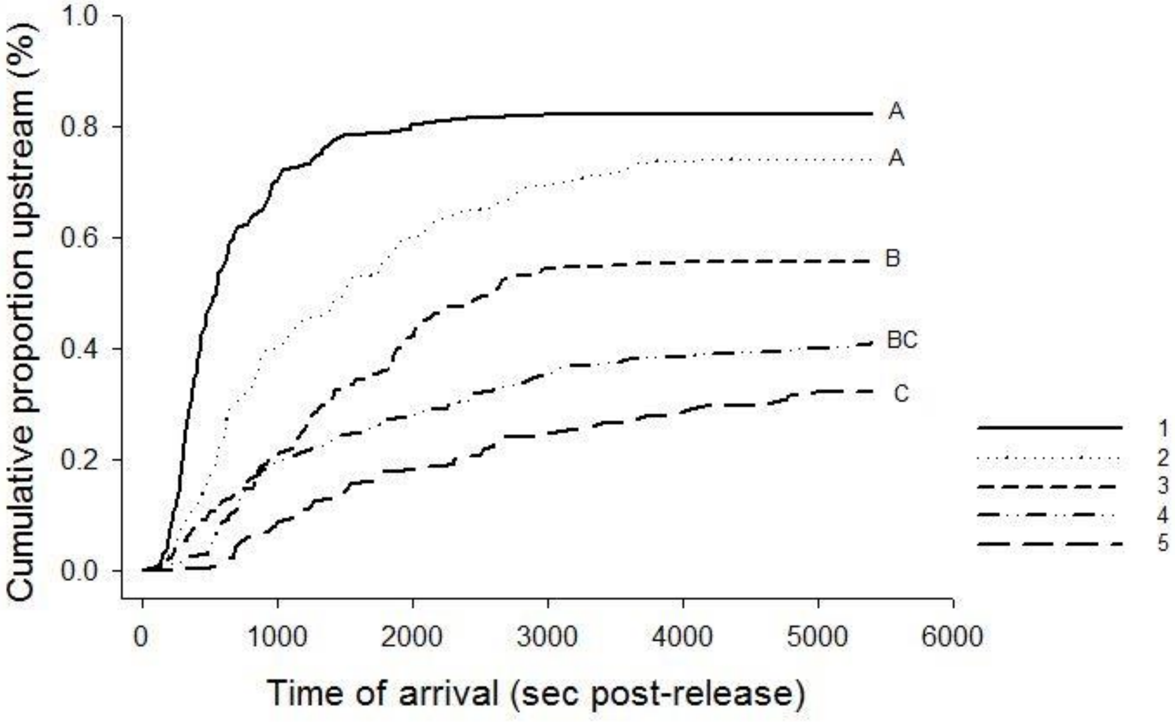


Figure 2.3: Cumulative proportion of animals detected at the PIT antennas as a function of time since release for each of the following five treatments: (1 = Larval odor vs. river water; 2 = Larval odor vs. Alarm cue; 3 = Larval odor + Alarm cue vs. Alarm cue; 4 = Alarm cue vs. river water and 5 = Alarm cue vs. Alarm cue). Treatments with different letters were found to be significantly different from post-hoc GLMM testing.

