THE SEA LAMPREY ALARM RESPONSE: FIELD AND LABORATORY INVESTIGATIONS

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A THESIS

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

MASTER OF SCIENCE

Fisheries and Wildlife

2012

ABSTRACT

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The sea lamprey (*Petromyzon marinus*), a devastating invasive species within the Laurentian Great Lakes, exhibits a spectacular alarm response to the odor emitted from dead conspecifics that may differ substantially in function from the well-characterized system in ostariophysan fishes. Here, we report the first field test and a series of laboratory experiments designed to characterize the behavioral responses of migratory-phase lampreys to a set of odors derived from conspecific and heterospecific tissues, determine whether sex or sexual maturation alters these responses, ascertain if the putative alarm substance derives from a particular region of the body, and evaluate how variation in dilution and previous exposure history influence the response. The field test clearly demonstrated that sea lampreys restrict their migration path by avoiding areas activated with conspecific alarm cues. A number of findings were consistent with prevailing predation-risk model for fishes in that dilute odors from conspecific skin elicited avoidance response, reactivity was a function of phylogenetic relatedness between species, and the response attenuated in females upon maturation but not in males. However, unlike alarm cues for other aquatic species, the sea lamprey alarm substance activates a larger space, is contained within multiple tissues, and is persistent over time (survives 96 h of decay). These features suggest a broader ecological function than the detection and avoidance of a predator. Given the strength and consistency of the response (>99% repellency at the highest concentration) these semiochemical(s) could provide a beneficial supplement to current sea lamprey management practices by redistributing the annual spawning run.

AKNOWLEDGEMENTS

I would like to thank my advisor Dr. C. Michael Wagner, and members of my graduate committee Dr. Jim Miller and Dr. Scott Peacor for their support and assistance. I thank all personnel at the United States Geological Survey Hammond Bay Biological Station, Millersburg, Michigan, and the United States Fish and Wildlife Service Marquette Biological Station, Marquette, Michigan, for their assistance in animal capture, housing, maintenance, and technical support during these projects. I would like to give special thanks to field technicians and colleagues for their hard work, dedication, and patience regarding these projects: Skye Fissette, Greg Byford, Carrie Kozel, Sarah Ptasnik, Trevor Meckley, Tyler Buchinger, Eric Willman, Cory Olaf Brant, Brett Diffin, and Amber Masters.

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INTRODUCTION

Organisms combat the pervasive force of risk by obtaining information regarding their current and future surroundings. Such, information can be obtained via personal information, often a time consuming enterprise, or by monitoring the success of others via social information (Dall et al. 2005). Chemically-mediated risk assessment is particularly important within aquatic environments (Chivers and Smith 1998; Ferrari et al. 2010). Here, molecules containing information regarding opportunities (e.g. food, mates, habitat) and perils (e.g. predation) are cast into the aqueous medium and can be transmitted great distances by currents and persist over time (Wisenden and Chivers 2006; Wisenden 2008; Ferrari et al. 2010; Thiel and Breithaupt 2011). These cues and signals are available to all individuals encountering the odor plume. As a result, many aquatic organisms rely on chemical information regulate behavioral decisions across a range of spatial and temporal scales (Lima and Dill 1990; Dodson et al. 1994; Barbin et al.1998; Døving et al. 2006).

Olfaction is the principle sensory system used to guide the nocturnal migration of the semelparous sea lamprey (*Petromyzon marinus*) into suitable riverine spawning habitat (Applegate 1950; Manion and Hansen 1980; Binder and McDonald 2007; Vrieze et al. 2010; 2011). Vrieze et al. (2010) demonstrated that anosmic lampreys failed to locate spawning streams. The migration does not terminate within the natal stream (Bergstedet and Seelye 1995; Waldman et al. 2008). Instead, adult lamprey select spawning tributaries based on the odor released passively from multiple generations of stream-resident larvae (ammocoetes) that confirms previous spawning success and the presence of suitable larval habitat (Teeter 1980; Wagner et al. 2009). Following stream selection, males release a pheromone to attract ovulating females to a constructed nest (Li et al. 2002; Johnson et al. 2012). Significant effort has been dedicated to elucidating the active components of larval odor (Sorensen and Vrieze 2003; Sorensen et al. 2005) the male mating pheromone (Li et al. 2002), and studying these attractants as potential alternative management strategies for the invasive population in the Laurentian Great Lakes (Johnson et al. 2005; Wagner et al. 2006; Johnson et al. 2009; Luehring et al. 2011; Meckley et al. 2012).

Wagner et al. (2011) suggested that risk information might also be important for regulating habitat selection during the sea lamprey migration. The authors demonstrated that sea lampreys are consistently repelled by dilute exposure to a composite odor collected from both freshly killed and decayed conspecifics under laboratory conditions (Wagner et al. 2011). In other aquatic organisms, conspecific alarm cues are a reliable indicator that a predator has attacked and consumed nearby conspecifics (Wisenden et al. 2004; Wisenden 2008). Concurrent exposure to alarm cues with a predatory encounter allows prey to adjust their behaviors in a manner that increases the probability of survival (Mathis and Smith 1993; Wisenden et al. 1999; Mirza and Chivers 2003). Accordingly, these cues activate a small area over a relatively short amount of time. For sea lampreys, the ultimate mechanism underlying the avoidance of alarm cues is less clear, because the odor can arise from three potential non-exclusive sources: (1) dead larvae, (2) dead migrants, or (3) dead spawners. These odors could indicate the risk of selecting a habitat (1) that is not conducive to the long term survival of their progeny, (2) where the probability of predatory attack is high, or (3) where mate availability is low because spawning has ceased within that tributary (Wagner et al. 2011).

Wagner et al. (2011) highlighted two opportunities presented by the discovery of a sea lamprey alarm cue. First, aquatic reproductive migrations typically rely on chemical information to locate spawning habitats (Døving and Stabell 2003; Moore and Crimaldi 2004; Døving et al. 2006; Keefer et al. 2006) rather than avoiding risky routes. The sea lamprey reproductive migration may be unique in that it is principally mediated by multiple sources of opposing chemical information. A flexible strategy would increase the probability that the sole reproductive bout will be successful. We are unaware of another strategy that incorporates current chemical information regarding the success of conspecifics across life-history stages to mediate habitat selection along the course of a reproductive migration. Quantifying trade-offs between risk and opportunity is difficult because animals are incorporating various forms of information across sensory modalities (Abrahams & Dill 1989; Bednekoff 1996). Determining the value of information requires an understanding of the animal's internal state (Webster and Laland 2011), social circumstances (Brown et al. 2006; Wisenden et al. 2010) and the motivation of an individual to respond (Metcalfe et al. 1987). Multiple facets of the sea lampreys reproductive migration indicate this species potential as a model to understand the importance of risk information during habitat selection and migration. First, prior to the onset of migration, lampreys cease feeding, thus they do not trade-off foraging opportunities with risk (Applegate 1950). Second, sea lampreys are semelparous, eliminating the potential trade-off between current versus future reproductive output. Third, lamprey movements are predictable in that they are constrained by the transition from a lake or ocean into a tributary where they proceed upstream. Fourth, lamprey movements are not influenced by proximity to other individuals (Siefkes et al. 2005). Finally, sea lampreys appear almost entirely reliant on information received over a single sensory channel, olfaction (Binder and McDonald 2007; Vrieze et al. 2010; 2011).

The second opportunity following the discovery of the sea lamprey alarm substance is the potential to achieve a novel form of invasive species control. The semiochemical(s) responsible for eliciting this response may provide a valuable supplement to the extensive sea lamprey control tactics underway within the Laurentian Great Lakes (Wagner et al. 2011). Sea lampreys are a devastating invasive species within the Great Lakes and are subject to a control program that relies on physical and electrical barriers to limit the extent of migration, trapping to remove adults, and lampricides to dispatch their stream dwelling progeny. Alarm cues could be used to prevent lampreys from entering certain tributaries, effectively concentrating the spawning run and increasing the efficiency of lampricide applications. The widely available alarm cues will allow us to verify their potential utility for pest management prior to undertaking the extensive process of elucidating the active chemical components (Wagner et al. 2011).

The goal for the first chapter of this thesis was to gain a basic understanding of the sea lamprey alarm response in order to predict under what circumstances the animal is likely to use these odors and anticipate how they might be deployed as a management technique. Using a previously published assay based on odor-mediated modification of space use (Wagner et al. 2011) we examined whether exposure to any of 17 dilute odors would alter the spatial distribution of single-sex groups of migratory-phase sea lampreys in a laboratory raceway in order to address four research questions. 1) *Are sea lampreys repelled by the odors of freshly killed and/or decayed adult and/or larval conspecifics?* 2) *Are sea lampreys repelled by the odor of dead and/or decayed heterospecifics?* 3) *Is the alarm substance contained in a particular tissue?* 4) *Are the responses of sea lampreys to the odor of dead and/or decayed adult* *conspecifics modulated by sex or maturation?* The second chapter further examines the expression of the alarm response by conducting laboratory experiments evaluating how variation in the frequency of previous exposure and dilution influences the expression of the alarm response. We also conducted the first field study with the aim of identifying whether sea lampreys modify their migration path in a natural environment in response to varying dilutions of conspecific alarm cues. The results shed light on the ecological function these cues serve and determine if alarm cues can be deployed as a supplement to the management of the invasive population.

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CHAPTER 1

Behavioral responses of sea lamprey (Petromyzon marinus) to a putative alarm cue derived from conspecific and heterospecific sources

Short title: Sea lamprey alarm response

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Keywords: Sea lamprey; Petromyzon marinus; Alarm substance; Risk; Conspecific and heterospecific odor; Public information; Habitat selection; Migration.

Abstract

The sea lamprey, *Petromyzon marinus*, exhibits a spectacular alarm response to the odor emitted from decayed conspecifics that may differ substantially in function from the well-characterized system in ostariophysan fishes. Here, we report a series of three laboratory experiments designed to characterize the behavioral responses of migratory-phase lampreys to a set of odors derived from conspecific and heterospecific tissues, determine whether sex or sexual maturation alters these responses, and ascertain if the putative alarm substance derives from a particular region of the body. A number of the findings were consistent with the prevailing predator-avoidance paradigm for fish alarm substances released from the skin after predator attack in that: 1) dilute odors derived from freshly ground skin were highly repellent; 2) the substance is contained in the organism early in life; 3) the odor derived from a close relative was avoided whereas those of a distant relative were not; and, 4) upon sexual maturity female response to the alarm substance was attenuated. Two interesting patterns arose that differed substantially from the prevailing paradigm: 1) conspecific odors remained repellent after 96 h of aerobic decay; and, 2) the cue was emitted from multiple areas of the body, not just the skin, and the repellency of the odor derived from any tissue increased in accordance with its mass. A persistent cue emitted from several sources suggests a broader ecological function than the detection and avoidance of a predator.

Introduction

Cues that reveal risk confer a fitness advantage to receivers capable of both accurately interpreting and properly responding to the information the cue contains. Consequently, alarm cues are ubiquitous constituents of animal public information strategies. Chemically-mediated risk assessment is particularly important in aquatic environments (Chivers & Smith, 1998; Kats & Dill, 1998; Ferrari et al., 2010). That fishes attend to alarm cues is implied by the organization of their olfactory system (Hamdani & Døving, 2007; Laframboise et al., 2007; Døving & Lastein, 2009) and evident in their behavioral responses to odors associated with predator presence (Mathis & Smith, 1993; Mirza & Chivers, 2001), predator attack and prey injury (Wisenden et al., 1999; Mirza & Chivers, 2003), startled prey (Wisenden et al., 1995; Bryer et al., 2001), and acquired predator recognition (Ferrari et al., 2006; Zhao et al., 2006; McLean et al., 2007). In fact, substantial effort has been expended to understand how a diverse array of vertebrates and invertebrates manage activity in space and time in response to predator-affiliated cues, and more recently, integrate that information into risk-informed decision-making (hereafter referred to as the predation-risk paradigm; McNamara & Dall, 2010; Schmidt et al., 2010). Considerably less attention has been paid to how alarm cues might be used to inform effective decision-making in important ecological circumstances that are not principally regulated by avoiding predation (e.g., the selection of reproductive habitat).

One particular class of alarm cues, the odors derived from dead and/or decayed conspecifics, may have originated as inadvertent social information released near the time of death that allows conspecifics to recognize risky circumstances (Yao et al., 2009; Wagner & Danchin, 2010).

Animal decision-making typically involves an active trade-off between risk and opportunity, mediated by information received across multiple sensory modalities (Bouwma & Hazlett, 2001; Blanchet et al., 2007; Kim et al., 2009). Consequently, the elucidation of the ecological role of any single piece of information often requires knowledge of the ecological and social circumstances (Brown et al., 2006; Wisenden et al., 2010), an animal's internal state as it relates to signal reception/perception (Rohr et al., 2002; Olsson et al., 2002; Lastein et al., 2008; Webster & Laland, 2011), motivation to respond (Metcalfe et al., 1987), and the relevant currencies. Quantifying these tradeoffs has proven difficult (Abrahams & Dill, 1989; Bednekoff, 1996). Wagner et al. (2011) recently demonstrated that sea lampreys (*Petromyzon marinus*) will avoid the odor emitted by dead conspecifics during the annual spawning migration. Several features of the sea lamprey reproductive migration relax a few of the complicating circumstances listed above and suggest this species will prove quite useful in the elucidation of the use of risk information as it pertains to habitat selection.

