MAKING A REPELLENT:
OVERCOMING PHYSIOLOGICAL IMPEDIMENTS TO GUIDING MIGRATORY SEA LAMPREY (*PETROMYZON MARINUS*) WITH AN ALAM CUE

By

Mikaela E. Hanson

A THESIS

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

Fisheries and Wildlife—Master of Science

2021

ABSTRACT

MAKING A REPELLENT:
OVERCOMING PHYSIOLOGICAL IMPEDIMENTS TO GUIDING MIGRATORY SEA LAMPREY (*PETROMYZON MARINUS*) WITH AN ALAM CUE

By

Mikaela E. Hanson

This thesis examined alarm cue application techniques to prevent habituation from occurring in sea lamprey (*Petromyzon marinus*), offering insights to sea lamprey's behavioral response to alarm cue in context of its use as behavioral management tool. Semiochemicals like alarm cues have the potential to be utilized by managers as behavioral tool as they can guide an animal's response and movement. Alarm cues are released from damaged tissues when an animal is injured, such as an attack, warning nearby conspecifics of a predation event. Sea lamprey, a species of management concern in the Laurentian Great Lakes, respond to their alarm cue with spatial avoidance and increased swimming speed, moving out of the affected area. Previous work has indicated that sea lamprey habituate to their alarm cue within four hours of continuous exposure. To examine habituation prevention, application techniques tested included modulating the strength of the alarm cue and the interval at which it was applied (i.e., pulsing the alarm cue). Overall, we found that the presence of alarm cue increased upstream movement and sea lamprey with slower upstream movement avoided the alarm cue side. This suggests the more time spent gathering information about the alarm cue, the better sea lamprey were able to avoid the predation risk. The application that had the best implications for management was a pulse conditioning treatment that altered alarm cue dilutions between 1 ppm and 10 ppm, every 15 min. After 4h of conditioning to this treatment, sea lamprey maintained their response to the alarm cue. A continuous pulsed treatment is practical for management purposes as the guiding odor, alarm cue will be continuously present in the system for sea lamprey to detect and respond to.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. C. Michael Wagner for his dedicated guidance, mentorship, and support. I would also like to thank my committee members, Dr. Brian Wisenden and Dr. Heather Eisthen for their expertise, feedback, and support. I would like to thank the United States Geological Survey at Hammond Bay Biological Station (HBBS) and the United States Fish and Wildlife Service for their assistance in animal collection and animal housing, use of facilities, maintenance, and support for these projects. I would especially like to thank the personnel at HBBS for installing safety measures during the 2020 COVID Pandemic and allowing us to collect data at HBBS. Additionally, I would like to thank members of my lab for their support of this project and data collection during the 2020 field season: Kandace Griffin, Emily Mensch, Taylor Haas, and John Hume. I would also like to thank Matt Zink and Tom Luhring for their help in R and statistical models built in this thesis. Thank you to Taylor Rupp for her help and guidance in BORIS, the behavioral software. Huge thank you to the technicians who helped to collect this data, Madison Perry, Kris Rygiel Anna Tursky, Emily Suominen, Grace Forthaus, and Tyler Welklin. I would also like to thank my friends and family for their continued support during this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Parasitic feeding by invasive sea lamprey (*Petromyzon marinus*) has been one of the greatest risks to native fish populations and the fishery-based economy surrounding the Laurentian Great Lakes. Sea lamprey are an external parasite that feeds on the blood and tissues of their host. Sea lamprey start their life as filter-feeding larvae buried in the sediment, which can last 3-10 or more years (Potter, 1980; Purvis, 1980). After this stage, they metamorphose (June-March) and out-migrate to lakes to feed on their host fish as a parasitic juvenile (12-18 months) (Renaud, 2011). From March through July, migratory phase (here after referred to as *sub-adult*) sea lamprey migrate upstream to tributaries to spawn (Larsen, 1980). Adult sea lamprey die shortly after spawning. Sea lamprey have contributed to the significant decline in native fish populations, especially Lake Trout (*Salvelinus namaycush*), which were nearly extirpated in the Great Lakes (Hansen et al., 2016; Siefkes, 2017). Not only does this impact have substantial ecological impacts, but loss of large game species also affects fishing-based industries and tourism worth $7 billion dollars annually to the regional economy (GLFC, n.d.). Sea lamprey populations in the Great Lakes are managed by the Great Lakes Fisheries Commission (GLFC) in partnership with government organizations from the U.S. and Canada as part of the Sea Lamprey Control Program.