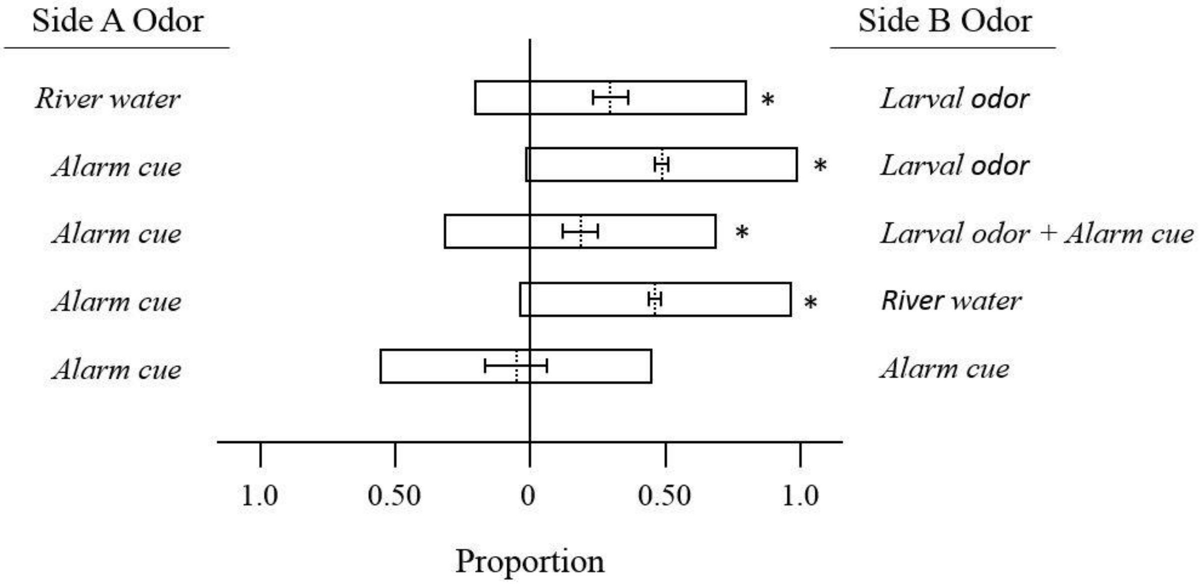


Figure 2.4: Side-by-side plots of the mean proportion of migrants actively swimming up each channel in regards to the five treatments (1 = Larval odor vs. river water; 2 = Larval odor vs. Alarm cue; 3 = Larval odor + Alarm cue vs. Alarm cue; 4 = Alarm cue vs. river water; and 5 = Alarm cue vs. Alarm cue). Z-tests were ran to see whether each treatments distribution of migrants was statistically different from a 50:50 distribution showing either attraction or repulsion (see Table 2.2). Treatments with an asterisk (*) were statistically different. Larval odor appeared attractive in all circumstances. A conspecific alarm cue restricted migrant's routes unless the cue was introduced to both channels.

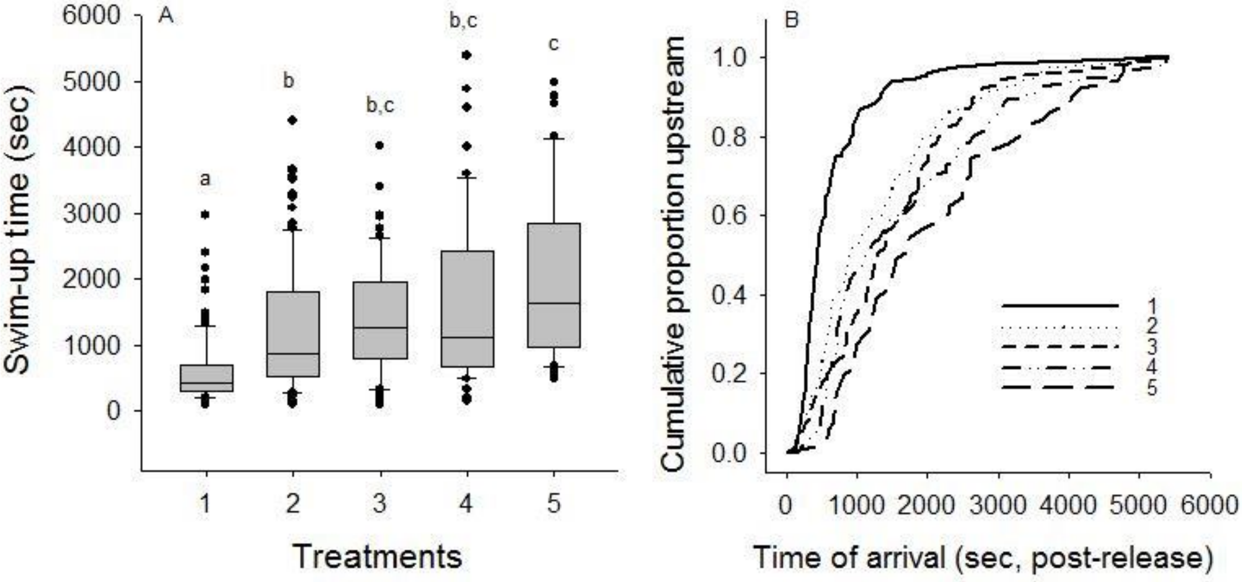


Figure 2.5: (A) Box-plot of the individual swim-up time (elapsed time of arrival at the PIT antennas) during the field experiment for each treatment (1 = Larval odor vs. river water; 2 = Larval odor vs. Alarm cue; 3 = Larval odor + Alarm cue vs. Alarm cue; 4 = Alarm cue vs. river water and 5 = Alarm cue vs. Alarm cue). Different letters indicate statistically different time per post-hoc pairwise Kruskal-Wallis test performed on rank data. (B) Cumulative proportion of animals detected at the PIT antennas as a function of time since release for each treatment. Only lampreys that swam up to the PIT antennas are included.