The sea lamprey undertakes a long-distance, nocturnal, terminal migration to reproduce and die in rivers (Manion & Hansen, 1980). Because sea lampreys cease feeding prior to the onset of the migration, they do not trade-off foraging with reproductive opportunities or risks. Further simplifying matters, they appear almost entirely reliant on information gathered from a single sensory modality (olfaction). Unlike salmonid fishes, sea lampreys do not return to their natal streams (Bergstedt & Seelye, 1995; Waldman et al., 2008). Rather, they depend on a set of olfactory cues to guide them into high quality reproductive habitat. Overlapping generations of larvae (ammocoetes) that reside in stream sediments release an odor that indicates past spawning success and allows migratory lamprey to locate habitats likely to support future offspring

(Sorensen et al., 2005; Wagner et al., 2009). Once in the proper area, sexually mature males present themselves on nests and attract mature females with a mating pheromone (Li et al., 2002; Johnson et al., 2009). Both odors are sources of public information that indicate opportunities and likely function to aggregate migrants into high-quality habitats and facilitate the identification of ready mates.

Wagner et al. (2011) suggested the alarm cue emitted by dead conspecifics adds risk assessment to the reproductive habitat-selection process. The odor of dead lampreys may originate from any of three sources: 1) dead larvae, indicating the risk of depositing offspring into habitat no longer conducive to survival of progeny; 2) dead migrants, indicating the presence of predators and the risk of attack; or, 3) dead spawners, indicating the cessation of spawning and the risk of maturing in a habitat with low mate availability. Thus, reproductive habitat selection by the sea lamprey may principally be regulated by odors of opposing valence received on a single sensory channel, and thereby highly vulnerable to experimental manipulation. Here, we report the results of a series of laboratory experiments designed to examine the expression of the sea lamprey alarm response by migrants in response to a number of odors and factors associated with these sources of risk.

Using a previously published assay based on odor-mediated modification of space use (Wagner et al. 2011) we examined whether exposure to any of 17 dilute odors would alter the spatial distribution of single-sex groups of migratory-phase sea lampreys in a laboratory raceway in order to address four research questions. 1) *Are sea lampreys repelled by the odors of freshly killed and/or decayed adult and/or larval conspecifics?* Wagner et al. (2011) used a mixture of odors derived from both freshly killed and decayed lampreys collected at four time-points post-mortem. Thus, it is unclear from their results whether the responsible semiochemical(s) were present in the living tissue or were a product of putrefaction. 2) *Are sea lampreys repelled by the odor of dead and/or decayed heterospecifics?* To ascertain whether the putative alarm substance derives from lamprey tissues, or any dead or decaying fish, we tested migratory sea lamprey responses to the odors derived from a distantly related fish, the bluegill sunfish (*Lepomis macrochirus*) and a closely related lamprey in the family Petromyzontidae, the silver lamprey (*Ichthyomyzon unicuspis*). 3) *Is the alarm substance contained in a particular tissue?* For fishes, it is believed that alarm substances are typically held within the epidermis, although examinations of other tissues are nearly absent from this considerable literature (Pfeifer, 1962; Smith, 1979; Chivers & Smith, 1998; Mathis, 2009; Mirza, 2009). We determined whether extracts from several tissues would repel migrants. 4) *Are the responses of sea lampreys to the odor of dead and/or decayed adult conspecifics modulated by sex or maturation?* The behavioral responses of fishes to alarm substances can vary between sexes and through ontogeny (Mirza et al., 2001; Williams et al., 2001; Golub and Brown, 2003; Harvey and Brown, 2004; Lastein et al., 2008; Gall & Mathis, 2011). We determined whether sexual maturity altered responses to the odors collected from fresh-killed or decayed adults for males and females.

Material and methods

To ascertain whether sea lampreys were repelled by, attracted to, or indifferent to each stimulus odor we evaluated space use in the raceway per the approach of Wagner et al. (2011) in three separate experiments. First, we examined the response of single-sex groups of migratory-phase sea lampreys to a solvent control, six conspecific odors derived from freshly-killed or decayed adult and larval lampreys, and four odors derived from freshly-killed or decayed heterospecifics, as outlined below (Experiment #1, Research Questions 1 & 2). Second, to determine whether the putative alarm substance is contained within a particular region of the body, we examined the response of migratory-phase female sea lampreys to odors extracted from three partial carcasses of freshly-killed or 96 h decayed conspecifics: 1) skin; 2) organs from a gutted specimen; and, 3) the soma, defined here as the muscle and skeletal remainder of a headless, skinless, gutted carcass (Experiment #2, Research Question 3). Finally, to determine whether sexual maturity alters the sea lamprey response to the odors from conspecific carcasses, we examined the response of groups of spermiating males or ovulating females to odors extracted from freshly-killed or 96 h decayed adult sea lampreys (Experiment 3, Research Question 4).

Test Subjects

Per previously published procedures (Wagner et al., 2006, 2009) we acquired adult migratory-phase sea lampreys from three tributaries to Lake Huron in Michigan, USA (the Cheyboygan, Ocqueoc, and St. Mary’s Rivers) from May - July 2011. Sea lampreys were live-trapped by the U.S. Fish and Wildlife Service as part of the annual sea lamprey control program and transported to the Hammond Bay Biological Station (Millersburg, Michigan, USA) where they were separated by sex, placed into 1000 L holding tanks receiving a continuous flow of fresh water from Lake Huron (100% exchange every 2 h). We held all sea lampreys at the station for at least 48 h prior to use, monitored them to ensure normal behavior, and physically examined each specimen for physical damage. Only robust lampreys were used in the trials, and each subject was used in a single trial. At the conclusion of each trial the adult lampreys were returned alive and unharmed to the U.S. Fish and Wildlife Service for final disposition. We acquired sea lamprey larvae from a captive population of recently captured wild animals maintained at the Hammond Bay Biological Station. Larvae were held in 1000 L flow-through tanks that were partially filled with sand and received a continuous supply of Lake Huron water. For heterospecific odor extractions we obtained adult bluegill sunfish via angling from Ocqueoc Lake, Michigan, USA and adult silver lampreys via electrofishing in several tributaries near the Hammond Bay Biological Station. All subjects used for extraction were euthanized via cervical dislocation. Use of fishes and all experimental procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF# 02/11-027-00).

We placed a portion of the sea lampreys into cages and held them in the nearby Ocqueoc River to facilitate maturation per the procedures of Luehring et al. (2011). These subjects were only used in the trials requiring sexually mature subjects (Experiment #3). We distinguished sexually mature from migratory-phase lampreys based on the emergence of sexual dimorphisms that accompany maturation (Richardson et al., 2010) and by the release of milt or eggs per the procedure of Siefkes et al. (2003). Once the animals reached sexual maturity we transferred them back to the Hammond Bay Biological Station where they were acclimated to Lake Huron water for a minimum of 48 h prior to use in the behavioral assay.

Apparatus

All trials took place at night in two linear concrete laboratory raceways per the general procedures of Wagner et al. (2011). The experimental section of each raceway was 5.0 m long and 1.84 m wide. The bottom was lined with white fiberglass to facilitate detection of lampreys against the background and a single IR-sensitive video camera equipped with two infra-red light arrays was mounted directly over each experimental section. The raceways received a continuous flow of water pumped directly from Lake Huron where the temperature ranged from 7° - 18° C over the course of the experiments. We maintained discharge at 0.006 - 0.01 $m^3 s^{-1}$ to simulate water currents in the lake. In order to simulate a natural day-night schedule we dimmed the lights of the room at sunset leaving a large window and the end of the raceway uncovered while natural light diminished. After 90 minutes we rendered complete darkness by covering the window. We observed lamprey movements in an adjacent room on video monitors and recorded their activity onto digital media.

During a trial the subjects experienced a dilute olfactory stimulus extracted from a single individual sea lamprey adult or from multiple individuals (silver lampreys, larval sea lampreys, or adult bluegill sunfish) necessary to approximate the weight of an adult sea lamprey (220-241 g). Prior to introduction, we mixed 45 ml of a stimulus odor into 450 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 during release. We introduced the test odor/lake water mixture into one side of each raceway at a rate of 15 ml min^{-1} via laboratory-grade peristaltic pumps (MasterFlex model 7533-20). The final dilution of raceway water to extracted odor was 200 000:1. To ensure no cross-contamination of odors we used a separate set of pump tubing for each stimulus odor. Following each trial we thoroughly cleaned the tubing by pumping 100% ethanol for ten minutes at a rate of 15 ml min^{-1}.

To ensure the odor was distributed only on one side of the raceway we conducted a series of eight dye-release tests with Rhodamine WT (two tests on each side of either raceway). On two separate dates we pumped dye into either side of both raceways per the procedure above and allowed it to distribute for five minutes. We measured Rhodamine concentration at every node in a 20 X 20 cm grid in the experimental sections with a Turner Designs DataBank logger equipped with a series Cyclops-7 Rhodamine WT probe (minimum detection 0.01 ppb). Dye distributions indicated the undisturbed plume was consistently confined to the nearest third of the raceway's width.

Odor Collection

Whole Carcass Extraction - We extracted the odor emitted by whole carcasses of individual adult female sea lamprey (234 – 310 g wet weight), sea lamprey larvae of indeterminate sex (220 g total wet weight; ~400 individuals), and adult bluegill sunfish of indeterminate sex (215-235 g wet weight; 2 or 3 individuals) at two time-points post-mortem (0 h and 96 h) via Soxhlet extraction. We also extracted the odor emitted by adult silver lamprey (241 g total weight; 2 individuals of indeterminate sex) at only a single time-point (96 h post-mortem) due to limited availability. We used a 1 L 71/60 Soxhlet apparatus with six bulb water-cooled Allihn condenser and a 1 L solvent reservoir heated with a hemispherical mantle to 75° - 80° C. Prior to extraction, we placed the carcass(es) in a 1 L HDPE bottle, and allowed aerobic decay to proceed at room temperature (this step was skipped for the 0 h fresh-killed extractions). We then extracted the carcass(es) in a 50:50 w/w solution of 200 proof ethyl alcohol and deionized water. Prior to placement in the apparatus, the carcass(es) were rinsed for 30 min with 150 ml of solvent. We added the remaining solvent (850 ml) into the solvent reservoir and loaded the carcass(es) into the extractor body. To prevent particulate matter from clogging the siphon we placed larvae into cellulose extraction thimbles and covered the siphon inlet with uncoated aluminum screen. For each extraction we cycled the extractor three times. Following extraction we filtered the rinsate and extract, combined the two, and stored the final extract at -80° C until use.

Tissue Extraction – Because both 0 h and 96 h whole-carcass extracts elicited similar responses from migratory-phase adults (Experiment #1), we collected and extracted the odor produced by partial carcasses of fresh-killed sea lamprey from a single time-point post-mortem (0 h) for additional testing. First, we euthanized a single adult female subject via cervical dislocation and immediately made a circular incision through the epidermis around the circumference of the organism behind the posterior-most gill opening. We removed the skin posterior to the incision in one piece with a pair of pliers, rinsed the skin with 500 ml of deionized water and placed the skin into 150 ml of extraction solvent (0 h skin). We ground the skin using a mortar and pestle for ten minutes and diluted the mixture with an additional 650 ml of solvent. The skinless, decapitated lamprey carcass was rinsed in a second 500 ml of deionized water and extracted per the procedure (0 h skinless carcass).

To further discern whether responses to different tissue-classes were related to odors emitting from a particular region of the body (Experiment #2), we dissected two additional adult female lampreys and separated the skin, organs, and soma, rinsed each tissue in 500 ml deionized water, and immediately placed it into a separate 1 L HPDE bottle. We allowed each tissue to decay for two time-points post-mortem (0 h and 96 h) and collected the extract following the same procedures described above for a whole carcass. Organs and skin were placed into cellulose thimbles prior to extraction.

Behavioral Assay

Six hours prior to the start of an evening's trials we transferred ten groups of ten lampreys each into separate holding cages, five cages per raceway, placed upstream of the experimental section to prevent any prior exposure to the stimulus. All groups within a raceway were the same sex to prevent any unanticipated responses to intersexual odors emitting from the cages. At the start of a trial we carefully and slowly moved a single holding cage into the middle of the experimental arena and lifted the cage to release the animals. Each experimental trial was 40 minutes long and consisted of a 20 min pre-stimulus period and a 20 min stimulus period during which the stimulus odor was introduced into one side of the raceway. We alternated the side of the raceway receiving the stimulus across replicates within each treatment such that each treatment received an equal number of trials (4 or 5) with the stimulus discharged on right and left sides of the channel. The pre-stimulus period began when a majority of the sea lampreys (≥ 6 fish) had detached from the bottom of the raceway and were actively swimming. Following the trial, we removed the lampreys from the raceway and recorded the lengths (total length, mm) and weights (wet weight, 0.01 g) of each subject.

In addition to the odors outlined above, we tested whether each class of respondent (migratory males, migratory females, ovulating females, or spermiating males) responded to the solvent mixture used during extractions. We recorded the position of each subject every 30 sec after the start of a trial by replaying the video and assigning each lamprey to one side of the experimental arena based on the position of its head. We analyzed sea lamprey distributions during the final ten minutes of the stimulus period. The pre-stimulus period was used as an acclimation period and the first ten minutes of the stimulus period was not analyzed to provide time for the distribution of the lampreys to stabilize after introduction of the stimulus.