The GLFC and its stakeholders have identified a need for more efficient management practices to control sea lamprey populations. The most widely used technique to control sea lamprey populations is the application of a lampricide, 3-trifluoromethyl-4-nitrophenol (TFM). TFM targets the larval life stage of sea lamprey in river systems. The larval stage is targeted for control as sea lamprey are concentrated in stream systems and have yet to metamorphose to feed on host fish (Siefkes, 2017). While the application of TFM has been effective in significantly reducing sea lamprey populations, its social license, acceptance of the practice by the stakeholders and the public, is lessening. TFM can affect non-target organisms such as foraging fish, young of the year, and olfactory responses in fishes (Dahl & McDonald, 2011; Sakamoto et al., 2016). Additional control techniques include barriers, such as dams to block upstream movement for spawning adults. However, barriers can also pose problems for non-target fishes as they can decrease connectivity in aquatic ecosystems (Siefkes, 2017). There is a need for alternative management tactics that could increase the effectiveness of the current sea lamprey control program, reducing the use of TFM and increasing connectivity in rivers.

An example of an emerging management technique that requires further exploration is behavioral manipulation through the application of attractants or repellants. This 'attract' or 'repel' information is detected by the animal via a sensory systems (i.e. visual, audio, olfactory, tactile, taste), processed and responded to (Greggor, Berger-Tal, & Blumstein, 2020). As animals use this sensory information to guide their behavioral responses, managers can apply the same information in the field to guide animals' responses in a favorable outcome (attract = towards, repel= away).

Sea lamprey heavily rely on attract and repel sensory information, especially during their spawning migration. Sea lamprey select (attract) spawning streams through the detection of larval odor (Sorensen et al., 2005) and the male sex pheromone (Li et al., 2002). Additionally, during their upstream migration, they must avoid the risk of predation as sea lamprey migrate into a narrower, shallow stream from a large, deep lake. To help evade this risk, sea lamprey migrate nocturnally, decreasing their risk of predation. They also detect and respond to a repellant, alarm cue. Alarm cues are released from damaged tissues of conspecifics and can signal a predation event. Through the application of attractants (larval odor, sex pheromone) and repellants (alarm cue) we may be able to manage sea lamprey more effectively as the detection of this information guides their behavioral response and ultimately directional movement.

The repellant, alarm cue may be an effective alternative management tactic. An alarm cue is a chemical mixture that is inadvertently released from damaged tissues when an animal is injured, such as an attack from a predator. This cue is public information and benefits only the receiver, conspecifics of a risk, not the one sending the information. When exposed to alarm cue, sea lamprey avoid the area where the cue is present and increase their swimming speed to move out of the area (Hume et al., 2015; Di Rocco et al., 2016; Luhring et al., 2016). As sea lamprey actively avoid areas activated with alarm cue, the cue may be used to guide them to a trap, a designated area for TFM application, or away from a selective fish passage device designed for native fish movement (Hume et al., 2015; Hume, Luhring, & Wagner, 2020).

As sea lamprey migrate during the nocturnal period, the ability to maintain the effect of the alarm cue response for 8-9 continuous hours will be necessary to maximize the number of individuals directed towards a targeted area. Long-term (24 h) conditioning studies have demonstrated a preserved response to alarm cue and predator odors (Imre et al., 2016). However, short-term conditioning studies have demonstrated with continuous exposure, sea lamprey decrease their response to alarm cue (Bals, 2012). Bals (2012) conditioned sea lamprey with alarm cue continuously for 0, 1, 2, 4, or 8h, and then tested their behavioral response to the alarm cue in a raceway. Researchers found that the response to the alarm cue began to attenuate after 2h of continuous exposure to a fixed concentration and the response was eliminated after 4h (Bals, 2012) (putatively a habituation response, Table 1). Additionally, after 4h of continuous exposure to the alarm cue, researchers removed the stimulus 0, 0.5, 1, or 2h and examined response to alarm cue in the behavioral assay. The response to alarm cue recovered between

0.5 and 1h (presumably spontaneous recovery) (Bals, 2012). Finally, Bals (2012) examined whether lamprey maintain their response to alarm cue when the exposure was not continuous. Lamprey swam in and out of the alarm cue plume in the behavioral assay over a period of 5h. In this experimental set up, sea lamprey maintained their response to the alarm cue (Bals, 2012). The results of these studies illustrate some of the constraints that need to be considered if repellants or attractants are to be implemented as a management technique, such as the perceived risk in the method of application of the alarm cue.

Table 1. Key Terms.

Term	**Definition**
Habituation	Learned. Decreased response to a stimulus due to repeated exposure with no consequences. Saves prey time and energy.
Sensory Adaptation	Decreased response to a stimulus via the sensory system (neurons/receptors/cells). Receptors adjust the sensitivity to signal.
Tolerance	Behavioral state. Intensity of a stimulus an individual permits without responding in a defined way (Bejder et al., 2009). In the context of habituation, tolerance levels are predicted to increase while tolerance levels are predicted to decrease for sensitization (Bejder et al., 2009).
Risk Assessment	Predation risk response depends on environmental context such as the duration of the risk or foraging or mating opportunities. Is the severity of the risk worth responding to in terms of the prey's time and energy? (Lima & Bednekoff, 1999; Ferrari et al., 2010)
Dishabituation	For a habituated response, a presentation of a second 'strong' stimulus, causes the response to the original stimulus to recover (Rankin et al., 2009; Blumstein, 2016).
Spontaneous Recovery	Recovery from habituation when stimulus is withheld. Recovery is on a scale, it can be partial to full response recovery (Rankin et al., 2009; Blumstein, 2016).
Sensitization	Opposite of habituation. Heightened and/or enhanced response to a stimulus due to repeated exposure (King, Douglas-Hamilton, & Vollrath, 2007; Bejder et al., 2009).