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CHAPTER 3: DO NATIVE LAMPREYS AND INVASIVE SEA LAMPREY SHARE AN ALARM CUE? IMPLICATIONS FOR USE OF A NATURAL REPELLENT TO GUIDE IMPERILED PACIFIC LAMPREY INTO FISHWAYS

ABSTRACT

Instream barriers affect anadromous lampreys worldwide by preventing access to spawning habitat, resulting in the decline of several species. Because lampreys rely heavily on olfactory cues to choose movement paths during upstream migration in rivers, the manipulation of these responses may be used to guide individuals into the vicinity of fish passage devices and thereby mitigate the impacts of barriers. However, because experimentation with imperiled species presents significant legal and ethical challenges, use of surrogate species that exhibits similar responses may prove very useful. Our laboratory study establishes that (1) the odor derived from dead Pacific Lamprey *Entosphenus tridentatus* elicits an avoidance response from invasive Sea Lamprey *Petromyzon marinus* in the Laurentian Great Lakes, and (2) the magnitude of this response does not differ from the conspecific alarm cue present in Sea Lamprey. By presenting the odor of the side of a river channel opposite a lamprey fish passage device, migrating lampreys of conservation concern may be guided to fishways, if the behavioral response to the cue has evolved in these taxa. Due to their availability and well-studied chemical communication system, Sea Lamprey may prove to be a useful surrogate for identifying and producing chemosensory cues for use in guiding Pacific Lampreys towards fish passage devices and away from intakes and screens.

INTRODUCTION

The use of an abundant species as a surrogate to develop the means to effectively manage a closely related imperiled species has considerable appeal (Caro et al. 2005; Wenger 2008). Rare and threatened species may be difficult to observe or collect and are subject to considerable legal protections that restrict experimentation. Researchers and managers have attempted to use surrogate species as response indicators to predict population changes after large-scale ecological or anthropogenic perturbation. The utility of surrogates at this scale has proven inconsistent due largely to a failure to verify that surrogate and target species exhibit similar population responses to complex ecological drivers (Lindenmayer et al. 2002; Mortelliti et al. 2009; Murphy et al. 2011). Alternatively, surrogate species may prove effective in addressing small-scale mechanistic questions where the responses of the individual animal are key to the creation of successful management practices. Here, the surrogate is more akin to a medical model species, and the underlying assumption of conserved biology (e.g., physiology, sensory capacity, behavior) may be more easily established.

Surrogate species may be particularly useful in the discovery of new means to guide imperiled migratory fishes into fishways at dams, where success or failure at the population scale is determined by numerous individual acts of passage through a manmade device. Barriers to upstream migration are a threat to anadromous lampreys worldwide, as they prevent migrants from accessing spawning habitat (reviewed in Maitland et al. 2015; Fig. 1). Of particular note, the Pacific Lamprey *Entosphenus tridentatus*, a species of significant cultural and ecological importance, has experienced a drastic population decline over the last 50 years (Close et al. 2002). The species is now considered at risk (ODFW 2006; WDFW 2015), and large-scale restoration projects are underway across the Pacific Northwest (Keefer et al. 2009; Maitland et al. 2015). In the Columbia River basin, 12 lamprey passage systems (LPS; a purpose-built ramp fishway designed to exploit the Pacific Lamprey's ability to climb wetted surfaces) have been installed at barriers to facilitate upstream passage. An LPS device is installed as a separate system at traditional fishways, and is often integrated into some element of the existing fishway (e.g., is placed into auxiliary water supply channels or adjacent to existing fishway entrances). Once entered, the passage efficiency of an LPS can be greater than 90% (Moser et al. 2011). However, too few migrating lampreys locate the relatively small LPS entrance to achieve population management goals, particularly in large river systems where encounter rates may be less than 40% (Moser et al. 2002a, 2002b; Keefer et al. 2013). There is a pressing need to increase encounter rates with LPS fishways to protect the Pacific Lamprey.

Guidance towards an LPS entrance may be achievable by manipulating the information the animal uses to select movement pathways in rivers. Migratory movements of lampreys are thought to respond to plumes of semiochemicals, conspecific odors that lead migrants towards high quality habitat and away from areas of risk (Buchinger et al. 2015). For example, the closely-related and heavily studied Sea Lamprey *Petromyzon marinus* relies extensively on two odors to guide movements during their nocturnal upstream migration in rivers. The odor emitted by conspecific larvae guides migrants into streams with suitable spawning and rearing habitat (Sorensen et al. 2005), and the application of this odor can be used to lead migrants into tributaries or near one bank of a river channel (Wagner et al. 2006, 2009). However, movement along river banks also brings the animal into potential contact with nocturnal shoreline predators (e.g., raccoon), increasing the risk of predation. A second odor, a natural repellent contained in the tissue of live lamprey that is released when the skin is ruptured (putatively an alarm cue), causes migrants to move to the opposite side of the channel while continuing to move upstream

(Bals and Wagner 2012; C.M. Wagner, unpublished data). By applying this natural repellent to one side of the channel to evoke migrants to the opposite bank during migration, Sea lampreys quickly encountered a trap entrance placed opposite the odor plume (Hume et al. 2015). The movement process associated with encountering and entering traps is similar to finding and entering a fishway (Bravener and McLaughlin 2013).