Data Analysis

We performed all statistical analyses in SAS (ver. 9.2). To ascertain which odors altered the spatial distribution of adult migratory-phase sea lampreys in response to conspecific and heterospecific odors (Experiment #1), we performed a two-way analysis of variance (ANOVA) with the proportion of animals on the stimulus side of the raceway as the response and sex and stimulus type as fixed factors in the model. We transformed the proportions with an arcsine (square root) transformation and tested for normality with Shapiro-Wilk's test. Each respondent type (migratory male or migratory female) was exposed to eleven odors including a solvent control, fresh-ground sea lamprey skin, fresh-ground bluegill sunfish skin, and eight Sohxlet-extracted odors (0 h whole sea lamprey carcass, 96 h decayed whole sea lamprey carcass, 0 h skinless sea lamprey carcass, 0 h sea lamprey larvae, 96 h decayed sea lamprey larvae, 96 h decayed whole silver lamprey carcass, 0 h whole bluegill carcass, and 96 h decayed whole bluegill carcass). We completed ten replicates for each combination of odor and respondent. We used Dunnett's test ($\alpha = 0.05$) to compare the response for each odor to that of the solvent control (50:50 w/w ethanol and deionized water). The transformed data were normally distributed (Shapiro-Wilk, all $W > 0.83$ and all $P > 0.35$).

To ascertain whether the alarm substance was contained within a particular region of the body (Experiment #2), we evaluated the responses of migratory-phase female sea lampreys exposed to six odors: 0 h (fresh) or 96 h (decayed) skin, organs, or soma (N = 8 for each). We directly compared the proportion of lampreys on the stimulus side during pre-stimulus and stimulus periods with separate paired t-tests for each odor (two-tailed, $\alpha = 0.05$; proportions were transformed with an arcsine (square root) transformation). We also investigated whether variation in responses to the odors could be explained by differences in the mass of the tissue types using linear regression with tissue mass as the independent variable and proportion of animals on the stimulus side of the raceway during the stimulus period as the dependent variable.

To determine whether sexual maturity influenced the expression of the alarm response (Experiment #3), we evaluated the responses of single-sex groups of mature females or mature males exposed to three odors: 0 h (fresh) and 96 h (decayed) whole sea lamprey carcass and the solvent control (N = 8 for each). We analyzed the data with a two-way ANOVA ($\alpha = 0.05$) as above (Experiment #1).

Results

Experiment #1: Responses to the Odors of Fresh-killed or Decayed Conspecifics and Heterospecifics by Migratory-phase Sea Lampreys (Research Questions 1 & 2)

The model results (ANOVA, $F_{21,198} = 11.97$, $P < 0.001$) clearly indicated the type of odor introduced into the raceway significantly influenced the tendency of animals to avoid the stimulus side of the channel (Type III SS = 3.833, $F_{10,198} = 23.14$, $P < 0.001$). The sex of the migratory lamprey had no effect on space use (Type III SS = 0.004, $F_{1, 198} = 0.02$, $P = 0.8768$) nor was the interaction between sex and the odor type significant (Type III SS = 0.225, $F_{10,198} = 1.36$, $P = 0.203$). Migratory-phase sea lampreys consistently avoided the odors extracted from freshly-killed and decayed, whole and partial sea lampreys relative to the control. Specifically, both male and female migrants avoided the odors emitted from fresh-killed and decayed whole adult and larval carcasses (0 h, 96 h), 0 h skin, 0 h skinless carcass, and 0 h larvae (Dunnett's, all $P < 0.001$; Fig.1B). Extracts obtained from 96 h decayed silver lamprey repelled migratory sea lampreys (Dunnett's test, $P < 0.001$, Fig. 1C) whereas odors obtained from bluegill sunfish did not alter the spatial distribution of migratory lampreys (Dunnett's test, 0 h carcass, $P = 1.00$, 96 h carcass, $P = 0.8281$, skin, $P = 1.00$; Fig. 1C)

Experiment #2: Responses to Tissue-specific Odors by Migratory-phase Sea Lampreys (Research Question 3)

Female migrants consistently avoided the odors emitted from the 0 h skin ($t_7 = 3.24$, $P = 0.01$), 0 h organs ($t_7 = 3.25$, $P = 0.01$, Fig. 2A), 0 h soma ($t_7 = 3.48$, $P = 0.003$), and 96 h soma ($t_7 = 6.95$, $P < 0.001$). The odors emitted by 96 h organs ($t_7 = 1.7$, $P = 0.06$) and 96 h skin ($t_7 = 1.77$, $P = 0.07$) did not significantly influence the distribution of migratory females compared to the pre-stimulus period (Fig. 2A). Time spent on the stimulus side was negatively related to the mass of the extracted tissue (linear regression, $r^2=0.25$, $P=0.003$, Fig. 2B).

Experiment #3: Effects of Maturation on the Sea Lamprey Response to Conspecific Odor (Research Question 4)

The model (ANOVA, $F_{5,42} = 3.08$, $P = 0.0185$) suggests that the sex of the lampreys significantly influenced the tendency of animals to either occupy or avoid one side of the channel (sex, Type III SS = 0.265, $F_{1,42} = 7.90$, $P = 0.0075$). The type of odor introduced was not significant (odor, Type III SS = 0.045, $F_{2, 42} = 1.48$, $P = 0.2401$) nor was the interaction between sex and the odor type (Type III SS = 0.766, $F_{2,42} = 2.28$, $P = 0.1145$). Although the interaction term was not significant at $\alpha = 0.05$, after visually examining the data plots we adopted the conservative approach of Littell et al. (2002) and investigated simple main effects of the influence of odor within sex. We compared each stimulus type back to the solvent control. Sexually mature male sea lampreys continued to avoid the odor from either fresh killed or 96 h decayed conspecific carcasses (simple main effect of stimulus type within sex comparing back to a solvent control; $P = 0.0198$ (0 h); $P = 0.0415$ (96 h); Fig 3A) whereas sexually mature females were repelled by neither ($P = 0.9449$ (0h); $P=0.724$ (96h); Fig. 3B).

Discussion

Alarm substances released by fishes have almost always been viewed through the specter of predation. The substance is most often released from specialized cells in the skin upon attack (Lima & Dill, 1990; Wisenden & Chivers, 2006), and as the threat of predation diminishes over time and space, so too does the behavioral reactivity of the substance (Hazlett, 1999; Ferrari et al., 2007; Wisenden et al., 2009). Here, we have demonstrated a number of attributes of the sea lamprey alarm substance that are consistent with the predator-avoidance paradigm in fishes. In particular: 1) dilute odors derived from freshly ground skin were highly repellent; 2) the substance is contained in the organism early in life; 3) the odor derived from a close relative was avoided whereas those of a distant relative were not; and, 4) upon sexual maturity female response to the alarm substance was attenuated. However, we also discovered two interesting patterns that differ substantially from other systems. First, repellency of conspecific odors was retained after 96 h of aerobic decay, suggesting the alarm response derives from reception of a stable odorant, or perhaps the existence of a set of alarm substances, one of which is a product of putrefaction. Second, the cue was emitted from multiple areas of the body, not just the skin, and the repellency of the odor derived from each tissue-type was related to the mass of the extracted tissue, suggesting larger tissues produced a more concentrated odor.

Are sea lampreys repelled by the odors of freshly killed and/or decayed adult and/or larval conspecifics?

The odors extracted from both fresh-killed (uninjured) and decayed adult and larval lampreys were highly repellent to migrants. Compounds emitted from decaying conspecifics are not included in traditional outlines of aquatic predator-prey interactions (Lima & Dill, 1990; Wisenden & Chivers, 2006), but do induce avoidance in certain invertebrates (Yao et al., 2009). Presumably, the threat of predation diminishes as a function of time since attack, distance from the attacker, and the social context (Wisenden & Chivers, 2006). Wisenden (2008) characterized these features, along with physical factors that determine odor advection, as determinants of the active space of an alarm cue. When the risk conveyed by the odorant is predator attack, the active space is typically small and the threat relatively short-lived. Consequently, fish alarm substances lose their efficacy with dilution (Marcus and Brown, 2003; Mirza and Chivers, 2003) and time since release (Wisenden et al., 2009). Lampreys may use this odor as an indication of circumstances unfavorable to successful reproduction that operate over considerably larger scales. Specifically, the odor, particularly when emitted from dead larvae, could facilitate the avoidance of habitats where larval survival has diminished. Live larvae produce an attractive odor that operates as a conspecific cue and attracts migrants into streams with habitat sufficient to support future offspring (Sorensen et al., 2005; Wagner et al., 2006, 2009). Migrating sea lampreys use behavioral mechanisms consistent with the localization of a large, diffuse odor source to find these streams (Døving et al., 1985; Moore & Crimaldi, 2004; Vrieze et al., 2011), and do not modulate their response to the concentration of the larval pheromone, only its detection (Wagner et al., 2009). In opposition, the odor emitted by dead and decaying larvae liberated from sediments during high spring flows may indicate overwinter mortality. Manion and Smith (1978) report the capture of decaying larvae during the migratory season in traps designed to capture downstream drifting larvae. A sea lamprey's entire lifetime fitness is determined in a single reproductive season. Thus, the alarm cue may oppose the larval pheromone and thereby allow migrants to avoid spawning in streams where future larval survival is uncertain.

Are sea lampreys repelled by the odor of dead and/or decayed heterospecifics?

The response of migrants to heterospecific odors appeared dependent upon the relatedness of the emitter species to sea lamprey. Sea lampreys did not avoid any of three odors (skin, fresh-killed and decayed carcasses) from a distantly related teleost fish whereas the sole odor from a confamilial agnathan (96 h decayed carcass) elicited avoidance from migratory adults. Diminished response to alarm cues with increasing phylogenetic distance has been observed in several taxa (Schütz, 1956; Mirza & Chivers, 2001; Mirza et al., 2001; Dalesman et al., 2007; Shabani et al., 2008; Briones-Fourzán & Lozano-Álvarez, 2008). Silver and sea lampreys share similar life histories and habitat requirements (Cochran & Lyons, 2004). Fine et al. (2004) reported a similar finding with respect to the larval odor, where water conditioned by larval silver lamprey proved attractive to migrating sea lampreys, and vice-versa. They report ten species of Petromyzontidae that each produce and release three lamprey-specific bile acids related to the production of pheromones. Sea lampreys were unresponsive to odors derived from bluegill sunfish. A member of the Centrarchidae, the bluegill is a non-ostariophysan fish and therefore may not emit the classic fish alarm substance (Schreckstoff; Smith, 1992); although another member of the genus *Lepomis*, the pumpkinseed sunfish (*Lepomis gibbosus*), does produce an alarm substance (Marcus & Brown, 2003). Our data add support to the general contention that production of certain semiochemicals, including the alarm cue, may be conserved across species in the Petromyzontidae.

Is the alarm substance contained in a particular tissue?

Both male and female migratory-phase lampreys strongly avoided the odor of freshly-ground skin (Experiment 1, Fig.1), whereas the odor extracted from unground skin was not as repellent (Experiment 2, Fig. 2A). Thus, it appears the substance is contained in the skin, though not solely, and is likely released as a consequence of damage. Alternatively, the application of heat to the extracted skin may have partially denatured or deactivated the active compound. This seems unlikely given the high reactivity of whole and partial carcass extracts that were similarly treated.

When migrating sea lampreys enter streams they transition from large, deep environments into relatively shallow, narrow ecosystems that render them vulnerable to shoreline predators (Applegate, 1950; Cochran et al., 1992). These terrestrial predators often fail to consume whole individuals when prey is seasonally abundant, leaving recently-attacked carcasses in the environment (Wigley 1959; Sih, 1980; Gende et al., 2001). Thus, the predominantly unidirectional downstream flow would quickly convey this cue to upstream-swimming migrants. In our experiments, lampreys that entered the plume activated with the odor of dead conspecifics reacted quickly by reversing direction and moving to the downstream end of the raceway in an apparent attempt to escape. During the annual migration sea lampreys will sometimes stall in stream reaches with cover for days to weeks (Almeida et al., 2002). Migrants may time their upstream movements to correspond with low-risk conditions.

In the well-studied ostariophysan fishes, the alarm substance is contained in epidermal club cells that release their contents only upon rupture (Pfeiffer, 1962; Pfeiffer, 1977; but see Carreau-Green et al., 2008). Upon attack the cue is passively released into the environment as public information and notifies local individuals of the prey species, and other taxa attuned to the cue, of the risk (Chivers & Smith, 1998). Lamprey skin also contains club cells that appear in the larval stage and may play a similar role (Pfeiffer & Pletcher, 1964; Downing & Novales, 1971). Decidedly unlike the ostariophysan model, the extracts collected from multiple tissues (freshly ground skin, extracted skin, skinless lamprey carcass, fresh or decayed soma, and fresh internal organs) all resulted in significant repellency. Our purpose in evaluating the response to partial-carcasses was to ascertain whether the response to a whole carcass could be replicated with the odor from a particular region of the body, and thereby demonstrate where in the organism the reactive compound was likely contained. Interestingly, the degree to which particular tissues repelled migratory females was linearly related to the mass of the extracted tissue. This suggests an intriguing possibility: the responsible compound may be more or less equally distributed throughout the body. Carreau-Green et al. (2008) demonstrated the presence of an alarm cue in larval fathead minnows (*Pimephales promelas*) too young to produce club cells, implying the alarm cue may not come from club cells, as traditionally believed. Because our extracts retained reactivity after 96 h of aerobic decay, it is also possible the responsible compound is chemically quite stable. The ostariophysan cue diminishes in efficacy within 3-6 h of release (Wisenden et al., 2009) and can be deactivated through the application of heat, raising the temperature slightly above that of our extraction solvent (90° C; Wisenden et al., 2009). Fish alarm cues are thought to be proteinaceous (Ferrari et al., 2010); however, Mathuru et al. (2012) more recently reported some active components of the alarm substance in zebrafish (*Danio rerio*) to be a mixture of chondroitin fragments. It is unlikely that a protein would have survived our Sohxlet extraction unaltered. An obvious alternative explanation is the presence of multiple cues; perhaps one that signifies attack or damage (recent death), another signifying decay (past death).

Are the responses of sea lampreys to the odor of dead and/or decayed adult conspecifics modulated by sex or maturation?

After sexual maturation, female lampreys did not respond to the odor of dead or decayed conspecifics whereas males did. Lastein et al. (2008) observed a similar pattern in Crucian carp (*Carassius carassius*) they attributed, in part, to reductions in the plasma concentrations of 17β-estradiol and testosterone in females that may be typical of the onset of ovulation in carps (Kobayashi et al., 2002). The plasma concentration of 17β-estradiol also decreases significantly in female sea lampreys at the time of ovulation, but peaks in males at spermiation (Sower et al., 1985). Whether these endocrine changes are responsible for the observed behavioral differences in risk-taking by lamprey remains speculative. After maturation, male sea lampreys typically present themselves on nests and chemically call to females by releasing a sex pheromone (Li et al., 2002; Johnson et al., 2009), a behavior that may expose males to an increased risk of predation. However, because our males were mature but had not yet attended a nest, we cannot determine whether the risk-averse behavior exhibited in the lab would persist after a nest is constructed and occupied. Females, on the other hand, are the responding sex. Ignoring risk-indicating public information during mate search may be a hallmark of the transition in priority from predator avoidance to the acquisition of mates, and reasonable given a likely concomitant reduction in the reliability of the cue. As the spawning season progresses, the presence of the alarm cue may become increasingly decoupled from any true source of risk (Smith, 1976). For example, nesting lampreys often exhibit sloughing skin after undertaking abrasive spawning behavior, including nest-building and male-male aggression (Applegate, 1950). Further, lampreys die within two weeks of maturation, and carcasses accumulate as spawning proceeds (Wigley, 1959). Eventually, the odor of deceased lampreys may become persistent, and absent the male sex pheromone, signal low mate availability to immature females. At that time, mature females may lack the energetic reserves necessary to effectively search for another spawning aggregation, whereas immature females may continue to search over large areas.

In summary, this work represents the first substantial investigation of the behavioral reactivity of the putative sea lamprey alarm substance previously reported anecdotally (Imre et al., 2010) and in a single laboratory test (Wagner et al., 2011). Our results suggest a system that differs substantially from previously described alarm substances in that the responsible compound(s): 1) survive the process of putrefaction; 2) may remain stable in the environment for long periods; and, 3) are liberated from multiple tissues. Sea lampreys may use the odor over large distances to ascertain reproductive habitat quality in addition to detecting recent predatory attacks on closely related taxa.

Acknowledgements

We are grateful to the staff at Hammond Bay Biological Station for providing access to the laboratory raceways and for creating an excellent research environment. Eric Stroud developed the protocol for Soxhlet extraction. Dan Hayes provided assistance with the statistical analysis. We would like to thank Peter Hubbard and two anonymous reviewers for several suggestions that substantially improved the manuscript. Skye Fissette, Brett Diffin, Greg Byford, Amber Masters, Eric Willman, and Trevor Meckley provided assistance in the laboratory. Funding for this project was provided by the Great Lakes Fishery Commission.

APPENDIX

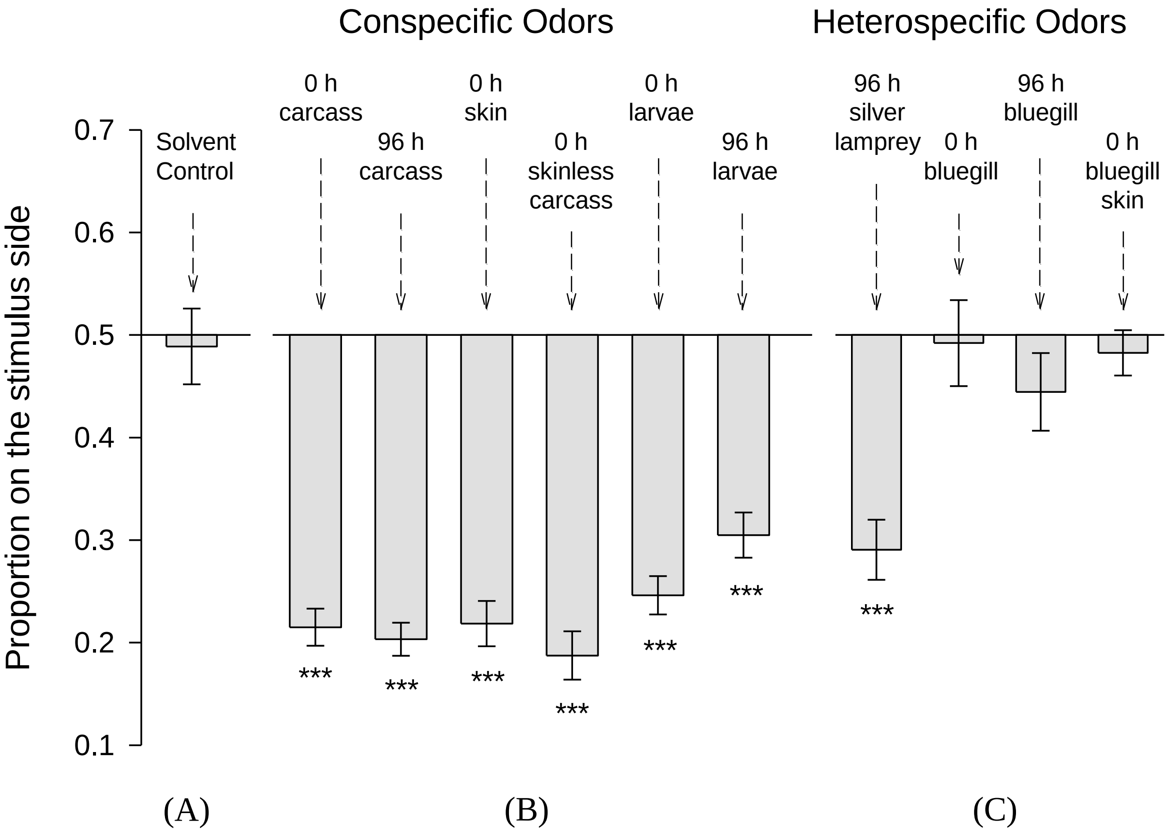

Fig.1. Mean (± 1 se) proportion of migratory sea lampreys on the stimulus side of the raceway after responding to (A) a solvent control, (B) conspecific odors, and (C) heterospecific odors collected from bluegill sunfish and silver lamprey. 0 h odors were collected from fresh-killed specimens, 96 h odors were collected after 96 h of aerobic decay. Responses to odors with an asterisk are significantly different from the solvent control. (ANOVA with Dunnett's test, $\alpha = 0.05$). N=20 for each bar. The analysis was performed on data that were arcsine (square root) transformed but are displayed as the observed proportions.

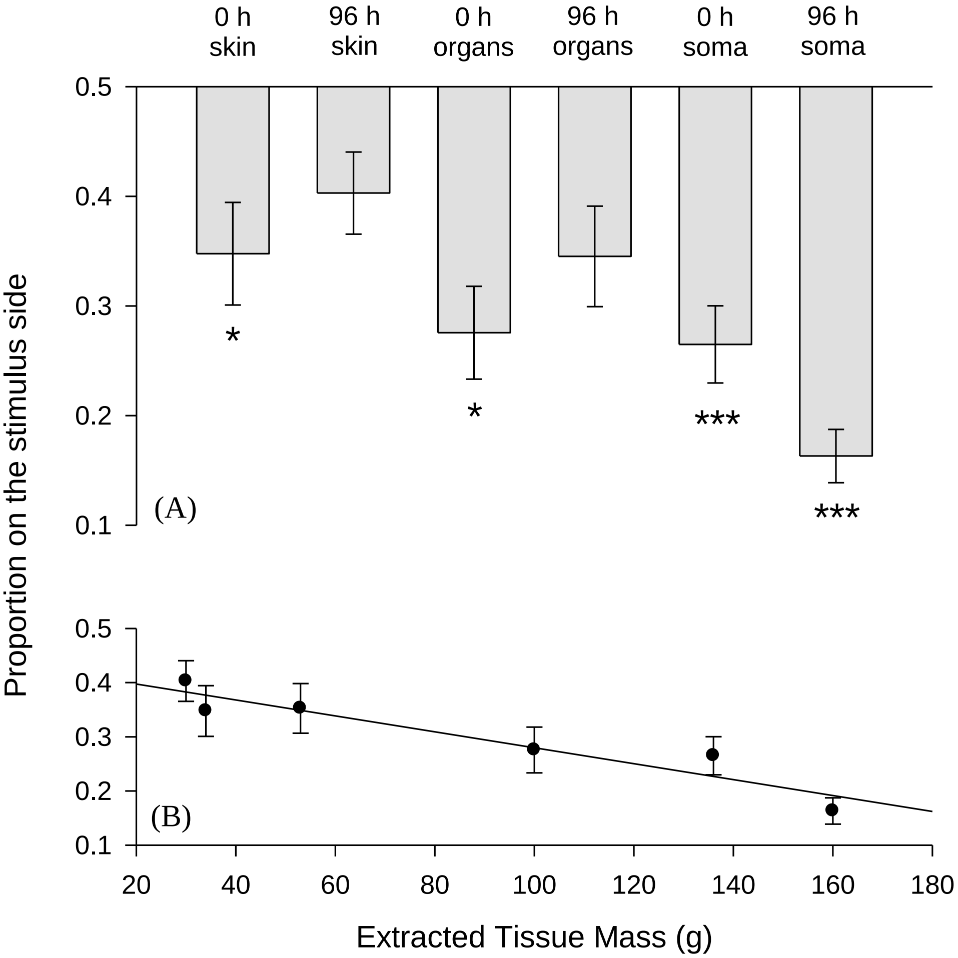

Fig.2. (A) Mean (± 1 se) proportion of migratory female sea lampreys on the stimulus side of the raceway after exposure to extracts resulting from three different tissue types (skin, internal organs, and soma) taken from fresh-killed and 96 h decayed conspecifics (N = 8). Asterisks indicate significant differences in distribution between the stimulus and non-stimulus sides of the raceway before and after addition of the odor (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B) A linear regression found a negative relationship between the average proportion of animals on the stimulus side and the mass of the tissue ($r^2=0.25$, $P=0.003$).

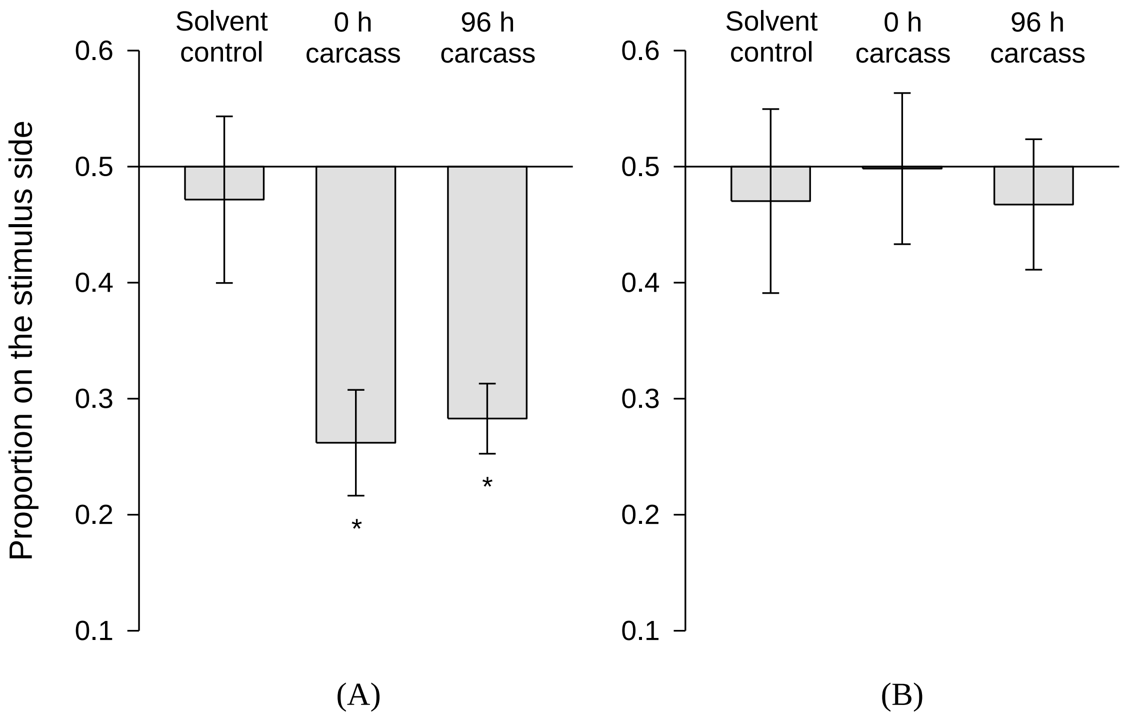

Fig.3. Mean (± 1 se) proportion of sexually mature male (A) or female (B) sea lampreys on the stimulus side of the raceway after responding to a solvent control and the odor extracted from fresh-killed and 96 h decayed whole carcasses. Responses to odors with an asterisk are significantly different from the solvent control (ANOVA with simple main effects of odor type within sex comparing odors to stimulus control, $\alpha = 0.05$). N = 8 for each bar. The analysis was performed on data that were arcsine (square root) transformed but are displayed as the observed proportions.