Overlap has been demonstrated amongst lampreys in their behavioral responses to attractive semiochemicals (Fine et al. 2004; Bals and Wagner 2012), including Sea and Pacific Lampreys (Robinson et al. 2009; Yun et al. 2011, 2014), suggesting Sea Lamprey may be a suitable candidate for surrogacy for the Pacific Lamprey. At least one other lamprey species (Silver Lamprey Ichthyomyzon unicuspis) produces the compound(s) that elicit the alarm response in Sea Lampreys. Whether any other lamprey has evolved the alarm response has not been investigated. However, alarm signaling is more likely to be conserved across related taxa (versus reproductive pheromones) because the risk of predation is often shared, whereas the benefit of reproductive opportunities is not (Mirza and Chivers 2001). If conserved, the alarm response could prove to be a very useful tool in guiding imperiled lampreys into the vicinity of fish passage devices by inducing them towards the river bank associated with the LPS entrance. Because experimentation with Pacific Lampreys is strictly controlled, we report an initial test of the hypothesis that the odor produced by dead Pacific Lamprey contains one or more natural repulsive compounds that elicits the known alarm response in migratory Sea Lamprey. We tested our hypothesis using a standard laboratory space-use assay to ascertain whether Sea Lamprey were repelled by either the death odors of Pacific Lamprey and/or Sea Lamprey (versus a control). Odors were applied to one side of a laboratory raceway and the resulting lamprey distribution was analyzed for evidence of repulsion or attraction. We predicted (1) that Sea

Lampreys would respond by avoiding extract derived from Pacific Lamprey, and (2) that the avoidance response would be similar in magnitude to the response from a conspecific extract.

METHODS

Odor collection

The odor of one fresh-killed adult male Pacific Lamprey (304 g; obtained from the lower Columbia River mainstem) and the odor of one fresh-killed adult male Sea Lamprey (238 g) were extracted separately by Sohxlet extraction following the procedures of Bals and Wagner (2012). Briefly, we used separate 1 L 71/60 Sohxlet apparatuses (Ace Glass Inc., Vineland, NJ) with a water-cooled Allihn condenser and a 1 L solvent reservoir heated by a hemispherical mantle to 75 – 80 °C. One liter of solvent (50:50 w/w of 200 proof ethyl alcohol and deionized water) was added into the solvent reservoir. A single carcass was then placed within the body of the extractor. Each extraction cycled three times. The resulting extracts were filtered, and stored at -20°C until use.

Apparatus

Because lampreys are nocturnal during their spawning migration all trials were conducted at night (2200 – 0300 h) between May 31 and June 26 2015. Trials took place at the Hammond Bay Biological Station (HBBS, Millersburg, Michigan, USA) in two 20 m x 1.84 m concrete raceways separated into an upstream holding area (7.5m), an experimental arena (5.0m), and a tailrace (7.5m) with net barriers (Fig. 2). Collimators were placed upstream of each experimental arena to promote smooth flow and distinct odor plumes. Each raceway received a continuous flow of water from Lake Huron through an offshore intake pump (temperature 7.4 – 11.9 °C). Discharge was maintained between 6.7 – 12 L sec^{-1}. Each experimental arena was equipped with one infra-red (IR)-sensitive video camera (Axis Communications; Q1604 Network Camera) placed directly overhead as well as two IR lights (Wildlife Engineering; Model IRLamp6). A live feed from the cameras was observed on video monitors in a separate room and trials were recorded onto digital media for analysis.

Stimulus odors were introduced into one side of the raceway via peristaltic pumps (MasterFlex model 7533-20) at a rate of at 20 mL min^{-1} at the upstream end of the experimental arena six inches from the left or right side. Prior to experimentation we mixed 8 mL of stimulus extract into 420 mL of lake water collected from the raceway in a 500 mL Erlenmeyer flask that was continuously stirred with a 2 cm magnetic stir bar. The final dilution of raceway water to extracted odor was 1 ppm (by volume), a dilution which achieved full repellency in conspecific trials (Bals and Wagner 2012). To ensure no cross-contamination of odors we used separate pump tubing, flasks and stir bars for each stimulus odor.