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CHAPTER 2

Sea lampreys restrict their migration route in response to chemosensory risk information

Short title: Sea lamprey alarm cues

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Keywords: Sea lamprey; *Petromyzon marinus*; Alarm substance; Risk; Social information; Habitat selection; Migration.

Abstract

Sea lampreys (*Petromyzon marinus*) possess an alarm substance that may function to regulate habitat selection along the course of a terminal reproductive migration by preventing lampreys from entering risky habitats. This may represent novel use of an odor traditionally viewed only in the light of predation. Several features of the lamprey's migration suggest the species will prove useful in evaluating the importance of risk information as it pertains to habitat selection. However, it is not known whether sea lampreys restrict their migration routes in response to conspecific alarm cues under natural conditions. Here, we perform the first field test aimed at identifying whether lampreys restrict their migration route in a natural stream across a range of risk levels (cue dilution) and laboratory experiments evaluating how variation in dilution and previous exposure history mediate the response. We report a strongly significant, all-or-nothing, response from the probability sea lampreys will enter a stream channel activated with conspecific alarm cues across a range of dilutions. This suggests that sea lampreys do not modulate behavior proportionally to the perceived amount of risk. Contrary to the field response, in the laboratory lampreys displayed a response proportional to cue dilution in the laboratory assay. Variation in frequency of previous exposure does influence the probability lampreys will respond, but only if the exposure is continuous. We conclude that sea lamprey alarm cues function as a discrete source of social information that serves to prevent adults from entering habitats that are too risky. Variation in the frequency of previous exposure to conspecific alarm cues influences the probability that risk-averse behavior will be expressed. Given the strength and consistency of the response, these semiochemical(s) could provide a beneficial supplement to management of the invasive sea lamprey population within the Laurentian Great Lakes.

Introduction

Organisms combat the pervasive force of risk by obtaining information that depicts their current and future surroundings (Schmidt et al. 2010; McNamara and Dall 2010). Such information can be obtained by personal experience or by monitoring the success of others via social information (Danchin et al. 2004; Dall et al. 2005). Within aquatic environments, molecules containing information regarding opportunities (e.g. food, mates, habitat) and perils (e.g. predation) are cast into the aqueous medium and can be transmitted great distances by currents and persist over time (Wisenden and Chivers 2006; Wisenden 2008; Ferrari et al. 2010; Thiel and Breithaupt 2011). This information is available to all individuals encountering the odor plume (Atema 1996; Weissburg et al. 2002). Accordingly, natural selection favors the development of sensory capabilities sufficient to transform this information into adaptive behavioral responses (Hamdani and Døving 2007; Laframboise et al. 2007; Dall 2010; Schmidt et al. 2010). As a result, many aquatic organisms rely on chemical information to manage risk and regulate decision making across a range of temporal and spatial scales (Lima and Dill 1990; Dodson et al. 1994; Barbin et al.1998; Døving et al. 2006).

Significant effort has been dedicated to understanding how variation in the magnitude and frequency of exposure to chemosensory risk information influences the expression of risk-averse behaviors (Lima and Bednekoff 1999; Sih et al. 2000; Brown et al. 2006; Ferrari et al. 2010). For aquatic vertebrates, conspecific alarm cues released following physical damage to epidermal cells activate relatively small spatial and temporal areas. Therefore, alarm cues function as a reliable form of public information; cue concentration provides graded information regarding the probability of a predatory encounter across space and time (Dall et al. 2005; Danchin et al. 2005; Wisenden et al. 2004; 2008). Accordingly, individuals are expected to match the magnitude of antipredator behavior with alarm cue concentration while trading-off with additional life history requirements (Helfman et al. 1989; Ferrari et al. 2005; Zhao et al. 2006; Ferrari et al. 2009). Alarm cue concentration is a function of both active space and time of the chemical information. These features are important because they reflect the selection gradient acting upon sensory systems and corresponding behaviors (Ferrari et al. 2007; Wisenden et al. 2008; Wisenden 2009). Also, variation in the frequency of risks can influence the degree that animals invest in risk mediating behaviors (generally predation; Foam et al. 2005; Brown et al. 2006). According to the risk allocation hypothesis (Lima and Bednekoff 1999), risk-mediating behavior should begin to diminish following periods of high perceived risk. Thus, variation in both the magnitude and frequency of chemical information can influence the probability and intensity of risk-averse behavioral responses (Helfman 1989; Sih et al. 2000; Brown et al. 2006; Ferrari et al. 2008; 2009).

Comparatively few studies have evaluated the importance of chemically-mediated risk-assessment under ecological circumstances outside of predation (Chivers and Smith 1998; Kats and Dill 1998; Ferrari et al. 2010). The sea lamprey (*Petromyzon marinus*) undertakes a long distance spawning migration which they rely on olfactory information to identify both risks and opportunities (Binder and McDonald 2007; Vrieze et al. 2010; 2011; Wagner et al. 2011). Sea lampreys possess an alarm substance that is notable in that is relatively stable (persists after 96 h of putrefaction), found within both larvae and adults, and is held within multiple tissues, not solely the skin (Bals and Wagner 2012). These features suggest that lamprey alarm cues may serve a function broader than solely regulating decision-making under the risk of predation. For sea lampreys, the mechanism underlying the avoidance of alarm cues is less clear, because the odor can arise from three potential non-exclusive sources: (1) dead larvae, (2) dead migrants, or (3) dead spawners. These odors could indicate the risk of selecting a habitat (1) that is not conducive to the long term survival of their progeny, (2) where the probability of predatory attack is high, or (3) where mate availability is low because spawning has ceased within that tributary (Wagner et al. 2011). Lampreys also rely on chemical information to identify potential opportunities along their spawning migration. The migration does not terminate within their natal stream (Bergstedet and Seelye 1995; Waldman et al. 2008). Instead, adult lamprey select spawning streams based on the presence of odor released passively from multiple generations of stream-resident larvae (ammocoetes; Teeter 1980; Wagner et al. 2006; 2009) and following stream selection, males release a pheromone to attract ovulating females to a constructed nest (Li et al. 2002; Johnson et al. 2012).

Aquatic reproductive migrations typically rely on chemical information to locate spawning habitats (Quinn 1993; Vickers 2000; Døving and Stabell 2003; Moore and Crimaldi 2004; Døving et al. 2006; Keefer et al. 2006) rather than avoiding risky routes. The sea lamprey reproductive migration may be unique in that it is principally mediated by multiple sources of opposing chemical information regarding the success of conspecifics across life-history stages to mediate habitat selection. This strategy would allow lampreys to incorporate up-to-date information to select spawning habitat.   The sea lampreys reliance on multiple forms of opposing information may prove useful for investigating how animals value risk information to regulate habitat selection. However, the ecological value of this information has not been validated; we have not determined whether sea lampreys avoid conspecific alarm cues in a natural environment where they are exposed to a plethora of additional information (Magurran et al. 1996; Wisenden et al. 2004; Sloman and Armstrong 2002; Johnson and Li 2010). Several lamprey-specific bile acids released by larvae have been found to be attractive in the laboratory (Sorensen et al. 2005) but not in the field (Meckley et al. 2012). Here, we present the results of the first field study with the aim of identifying whether sea lampreys modify their migration path in a natural environment in response to varying dilutions of conspecific alarm cues. We also performed laboratory experiments evaluating how variation in alarm cue dilution and exposure frequency influences the avoidance response. The results shed light on the ecological function these cues serve and determine if alarm cues can be deployed as a supplement to the management of the invasive population (Wagner et al. 2011).

We performed three experiments designed to evaluate the expression of the sea lamprey alarm response in both the field and laboratory settings. We hypothesize that sea lampreys use the presence of conspecific alarm cues to avoid risky migratory routes. For the first experiment, we tested this hypothesis by releasing groups of migratory-phase lampreys downstream of a river section divided into two channels. One channel was activated with conspecific alarm cues and the other with a solvent control and equal concentrations. We predict that sea lampreys will avoid swimming into stream channels activated with conspecific alarm cues to limit the aforementioned risks. If this cue represents an antipredator adaptation, we would expect lampreys to match the intensity of their response with cue concentration. Conversely, if the cue is used a proxy for habitat quality, we would expect to observe a threshold response. The second experiment evaluated the active space of sea lamprey alarm cues collected from whole carcass and skin extracts. Because we hypothesized that sea lampreys use these odors for habitat selection over a considerably larger scale than other aquatic organisms, we would expect the active space of the chemical alarm cue to be much greater compared to species that use these odors solely for limiting predation risk (Wisenden 2008). The third and final experiment evaluated whether previous exposure to conspecific alarm cues influenced the behavioral response. We would not expect the response to attenuate following previous exposure because it would limit the ability of lampreys to obtain accurate information regarding habitat quality as they proceed upstream through risky habitats.

Materials and Methods

Experimental subjects

We acquired adult migratory-phase sea lampreys from four tributaries to Lake Huron and Lake Michigan in Michigan, USA (the Cheyboygan, Ocqueoc, Manistee, and St. Mary's Rivers) from May - July 2011. The U.S. Fish and Wildlife Service captured sea lampreys as part of the annual sea lamprey control program and transported to the subjects to the Hammond Bay Biological Station (Millersburg, Michigan, USA) where they were separated by sex and placed into 1000 L holding tanks receiving a continuous flow of fresh water from Lake Huron (100% exchange every 2 h). We held all sea lampreys at the station for at least 48 h prior to experimental use, monitored them to ensure normal behavior, and physically examined each individual for signs of physical damage. We only used robust lampreys and individuals were only used in a single trial. At the conclusion of each laboratory trial the adult lampreys were returned alive and unharmed to the U.S. Fish and Wildlife Service for final disposition. Lampreys released into the stream were not recovered. Use of fishes and all experimental procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF#######).

Alarm Cue Collection

We extracted the odor released from freshly killed adult male and female sea lamprey carcasses via soxhlet extraction (Wagner et al. 2011; Bals and Wagner 2012). The soxhlet apparatus consisted of a 1 L 71/60 soxhlet body, a six bulb water-cooled Allihn condenser and a 1 L solvent reservoir heated with a hemispherical mantle to 75^{o} – 80^{o} C. Prior to performing the extraction, we prepared 1 L of solvent (50:50 w/w solution of 200 proof ethyl alcohol and deionized water). We euthanized a single adult sea lamprey via cervical dislocation with a razor blade. We rinsed the carcass for 30 min in 150 ml of solvent prior to placing the carcass into the soxhlet body. We placed the remaining 850 ml of solvent into the solvent reservoir and loaded the rinsed carcass into the soxhlet body. We placed uncoated aluminum screen in front of the siphon inlet to prevent particulate matter from clogging the siphon and allowed the extractor to cycle three times for each extraction (approximately 6 hours). After cooling, we filtered both the extract and the rinsate, combined them and stored the final extract at -80^{o}C until use.

Field Study

Study Site

We conducted all trials in the Ocqueoc River, a tributary to Lake Huron in Northern Michigan, USA. The experimental river section is located upstream of a lamprey barrier, therefore we were only permitted to release females to prevent establishing a new population of larval lampreys above the barrier. Also, this ensured there was no background larval odor present in the system that would confound our results. Bals and Wager (2012) demonstrated no difference in the response between migratory phase male and female sea lampreys to conspecific alarm cues. We divided the stream into two equally sized stream sections using a 6.4 m block net (Fig 4.; Wagner et al. 2009; Meckley et al. 2012). We monitored lamprey movement using passive integrated transponder (PIT) tags and four fixed PIT antennas (Oregon RFID, Portland, OR, USA). Temperature in the stream ranged from $12^{o} – 24^{o}$ C.

Tagging Female lampreys

The day prior to a trial, we internally tagged 40 migratory-phase female sea lampreys with a 23 mm PIT tag (Oregon RFID, Portland, OR, USA) by making a 3 mm lateral incision along the lower abdomen below the first dorsal fin. After inserting the tag the incision was sealed with Vet-Bond tissue adhesive (###).

Alarm cue collection

We collected the odor from 74 male and female (average weight of 235 g) sea lampreys via soxhlet extraction per the procedures described above. Following extraction, the extracts were combined, separated into 1 L aliquots and stored at -80^{o}C until use in the behavioral assay. The final extract had a concentration of 0.235 g tissue per ml solvent.

Experimental procedures

Twenty hours prior (approximately 02:00) to the start of the trial we stocked 40 PIT tagged female lampreys into two metal holding cages (20 in each cage) at the downstream end of the study site. Discharge was estimated with a flow meter (Marsh-McBirney Flow Mate 2000) using the velocity-area method prior to each trial. Discharge ranged from 1.08 – 2.43 $m^3 s^{-1}$. Each trial lasted 3 hours. We began pumping either alarm cue or solvent control to activate half of the stream to the desired concentration (10^{-6}, $2x10^{-7}$, or 10^{-7}; ml stream water: ml alarm cue extract) 15 min after sunset. The other stream channel was activated with an equivalent concentration of the solvent (50:50 Ethanol:DI water). Four trials were completed per dilution. We alternated the channel receiving the alarm cue across replicates within each treatment such that each treatment received an equal number of trials (2) with the stimulus discharged on right and left sides of the channel. We applied the stimulus to the stream using a laboratory grade peristaltic pump (MasterFlex model 7533-20). The tagged female sea lampreys were released from the cages 30 min after sunset and lamprey movements across the PIT antennas were recorded using a PDA attached to a single multiplexer. Following each trial, we downloaded the data to a computer for analysis.

Laboratory study

Alarm Cue Collection

We collected the odor from one male (255 g) and one female (192 g) via soxhlet extraction per the procedures described above. Following extraction, the two extracts were combined, separated into 200 ml aliquots and stored at -80°C until use in the behavioral assay. The final extract had a concentration of 0.232 g tissue ml solvent^{-1}.