Experimental subjects

Wild male Sea Lamprey (365 – 578 mm, mean 482 mm ± 2.62) were obtained during May and June 2015 while actively migrating into two tributaries of Lake Huron (Cheboygan and Ocqueoc Rivers, Michigan, USA) and one of Lake Michigan (Manistique River, Michigan, USA) by the U.S. Fish and Wildlife Service as part of an annual Sea Lamprey monitoring program. During migration, male and female Sea Lamprey exhibit equal responses to the death odor containing a putative alarm cue (Bals & Wagner 2012). Following capture the lampreys were transported to the station, were separated by sex using external characteristics per Siefkes et al. (2003), and were placed into 1000 L holding tanks receiving a continuous flow of fresh water from Lake Huron (100% exchange every 2 h). All experimental subjects were held at HBBS for a minimum of 48 h prior to use and observed to ensure normal activity. Only specimens lacking external injury were used, and each lamprey was used in a single trial. All procedures for subject handling and experimentation were approved by the Michigan State University Institutional Animal Care and Use Committee (permit # AUF 01/14-007-00).

Experimental procedure

Prior to the beginning of a trial (approximately 1500 h), four groups of immature male Sea Lamprey (ten individuals per group) were placed into separate holding cages in upstream section of each raceway. At 2200 hours, a trial began when a single cage was moved into the middle of the experimental arena of a raceway and the subjects were released. Trial duration was 30 minutes, comprising a 10 minute pre-exposure period and a 20 minute exposure period. The pre-exposure period began when a majority (≥6) of Sea Lamprey were actively moving. During the exposure period, one of three stimulus odors, extracted Pacific Lamprey death odor, extracted Sea Lamprey death odor or a solvent control, was introduced into one side of each raceway. We completed eight replicates for each stimulus odor. In order to disperse any effect of raceway identity (channel one or two) or odor application side (left or right), each odor received an equal number of replicates in either raceway (four in channel one, four in channel two), and the application side was alternated across replicates within each raceway (i.e., for a single treatment four replicates were performed in each raceway, with the odor pumped twice on the left side and twice on the right side within a raceway). At the end of each trial, we recorded the length (total length, mm) and weight (wet weight, g) of each individual.

Data analysis

From the video recordings we determined the position of each individual's head every 30 s after the start of the trial. Positions were assigned to either the stimulus (control or death odor application side) or non-stimulus side of the raceway. Only the final ten minutes of the stimulus period were used for analysis to provide time for the distribution of lamprey to stabilize after introduction of the stimulus. To confirm no effect of raceway or side of the raceway receiving the stimulus odors, we first ran a one-way ANOVA ($\alpha = 0.05$) with odor identity, raceway, and treated side as fixed factors, and transformed proportion on the stimulus side as the response. The results confirmed an effect of odor identity ($F_{2, 19} = 10.24$, $P < 0.001$) and no effect of raceway ($F_{1, 19} = 0.18$, $P = 0.67$) or side ($F_{1, 19} = 3.03$, $P = 0.09$). Raceway and treated side were omitted from subsequent analysis. To determine whether space use differed in response to stimulus odors (vs. control), data were analyzed with a second one-way ANOVA ($\alpha = 0.05$). The transformed proportion of animals on the stimulus side was the response variable and odor identity (Sea Lamprey extract, Pacific Lamprey extract, or solvent control) was a fixed factor, with water temperature and discharge as random covariates. Post-hoc pair-wise Tukey's Honestly Significant Different (HSD) tests ($\alpha = 0.05$) were performed to test for significant differences in the proportion of animals on the stimulus side in all stimulus odor pairs. Prior to analysis all proportions were arcsine transformed and tested for normality via Shapiro-Wilk's test. The transformed data were normally distributed ($W > 0.95$, all $P > 0.38$). All statistical analyses were performed in SPSS.

RESULTS

The overall statistical model was significant (ANOVA, $F_{4,\,19}$ = 4.59, P = 0.009), with a significant effect of odor identity on the distribution of animals within the raceway ($F_{2,\,19}$ = 6.08, P = 0.009), but no effect of water temperature ($F_{1,\,19}$ = 0.23, P = 0.635) or discharge ($F_{1,\,19}$ = 0.34, P = 0.567; Fig. 3). Post-hoc pairwise comparisons of odors confirmed the predicted patterns in that: (1) the extracted odors of dead Pacific Lamprey (Tukey's HSD, P = 0.004) and dead Sea Lamprey (Tukey's HSD, P = 0.002) significantly repelled Sea Lampreys vs. the solvent control; and, (2) the magnitude of the repulsion elicited by Pacific Lamprey death odor and Sea Lamprey death odor did not differ (Tukey's HSD, P = 0.93). Generally, during the pre-stimulus period of trials Sea Lampreys actively swam around the edges of the experimental arena in either a clockwise or counter-clockwise fashion, spending less time swimming in the middle of the arena. When exposed to the solvent control, the same patterns of movement occurred. However, when either of the death odors (Pacific or Sea Lamprey) were introduced, individuals who encountered the plume altered their trajectories by turning away from the activated side and either moving towards the non-stimulus side or downstream to the block net in an apparent attempt to escape. The magnitude of responses varied from simple turning and avoidance to a striking "flight" response where the animal burst-swam downstream.

DISCUSSION

Migratory Sea Lamprey strongly and consistently avoided the odor extracted from fresh-killed Pacific Lamprey, indicating a repulsive compound or compounds, putatively an alarm cue (Imre et al. 2010; Bals and Wagner 2012), may be present in both taxa. Regardless of the chemical nature of the material (an alarm cue or other natural repellent), the observed response establishes the potential viability of using invasive Sea Lamprey from the Great Lakes as a source of material for testing guidance of live Pacific Lamprey towards fishway entrances. Utilizing Sea Lamprey as a behavioral surrogate species for Pacific Lamprey responses to semiochemicals and other odors requires further evaluation and rigorous hypothesis-testing as differences in their biology are known (Clemens et al. 2010). However, olfactory receptor morphotypes appear conserved from lampreys to teleost fishes, and it is likely their behavioral affiliations in reproduction, food acquisition, and risk perception are similarly conserved (Hamdani and Doving 2002; Hansen and Zielinski 2005; Laframboise et al. 2007; Doving and Lastein 2009). Sea Lamprey also avoid the odor extracted from dead Silver Lamprey *Ichthyomyzon unicuspis* (Bals and Wagner 2012), suggesting the cue is present in the two major clades of Petromyzontiformes (Fig. 1). As both Sea and Silver Lampreys are basal to Pacific Lamprey (Potter et al. 2015), the cue inducing this alarm response may have been present in a common ancestor.

Importantly, whether Pacific Lamprey have evolved a similar alarm response is yet to be determined. The evolution of an alarm response to chemical cues released upon injury in teleost fishes is hypothesized as a secondary function of the compounds involved. Specifically, the substances contained in ostariophysan club cells (located in the skin) function as anti-microbial agents in response to epidermal infections, and protective agents against ultraviolet light exposure, with olfactory perception of the compounds as injury-related odors evolving later (Chivers et al. 2007; Ferrari et al. 2010). Thus, evolution of the alarm response may be derived from ecological circumstances that dictate selective pressures. Here, those circumstances may include the non-homing migration, which requires movement into and through unfamiliar areas with new predators where alarm cues may be important signals of predation risk. Lampreys rely extensively on olfactory cues to locate habitat and mates while avoiding predation during their terminal migration (reviewed in Johnson et al. 2015). If the alarm response that is clearly demonstrated by Sea Lamprey is conserved across lamprey taxa, it could be applied in situations where behavioral manipulation, such as guiding migrants to fish passage devices, is used to protect species of conservation concern. Clearly, the next step is to evaluate whether alarm responses have similarly arisen in other anadromous lamprey species, particularly Pacific Lamprey.

In addition to successful upstream passage, lampreys newly transformed into the parasitic life phase must survive a downstream migration through a gauntlet of threats caused by in-stream barriers. During downstream migration, juvenile Pacific Lamprey can become impinged on intake screens, or entrained in irrigation canals, and may experience other physical injury resulting in direct mortality (Moser et al. 2015). Prior research has considered aversive stimuli to direct juvenile lampreys including electrical guidance (Applegate et al. 1952; Johnson and Miehls 2014), and other non-physical stimuli including carbon dioxide barriers (Dennis III et al. 2016). These technologies often require costly infrastructure, making whole-channel implementation difficult and expensive. Further, they tend not to be species-specific in action, and thus may have negative effects on non-target fishes. If the chemical alarm signaling system of lampreys is present across multiple life stages, application of the natural repellent upstream of entrainment/impingement areas may guide downstream migrants away from the dangerous areas. Evidence for the conservation of the alarm signaling system and behavioral response exists within Sea Lamprey larvae (Perrault et al. 2014) and newly transformed out-migrants have been observed to respond to the cue (C.M. Wagner, *unpublished data*). Thus, if conserved among Petromyzontid lampreys and their life stages, the lamprey repellent may be used to direct adult migrants toward an LPS for fish passage, and direct out-migrating juveniles away from danger.

The Sea Lamprey is currently a model organism in the fields of genomics, (McCauley et al. 2015), medicine (Cai et al. 2013) and biomimetics (Ijspeert et al. 2013). Our results add to a body of work suggesting the invasive population in the Great Lakes may prove useful as a model of behavioral, physiological and ecological responses to olfactory cues during odor-mediated migration. In particular, the availability of invasive Sea Lamprey as experimental subjects and a source of experimental olfactory cues, and the substantial research infrastructure supported primarily by the Great Lakes Fishery Commission, creates a circumstance whereby this invasive pest may become a valuable surrogate for lampreys of conservation concern. Confirming a behavioral alarm response within Pacific Lamprey and other lampreys of conservation concern, and identifying the chemical structure of the repulsive compound(s), will be important next steps.