To collect the odor released from sea lamprey skin we euthanized one male (skin weight 40.4 g) and one female (skin weight 31.9 g) sea lamprey via cervical dislocation with a razor blade. After euthanizing the animal, we made an incision around the circumference of the animal posterior to the final gill opening. We removed the skin in one piece and rinsed the skin in 1 L of DI water. We ground the skin with a mortar and pestle for 15 min in 100 ml of solvent (50:50 w/w solution of 200 proof ethyl alcohol and deionized water). After filtering the extract, we diluted each extract with 900 ml for a final volume of 1000 ml. The extract from the male and female were combined, separated into 200 ml aliquots and stored at -80°C until use in the behavioral assay. The final extract had a concentration of 0.036 g tissue ml solvent^{-1}.

Test subjects

Only male sea lampreys were used for studies in the laboratory. Sex does not influence the behavioral response of migratory-phase sea lampreys to conspecific alarm cues (Bals and Wagner 2012). However, upon maturation the response attenuates in females but not males (Bals and Wagner 2012). Using male sea lampreys throughout laboratory study would prevent maturation level from confounding our results.

Apparatus

All trials took place at night in two linear concrete laboratory raceways per the general procedures of Wagner et al. (2011) and Bals and Wagner (2012). The experimental section of each raceway was 5.0 m long and 1.84 m wide. We lined the bottom with white fiberglass to facilitate detection of lampreys against the background and illuminated each experimental section using two infra-red light arrays mounted directly over each experimental section and recorded lamprey movements using a single IR-sensitive video camera. The raceways received a continuous flow of water pumped directly from Lake Huron where the temperature ranged from 7° - 16° C over the course of the experiments. We maintained discharge at 0.01 m^3 s^{-1} to simulate water currents in the lake. Discharge was estimated with a flow meter (Marsh-McBirney Flow Mate 2000) using the velocity-area method prior to each night. In order to simulate a natural day-night schedule we dimmed the lights of the room at 19:00 leaving a large window and the end of the raceway uncovered while natural light diminished. After 90 minutes we rendered complete darkness by covering the window. The first trial began at 22:00. A single trial lasted for 30 min and consisted of a 10 minute pre-stimulus period and a 20 minute stimulus period when we introduced the odor. We held lampreys for subsequent trials upstream of the experimental section in holding cages. We observed lamprey movements in an adjacent room on video monitors and recorded their activity onto digital media. To analyze lamprey distributions, we recorded the position of each subject every 30 sec after the start of a trial by replaying the video and assigning each lamprey to one side of the experimental arena based on the position of its head. We analyzed sea lamprey distributions during the final ten minutes of the stimulus period to obtain a mean proportion of animals on the stimulus side of the raceway for each trial. The pre-stimulus period was used as an acclimation period and the first ten minutes of the stimulus period was not analyzed to provide time for the distribution of the lampreys to stabilize after introduction of the stimulus.

Experiment 1. Dilution

Prior to introducing the odor into the raceway, we mixed 0.08 – 80 ml of a stimulus odor into 400 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 during release. We introduced the test odor/lake water mixture into one side of each raceway at a rate of 15 ml min^{-1} via laboratory-grade peristaltic pumps (MasterFlex model 7533-20). We introduced both the whole carcass and skin extracts at 4 dilutions increasing in order of magnitude from 10^5:1 – 10^8:1 (ml stream water: ml extract). An additional treatment of $2x10^{-7}$ was run for whole carcass extract. The solvent was pumped into the raceway at a concentration of 10^{-5} as a control (N = 10 trials for all dilutions). To ensure no cross-contamination of odors we used a separate set of pump tubing for each stimulus odor. Following each treatment we thoroughly cleaned the tubing by pumping 100% ethanol for ten minutes at a rate of 15 ml min^{-1}.

Experiment 2. Previous Exposure

To determine if previous exposure influenced the likelihood sea lamprey would respond to alarm cues we proceeded via the pre-described general procedures. We diluted 8 ml of sea lamprey extract into 400 ml of lake water and pumped at a rate of 15 ml min^{-1} (dilution of 10^{-6}; ml water: ml alarm cue). We held the holding cages within the odor plume for a desired time (0, 60, 120, 240 or 480 min). Once the time had been reached, we released the lampreys into the raceway and recorded the behavior for 20 min. We did not include a pre-stimulus period for this set of trials because we were aiming to see if the response was maintained following continuous exposure.

Experiment 3. Previous Exposure and removal

Once we determined a time at which sea lampreys stopped responding to the odor we aimed to examine whether or not the response returned. We exposed sea lampreys to alarm cues for four hours via the methods described above. We then removed the animals from the odor plume and held them in water lacking alarm cues for 0, 30, 60 and 120 min. Once the desired time was reached we proceeded with a 20 min trial.

Experiment 4. Extended laboratory assays

We wanted to know if lampreys would continually avoid alarm cues if they repeatedly encountered the stimulus over the course of a night. We conducted raceway trials using the described methods except each trial lasted for five hours (22:00 – 03:00). We introduced the alarm cues at a dilution of 10^{-6} throughout the length of the trial. We analyzed every other ten minute period over the course of five hours.

Analysis

Field Assays

We analyzed channel choice and proportion of animals swimming up to the PIT antennas of sea lampreys by fitting a main effects two-way multivariate analysis of variance (MANOVA) with the mean proportion of respondents (animals that swam upstream into the experimental section) entering the stimulus channel and the proportion of the released animals swimming up to the PIT antennas as response variables and alarm cue dilution and stimulus side (left or right channel) as fixed factors in the model. We transformed the proportions with an arcsine (square root) transformation and tested for normality using Shapiro-Wilk's test (all data met the assumptions required for hypothesis testing, all $W > .95$, all $P > 0.05$). The same data transformation and normality test was also used for the laboratory experiments. We used Tukey's HSD ($\alpha = 0.05$) to examine differences in repellency between the solvent control and dilutions of sea lamprey alarm cue. All statistical analyses were performed using SAS (v 9.2).

Laboratory Assays

Experiment 1. Dilution. To determine the dilution of sea lamprey alarm cue that repelled migratory phase sea lampreys, we performed a two-way analysis of variance (ANOVA) with the proportion of animals on the stimulus side of the raceway as the response and alarm cue dilution and source (skin or whole carcass) as fixed factors in the model. We performed ten trials of each dilution and use Tukey's HSD ($\alpha = 0.05$) to examine differences between concentrations.

Experiment 2. Previous exposure. To determine if sea lampreys stopped responding to the alarm cue after multiple pre-exposure times we performed a one-way ANOVA with the proportion of animals on the stimulus side of the raceway as the response and the pre-exposure time as a fixed factor. We performed ten trials using each pre-exposure time (0 min, 60 min, 120 min, 240 min, and 480 min). We used Tukey's HSD ($\alpha = 0.05$) to examine differences between the pre-exposure times.

Experiment 3. Previous exposure and removal. After determining that sea lamprey would stop responding to alarm cues we were interested in examining whether or not the response would return once the animals were removed from the odor. After exposing the lampreys to the odor for four hours we held them out of the odor for four time periods (0 min, 30 min, 60 min, and 120 min). We used Tukey's HSD ($\alpha = 0.05$) to examine differences between the four time periods.

Experiment 4. Extended trial. We performed a two-way repeated measures ANOVA with mean proportion of animals on the stimulus side of the raceway as the response variable and stimulus type (ethanol and alarm cue) as a fixed factor and time period as a repeated measure.

Results

Field Experiment

The model (MANOVA, $F_{4, 10} = 35.79$, $P < 0.001$) indicates that the concentration of odor pumped into the channel significantly influences channel choice by migratory lampreys within a stream (Type III SS = 1.28, $F_{3, 11} = 44.38$, $P < 0.001$). The side the alarm cue was being administered did not influence channel selection (Type III SS = 0.03, $F_{1, 10} = 3.02$, P = .1151). Tukey's post-hoc comparison indicates that lampreys avoided the channel activated with alarm cue when the dilution was 10^{-6} and $5x10^{-6}$ but not 10^{-7} (Fig. 5; treatments sharing letters were not different using Tukey's post-hoc test; $\alpha = 0.05$). The model (MANOVA, $F_{4, 10} = 2.27$, P = 0.1341) also suggests that neither alarm cue concentration (Type III SS = 0.226, $F_{3, 11} = 2.99$, P =

0.083) or side of application (Type III SS = 0.002, $F_{1, 10} = 0.06$, P = 0.81) influenced the proportion of animals swimming up to the PIT antennas (Fig 5).

Laboratory Experiments

Experiment 1. Dilution. The model (ANOVA, $F_{9,99} = 8.95$, P <0.001) suggests that the dilution of odor pumped into the raceway significantly influenced the likelihood animals would swim within the stimulus side (Type III SS = 1.0096, $F_{5, 99} = 14.76$, P < 0.001; Fig. 6, treatments sharing letters were not different using Tukey's post-hoc test; $\alpha = 0.05$). While stimulus source (whole carcass or skin; Type III SS = 0.07, $F_{1, 99} = 2.97$, P = 0.08) and the concentration by source interaction (Type III SS = 0.087, $F_{3,99} = 1.21$, P = 0.031) did not significantly influence the mean proportion of lampreys on the stimulus side of the raceway.

Experiment 2. Previous Exposure. The model (ANOVA, $F_{4,45} = 12.77$, P <0.001) indicates that the time of previous exposure influences the likelihood that lampreys will respond to conspecific alarm cues. Tukey's post-hoc comparisons indicate that lampreys held within the alarm cue for 60 min and 120 min exhibit a response equivalent to no pre-exposure. However, after 240 min lamprey no longer avoided the area of the raceway treated with conspecific alarm cues (Fig. 7A; treatments sharing letters were not found to be significantly different using Tukey's test; α = 0.05).

Experiment 3. Previous Expsoure. The model (ANOVA, $F_{4,45} = 13.35$, P <0.001) suggests that after lampreys have been immersed in alarm cues for four hours the time since they have experienced the stimulus strongly influences the probability they will respond. Results of Tukey's post-hoc test indicates that lampreys do not respond after being removed from the odor for 30 min, however the response returns after 60 min of being held in water void of the odor (Fig. 7B; treatments sharing letters were not found to be significantly different using Tukey's test; $\alpha = 0.05$).

Experiment 4. Extended trial. The model (ANOVA, $F_{27, 196} = 9.01$, $P<0.001$) suggests that only stimulus type ($F_{1, 196} = 231.65$, $P<0.001$) significantly influences the probability of lampreys occupying one side of the raceway while time ($F_{13, 196} = 0.3$, $P = 0.9918$) and the odor type*time interaction ($F_{13, 196} = 0.6$, $P = 0.8504$) does not. Lampreys avoided the odor throughout the duration of trial (Fig. 8).

Discussion

This is the first demonstration of an aquatic vertebrate restricting their migration route in response risk-indicating chemical information. Lampreys did not display threat-sensitive behavior proportional to the magnitude of risk (alarm cue concentration) in the stream bioassay (Fig 3). The apparent all-or-nothing response indicates that, in the absence of additional chemical information, alarm cues function as a discrete source of social information. A concentration above a threshold disqualifies a migration path as too risky (Dall et al. 2005; Doligez et al. 2003). Lampreys are expected to display risk-averse tendencies because individuals that select tributaries lacking suitable larval habitat, are consumed by a predator, or cannot find a mate will relinquish their entire fitness. Similarly, lampreys are reluctant to swim in waters lacking larval odor when presented with the option, a behavioral tactic which prevents adults from selecting spawning tributaries lacking suitable larval habitat (Bjerselius et al., 2000; Wagner et al. 2006; 2009). Generally speaking, aquatic animals undertaking long-distance spawning migrations rely on chemical cues as a guide to breeding grounds (Quinn 1993; Baker and Hicks 2003; Moore and Crimaldi 2004; Debose and Nevitt 2008; Stabell 2012). Sea lamprey possess a potentially novel system in which habitat selection during a spawning migration is regulated by multiple sources of inadvertent social information representing both risk and opportunity that are perceived over a single sensory modality. Such plasticity increases the probability of spawning success by allowing individuals to incorporate up-to-date information regarding habitat quality and the success of conspecifics (Van Buskirk and Relyea 1998; Agrawal 2001).

Contrary to the field assay, lampreys displayed a graded increase in the intensity of avoidance behavior proportional to cue concentration in the laboratory (Fig. 6). This is typical of most aquatic species, as alarm cue concentration represents both the spatial and temporal distance from the source of risk (Ferrari et al. 2005; Zhao et al. 2006; Mirza et al. 2006; Ferrari et al. 2009; Ferrari et al. 2010). In a recent review, Ferrari et al. (2010) identified seven studies evaluating the behavioral response of aquatic organisms to varying concentrations of alarm cues in which the tests subjects displayed a response proportional to the concentration of alarm cue (Jachner and Rydz 2002; Ferrari et al. 2005; Mirza et al. 2006; Zhao et al. 2006; Kesavaraju et al. 2007; Ferrari et al. 2008; and Ferrari et al. 2009). We would have reached the same conclusion had we not investigated the response in the natural setting. Bals and Wagner (2012) mapped the odor plume within these raceways and found the plume was not evenly distributed across the stimulus half of the raceway. The odor was much more concentrated along the near wall than towards the center. Our dilution value represents the average across the stimulus half of the raceway, however, the cue was not evenly mixed. The more concentrated odor activates a larger proportion of the raceway above the detection threshold and likely accounts for increasing repellency with concentration. The heterogeneous concentration gradient across the stimulus half of the raceway could also explain discrepancies in sensitivity between field and laboratory studies. The dilutions of $2x10^{-7}$ and 10^{-7} displayed equivalent repellency in the laboratory (Fig. 6) but dramatic difference in the field (Fig 5). Although the laboratory experiment does not fully encapsulate the nature of the field response, it is useful for identifying odors which lampreys avoid and will be an essential tool for identifying the active components of the sea lamprey alarm cue (Bals and Wagner 2012). This is the first study evaluating behavioral response of aquatic organisms to a gradient of alarm cue dilutions in both laboratory and field settings and highlights the importance of replicating experiments under the natural conditions which the behaviors evolved (Ferrari et al. 2010; Johnson and Li 2010).