ACKNOWLEDGEMENTS

We are grateful to the staff at HBBS for providing access to their facilities. We would like to thank Alexa Maine (Confederated Tribes of the Umatilla Indian Reservation), Ralph Lampman (Yakama Nation) and Sam Onjukka (Oregon Department of Fish and Wildlife) for providing Pacific Lamprey as well as Fawn Fisher and Erica Adams for their late night work in the raceway. Funding for this project was provided by a grant from the Great Lakes Fishery Commission to C.M. Wagner.

APPENDIX

FIGURES

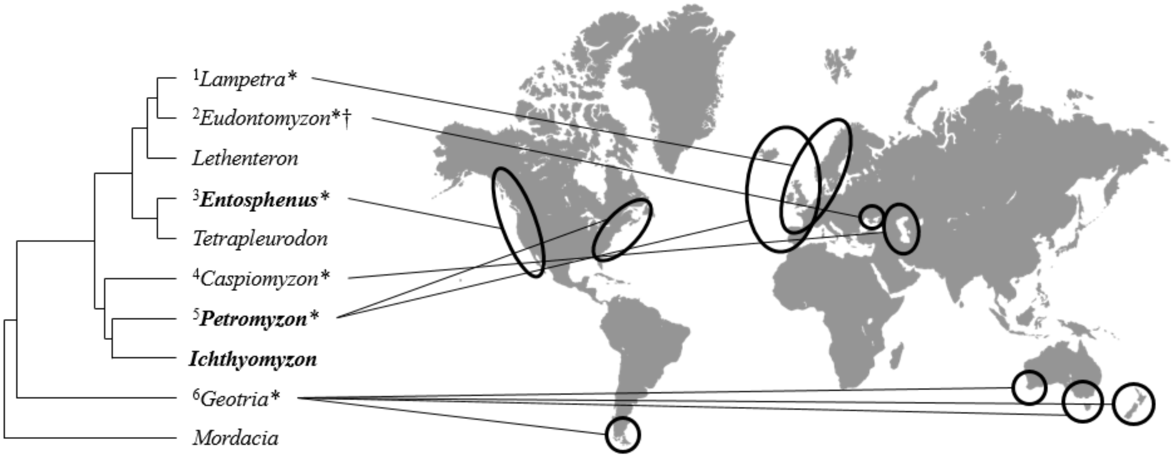

Figure 3.1: Cladogram based on *Cyt b* molecular data (derived from Potter et al. 2015) representing all extant lamprey genera. Black ellipses indicate geographical areas where a single anadromous species (indicated by an asterisk) belonging to that genus is currently threatened by in-stream barriers, resulting in the adoption of conservation legislation. Abbreviations: (†) indicates an anadromous species (*Eudontomyzon* sp. nov. migratory) now extinct, partially as a result of in-stream barriers (Kottelat et al. 2005); (*1*) *Lampetra fluviatilis* (IUCN, 2013; JNCC, 2010); (2) *E.* sp. nov. migratory (Kottelat et al. 2005); (3) *Entosphenus tridentatus* (ODFW, 2006); (4) *Caspiomyzon wagneri* (IUCN, 2008); (5) *Petromyzon marinus* (JNCC, 2010; OSPAR, 2009); (6) *Geotria australis* (IUCN, 2014). Genus names in bolded font are known to possess a putative alarm substance.

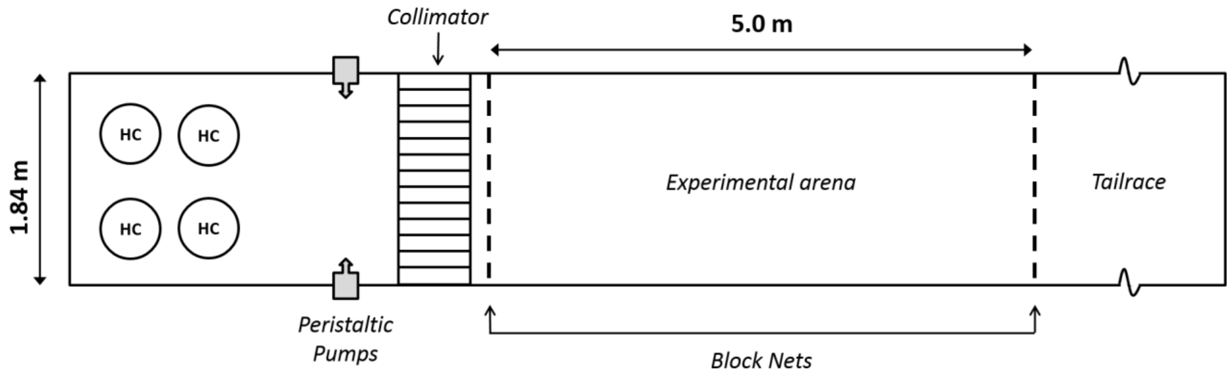

Figure 3.2: Overhead view of the raceway setup for behavioral space-use testing. Each raceway was separated into three zones: upstream holding area (7.5m long), experimental arena (5 m long), and downstream tailrace (7.5 m long). Water flowed from left to right in the figure. Prior to introduction into the experimental arena, groups of lampreys were held in holding cages (HC) in the upstream area. The odors were introduced via peristaltic pumps on the left or right side of each raceway. The bottom of each raceway was lined with white plastic and marked into four equal sized rectangles to assign the position of each animal to a side of the raceway from the recorded video.

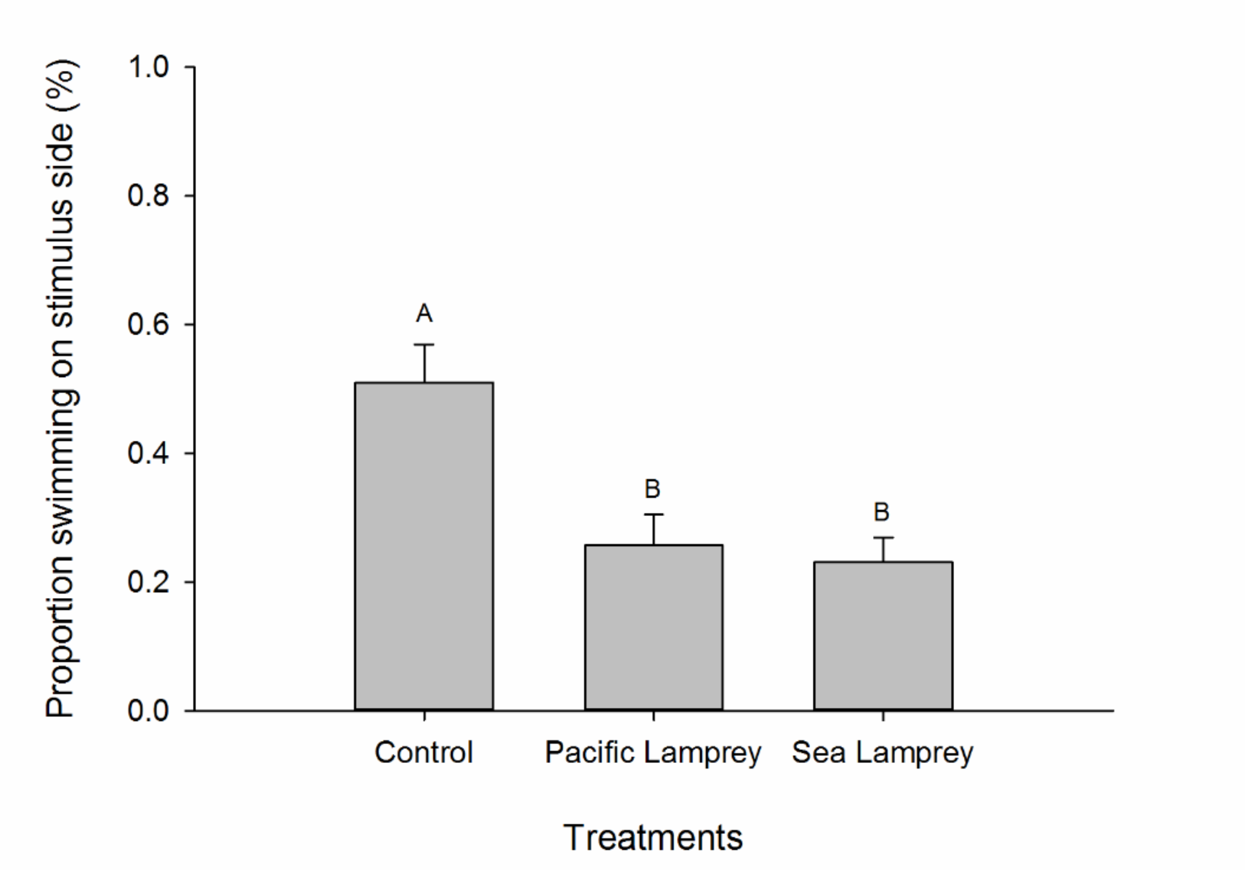

Figure 3.3: Mean (±1 SE) proportion of migratory male Sea Lamprey on the stimulus side of the raceway after exposure to heterospecific putrefied odors collected from whole carcasses (Sohxlet extraction). Treatments sharing letters are not found to be significantly different using Tukey's HSD ($\alpha = 0.05$). N = 8 for each bar. The analysis was performed on data that were arcsine transformed but are displayed as the observed proportions.

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