For such behavioral flexibility to be an adaptive strategy the cues must contain reliable information (Agrawal 2001; Bonnie and Earley 2007; McNamara and Dall 2010). It is clear that larval odor and the mating pheromone provide information regarding habitat suitability and mate location respectively (Li et al. 2002; Wagner et al. 2009). What information are lampreys gleaning from the odor emanating from dead conspecifics? For most species, behavioral responses to alarm cues are believed to be antipredator adaptations. When an individual is attacked by a predator, specialized epidermal club cells (Pfieffer 1962; 1977; but see Carreau-Green et al. 2008) are ruptured and release a cue that alerts nearby conspecifics or closely related heterospecifics of a predation risk over a relatively small area and a short amount of time (less than 6 h; Ferrari et al. 2007; Ferrari et al. 2008; Wisenden 2008). The lamprey alarm cue is notably different from other species because active component(s) remain repellant after 96 h of aerobic decay of a lamprey carcass, are present within multiple tissues (Bals and Wagner 2012), and activate a space much larger than that of ostariophysan fishes. For instance, Wisenden (2008) demonstrated that 2 cm^2 of skin collected from fathead minnows (*Pimephales promelas*), northern redbelly dace (*Phoxinus eos*), and brook sticklebacks (*Culaea inconstans*) activates an area with a radius of approximately 2 m (12 560 L). For comparison, 1 g of sea lamprey skin activates at least 285 700 L of water (Fig. 6). Active space of a chemical cue is important because it reflects the selection gradient acting upon sensory systems and corresponding behaviors. Active space is determined by cue concentration, receptor sensitivity, and social information (Lawrence and Smith 1989; Stacey and Sorensen 2002; Wisenden 2008). Siefkes et al. (2005) demonstrated that migratory movements are independent of other lampreys. Thus it appears sea lampreys have evolved either (1) greater receptor sensitivity to conspecific alarm cues or (2) the semiochemical(s) are more concentrated within the lamprey tissue compared to other fishes. These attributes suggest a function broader than the identification of a predator (Wisenden 2008).

Wagner et al. (2011) proposed that lampreys may have evolved to evade these odors to reduce the risk of depositing their offspring in habitats with high larval mortality or entering a tributary where the majority of spawning adults have perished and mate availability is low. Both scenarios would place strong selective pressures on lampreys to evolve greater sensitivity to an odor that is long-lived and present within multiple tissues. Our interpretation of active space and naturally plausible concentrations of sea lamprey alarm cue is ultimately limited because we are unaware of the semiochemical(s) responsible for eliciting this behavior. Lamprey spawning aggregations within tributaries can consist of thousands of animals in a short spawning window and following death lamprey carcasses tend to drift downstream less than one mile and accumulate within deep pools (Applegate 1950; Wigley 1959). Also, larval lamprey densities can reach up to hundreds per m^2 under ideal conditions and significant numbers of dead ammocoetes have been found in the spring during the migratory season (Manion and Smith 1978; Morman 1979; Murdoch et al. 1992). Significant amounts of odor could emanate from the sediments in areas with high over-winter mortality of larvae or large senescing mating aggregations.

The temporal and spatial scales at which sea lamprey require information likely attributes to discrepancies displayed between the sea lamprey alarm response and that of other aquatic organisms. For non-migrating species, persistent chemical information released from dead or injured conspecifics hundreds of meters upstream may not be a reliable indicator of risk, and responding to such information could result in lost foraging or reproductive opportunities (Lima and Bednekoff 1999; Sih et al. 2000; Brown et al. 2006; Wisenden 2008). Sea lampreys are constrained by a unidirectional migration upstream through shallow lotic habitats where they become vulnerable to shoreline predators (Applegate 1950; Cochran et al. 1992). Early detection of an upstream threat would allow lampreys to adjust the spatial and/or temporal course of their migration in order to minimize the risk. Even if a lamprey detects a significant risk upstream they are still going to confront the source, as alarm cue concentration did not significantly influence the proportion of animals swimming upstream (Fig 5). Confronting a predator in a confined stream may come with dire consequences. Conversely, approaching the confluence of a tributary with high larval mortality or ceased spawners would lack immediate risk. Migrating sea lampreys will delay upstream progress for several weeks while staying in one location (Almeida et al. 2002). During the stream assay, lampreys were confronted with a continuous source of alarm cues originating from upstream. These circumstances do not mimic conditions of a predatory attack, which are ephemeral threats; rather they are consistent with slow release from large quantities of dead larvae or spawners. A compelling question remains. Would lampreys abandon upstream movement if the entire river was activated with conspecific alarm cues?

Variation in frequency of previous exposure does influence the probability lampreys will respond, but only if the exposure is continuous. In the laboratory, repellency began to diminish after 120 min and was fully attenuated following 240 min of continuous exposure (Fig. 7A). The tempered behavioral response following extended exposure can be attributed to either the saturation of sensory receptors or learning/adaptive risk assessment by the organism (Lima and Bednekoff 1999; Ferrari et al. 2009; Wong et al. 2010), although the latter is unlikely because lampreys continuously avoided the odor during 5 h of repeated exposure over the course of one night (Fig. 8). These findings support the risk allocation hypothesis, following prolonged periods of high perceived risk the expression of risk-averse behavior diminished. Traditionally, this model examines tradeoffs between anti-predator behaviors and foraging opportunities. However, sea lampreys cease feeding prior to onset of migration. Sea lampreys may show decreased avoidance behavior following periods of prolonged risk exposure in order to continue their upstream migration.

Does habituation limit an individual's ability to obtain information? The attenuation of the response may be an adaptation to conditions typical of spawning streams late in the migration.

As the spawning season progresses, the condition of spawning individuals rapidly deteriorates as abrasive spawning behavior removes skin and lamprey carcasses will accumulate around spawning sites (Applegate 1950; Wigley 1959). At this time, the cue may become uncoupled with the source of risk if it provides information regarding larval survival or predation. Also, habituation may be a mechanism to allow spawning to proceed uninterrupted. We were working with concentrations that would be considered relatively ‘high’ (Fig. 6) which could have resulted in receptor saturation faster than what would naturally occur. Our goal was to determine if sea lamprey would habituate to the odor. The response returned after only 60 min of removal from the plume and lampreys consistently avoided the odor following repeated exposure over the course of an entire night (Fig. 7B; Fig. 8). So, even though lampreys stopped responding to the odor, there may not be a natural circumstance in which they will remain within a plume long enough to become habituated (Fig. 8). The return of the response following habituation and repeated avoidance following sequential exposures would allow lampreys to subsequently sample multiple tributaries and gain accurate information regarding habitat quality even after multiple exposures to the odor. Cane toad tadpoles (*Bufo marinus*) display a similar pattern following continuous exposure to conspecific alarm cues. Behavioral responses to the odor of crushed conspecifics began to diminish after 120 min and were not detectable following 240 min of repeated exposure (Hagman and Shine 2009). Whether the similarities are a result of convergent selection pressures and/or similarities in the olfactory receptors is not known.

Sea lampreys are an invasive parasite within the Laurentian Great Lakes and have contributed to dramatic declines in native and commercially valuable fisheries (Smith and Tibbles 1980; Eshenroder et al. 1992). Consequently, they are subject to an extensive integrated pest management strategy that relies on a combination of physical and electrical barriers to limit the distribution of spawning adults, trapping to remove adults from streams, and lampricides to dispatch stream-resident ammocoetes (Brege et al. 2003; Lavis et al. 2003; Jones 2007; Li et al. 2007). The sea lampreys reliance on tractable chemical information makes them vulnerable to behavioral manipulation for management purposes. Repellents (alarm cues) could be used to exclude lampreys from tributaries and attractants (larval odor and mating pheromone) to lure them into others (“Push-Pull” approach; Miller and Cowles 1990; Cook et al. 2007; Wagner et al. 2011). In turn, this would produce a greater number of lampreys over a smaller area; reducing the reliance on barriers and increase the efficiency of trapping and lampricide applications. The alarm cue could also be used to ‘plug’ barriers rendered temporarily porous such as locks and dams or deteriorating lamprey barriers.

The characteristics of a chemical barrier make the application a beneficial alternative to traditional barriers for multiple reasons (Noatch and Suski 2012). First, application of alarm cues would not disrupt flow of water or navigation. Second, the barrier is not permanent and can be relocated or shut off either seasonally or annually as adaptive management strategies change. Third, the application of odors does not pose a risk to the health of humans or other terrestrial animals susceptible to electricity or pesticides. Finally, physical and electrical barriers are not species specific and prevent any organism from passing, while reactivity to heterospecific alarm cues is generally a function of phylogenic relatedness between species (Huryn and Chivers 1999; Mirza and Chivers 2001; Dalesman et al. 2007; Turner 2008; Ferland-Raymond and Murray 2008; Bals and Wagner 2012).

In summary, we have demonstrated that sea lamprey conspecific alarm cues function as a discrete source of social information that serves to prevent adults from entering habitats that are too risky. Variation in the dilution and frequency of previous exposure to conspecific alarm cues influences the probability that risk-averse behavior will be expressed. The nature of that risk is currently undetermined because the odor can arise from three non-exclusive scenarios that would be detrimental to the fitness of migrating lampreys. We now know that migratory lampreys regulate their use of space in response to multiple olfactory cues providing information regarding success across life history stages. Future studies can begin to discern how lampreys use multiple forms of inadvertent social information to regulate habitat selection along the course of a terminal spawning migration. Given the strength and consistency of the response (>99% repellency at the highest concentration) these semiochemical(s) could provide a beneficial supplement to current sea lamprey management practices. Ultimately, ecological inquiries and the management utility will hinge on our ability to identify the active chemical components of the various cues sea lamprey rely on to guide their migration.

Acknowledgements

We are grateful to the staff at Hammond Bay Biological Station for providing access to the laboratory raceways and for creating an excellent research environment. Eric Stroud developed the protocol for Soxhlet extraction. Skye Fissette, Greg Byford, Sarah Ptasznik, Carrie Kozel, and Trevor Meckley provided assistance in the laboratory. Funding for this project was provided by the Great Lakes Fishery Commission.

APPENDIX

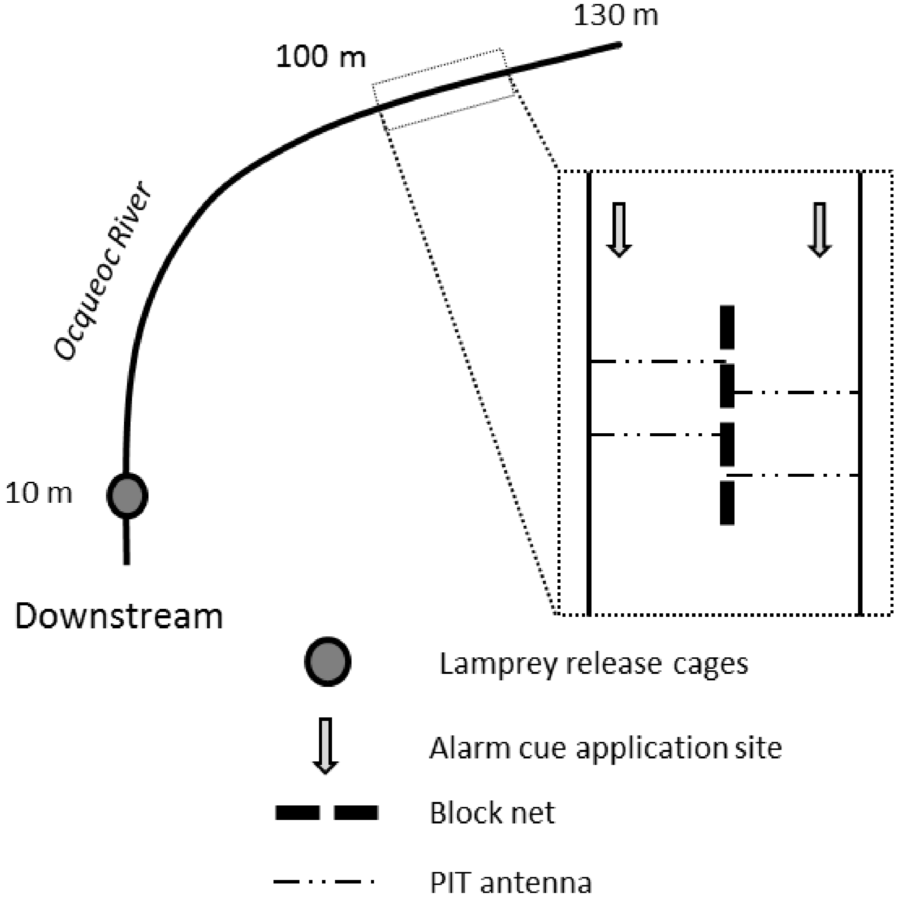

Figure 4. Diagram of Ocqueoc River field site. Sea lampreys were held in cages approximately 100 m downstream of the experimental arena. Lamprey movements were monitored with paired Passive Integrated Transponder (PIT) antennas as they swam upstream past the alarm cue application sites. Alarm cue was introduced 3 m above the most upstream PIT antenna. All distances displayed as distance upstream from the most downstream most point of the site.

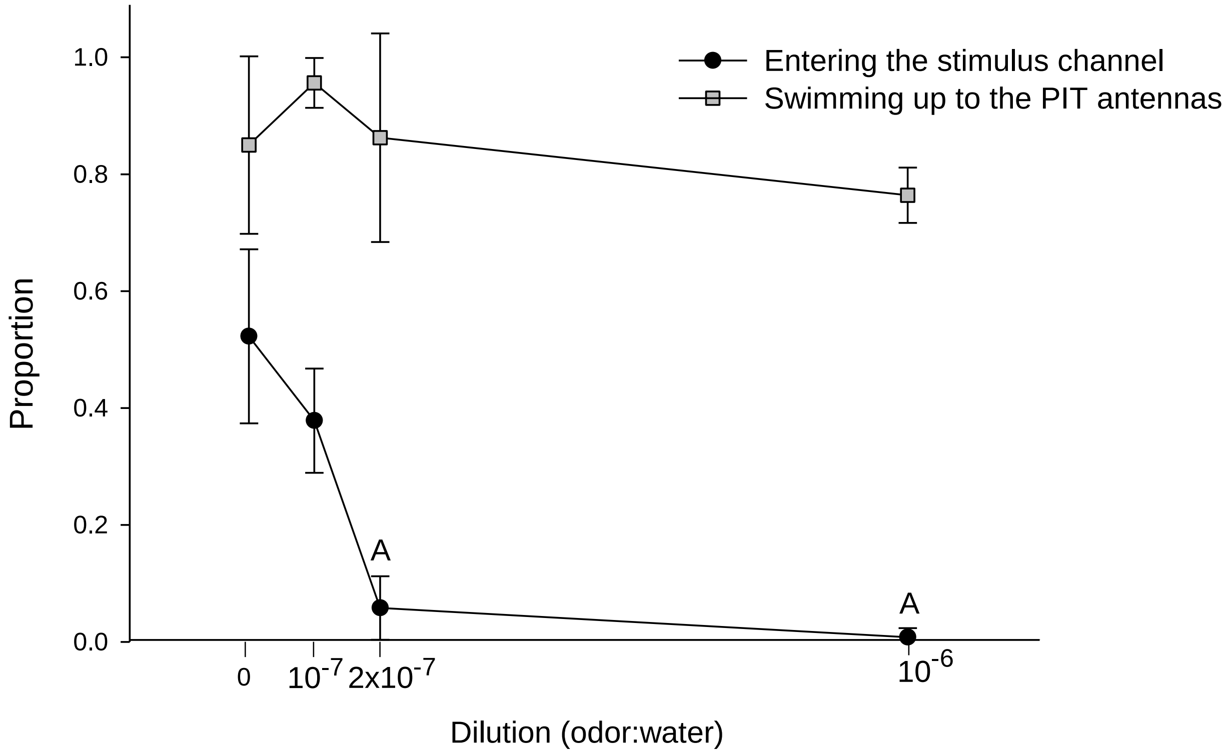

Fig. 5. (A) Mean (± 2 se) proportion of migratory female sea lampreys entering the stimulus channel (black) or swimming up to the PIT antennas (gray). Treatments sharing letters are not found to be significantly different using Tukeys HSD ($\alpha = 0.05$). N=4 for each point. The analysis was performed on data that were (A) arcsine (square root) or but are displayed as the observed proportions. The extract had an average concentration of 0.235 g tissue ml of solvent^{-1}.

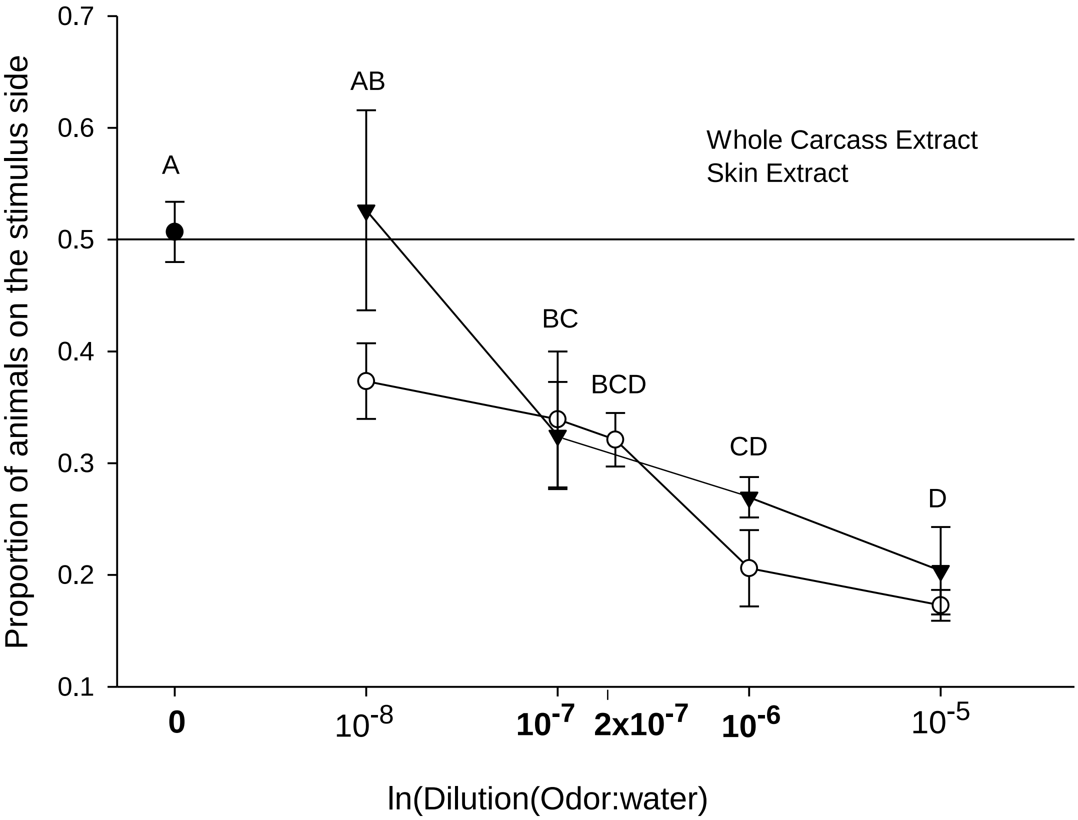

Fig. 6. Mean (± 1 se) proportion of migratory male sea lampreys on the stimulus side of the raceway after exposure to conspecific alarm cues collected from whole carcass extracts (soxhlet extraction) and ground skin extract. Treatments sharing letters are not significantly different. Treatments sharing letters are not found to be significantly different using Tukeys HSD (α = 0.05). The N=10 for each point. The analysis was performed on data that were arcsine (square root) transformed but are displayed as the observed proportions. Dilutions that are bolded were also used in the field assay. The whole carcass and skin extracts consisted of 0.232 and 0.036 g tissue ml of solvent^{-1} respectively.

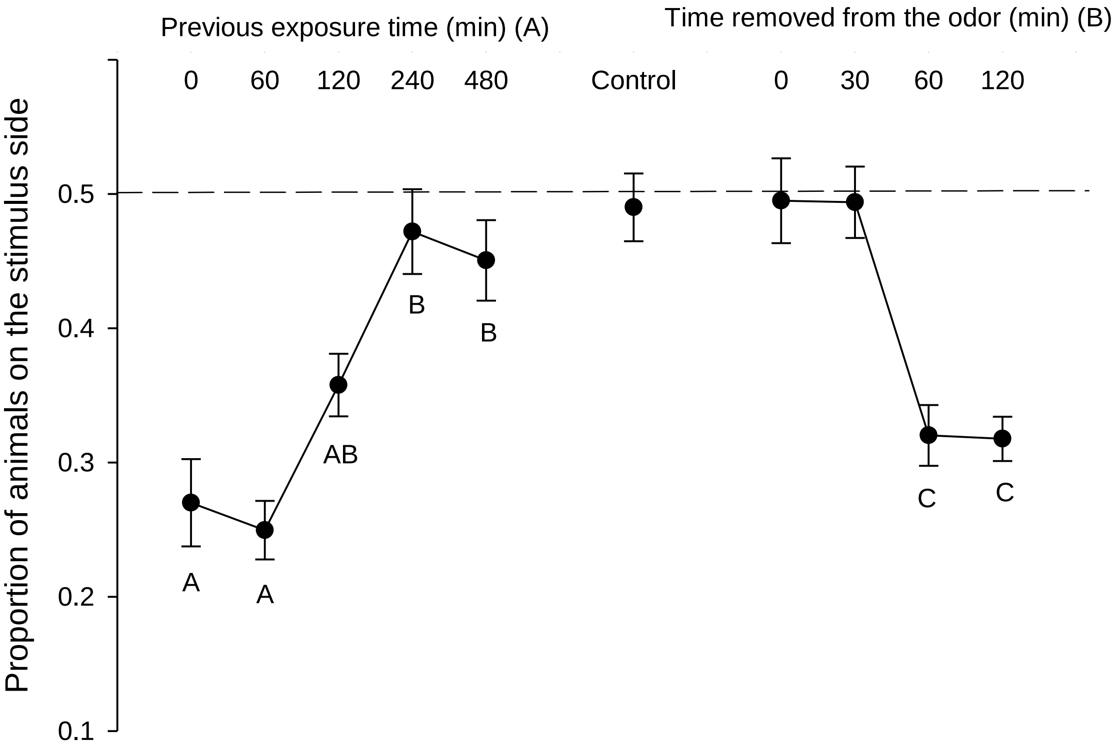

Fig 7. (A) Mean (± 1 se) proportion of migratory male sea lampreys on the stimulus side of the raceway after exposer to conspecific alarm cues following a prior exposure time. (B) Mean (± 1 se) proportion of migratory male sea lampreys on the stimulus side of the raceway after exposer to conspecific alarm cues at a dilution of 10^{-6} following a prior exposure time of four hours and a subsequent time removed from the odor. Treatments sharing letters are not found to be significantly different using Tukeys HSD (α = 0.05). The analysis was performed on data that were arcsine (square root) transformed but are displayed as the observed proportions.

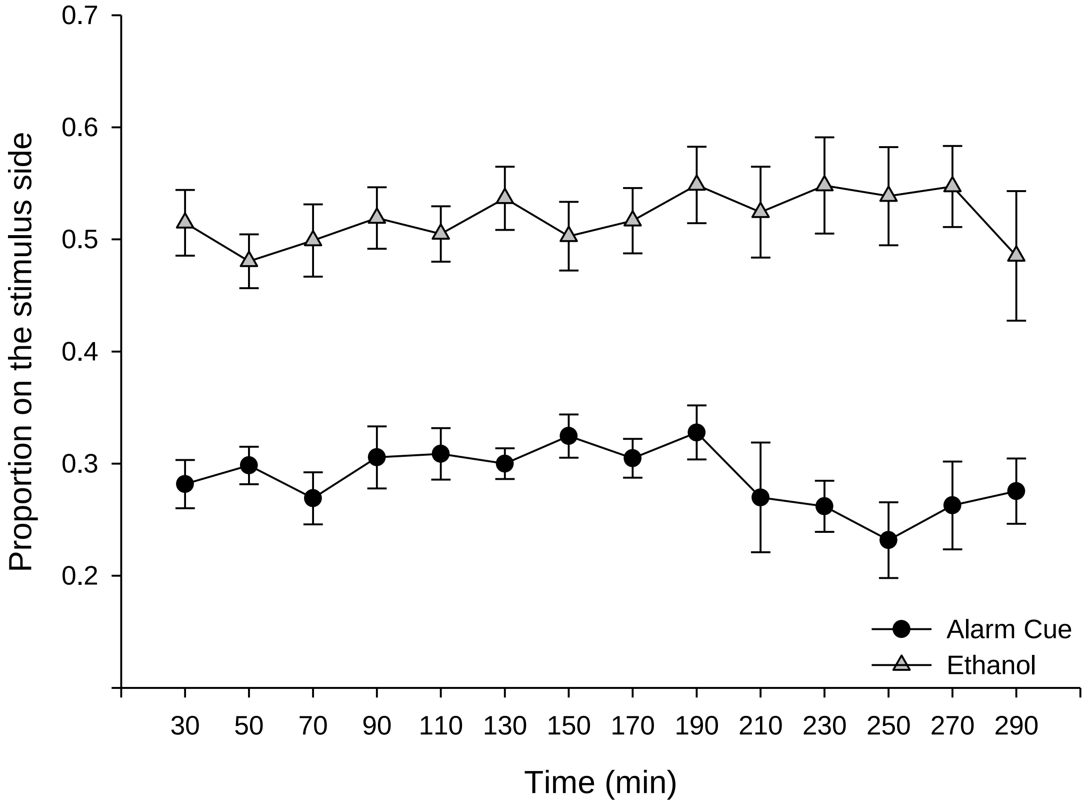

Fig. 8. Mean (± 1 se) proportion of migratory male sea lampreys on the stimulus side of the raceway after exposer to conspecific alarm cues and a stimulus control over the course of five hours (N = 10). The proportion of animals on the stimulus side was significantly different between ethanol and alarm cue applications and the response was not influenced by time. The analysis was performed on data that were arcsine (square root) transformed but are displayed as the observed proportions.

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