The objectives of my thesis were to discover and test application techniques that will maintain avoidance of the alarm cue in sea lamprey for 8h or more (a full night) in a river. Both pulse modulation and concentration application techniques were tested. The stimulus interval and intensity can determine the rate at which an animal habituates to a stimulus (or doesn't). The quicker the interstimulus interval (ISI), the more rapid the habituation (Rankin et al., 2009; Mcdiarmid, Yu, & Rankin, 2019)(Rankin et al., 2009; Mcdiarmid, Yu, & Rankin, 2019). In contrast, a slow ISI may lead to slow or no habituation (Rankin et al., 2009; Mcdiarmid, Yu, & Rankin, 2019). Additionally, when the sea lamprey swam in and out of the alarm cue over 5h (like a pulse), their response was maintained (Bals, 2012). Modulating the concentration of the cue is set within the context of what naturally occurs in the animal's environment. Sea lamprey may be continuously exposed to alarm cue at various concentrations as its migrating upstream. The strength of the stimulus applied also has an impact on habituation. Relatively weak stimuli lead to a more rapid, pronounced response attenuation, while stronger stimuli may lead to little or no response attenuation (Rankin et al., 2009; Blumstein, 2016).

The application techniques were tested in two different behavioral assays. The first assay was a Y-maze and measured upstream movement (yes/no), time to event, and side chosen. We found that upstream movement, an indicator of activity, was a stronger metric than spatial avoidance and switched assay designs to a smaller activity-based assay the following year. The second assay video recorded behavioral responses to the alarm cue and was quantified with an ethogram. From the ethogram, we created an index of activity to evaluate behavioral responses to the alarm cue.

REFERENCES

REFERENCES

Bals, J. (2012). The sea lamprey alarm response: field and laboratory investigations. MS Thesis. Michigan State University.

Bejder, L., Samuels, A., Whitehead, H., Finn, H., & Allen, S. (2009). Impact assessment research: use and misuse of habituation, sensitisation and tolerance in describing wildlife responses to anthropogenic stimuli– Mar. Ecol. Prog. Ser. 395: 177–185.

Blumstein, D.T. (2016). Habituation and sensitization: new thoughts about old ideas– Anim. Behav. 120: 255–262.

Dahl, F.H. & McDonald, R.B. (2011). Effects of Control of the Sea Lamprey (*Petromyzon marinus*) on Migratory and Resident Fish Populations– https://doi-org.proxy1.cl.msu.edu/10.1139/f80-230 37: 1886–1894.

Di Rocco, R.T., Johnson, N.S., Brege, L., Imre, I., & Brown, G.E. (2016). Sea lamprey avoid areas scented with conspecific tissue extract in Michigan streams– Fish. Manag. Ecol. 23: 548–560.

Ferrari, M.C.O., Elvidge, C.K., Jackson, C.D., Chivers, D.P., & Brown, G.E. (2010). The responses of prey fish to temporal variation in predation risk: Sensory habituation or risk assessment?– Behav. Ecol. 21: 532–536.

GLFC (n.d.). Great Lakes Fishery Commission - What is at Risk? http://www.glfc.org/what-is-at-risk.php.

Greggor, A.L., Berger-Tal, O., & Blumstein, D.T. (2020). The Rules of Attraction: The Necessary Role of Animal Cognition in Explaining Conservation Failures and Successes– Annu. Rev. Ecol. Evol. Syst. 51: 483–503.

Hansen, M.J., Madenjian, C.P., Slade, J.W., Steeves, T.B., Almeida, P.R., & Quintella, B.R. (2016). Population ecology of the sea lamprey (*Petromyzon marinus*) as an invasive species in the Laurentian Great Lakes and an imperiled species in Europe– Rev. Fish Biol. Fish. 2016 263 26: 509–535.

Hume, J.B., Luhring, T.M., & Wagner, C.M. (2020). Push, pull, or push–pull? An alarm cue better guides sea lamprey towards capture devices than a mating pheromone during the reproductive migration– Biol. Invasions 22: 2129–2142.

Hume, J.B., Meckley, T.D., Johnson, N.S., Luhring, T.M., Siefkes, M.J., & Wagner, C.M. (2015). Application of a putative alarm cue hastens the arrival of invasive sea lamprey (Petromyzon marinus) at a trapping location– Can. J. Fish. Aquat. Sci. 72: 1799–1806.

Imre, I., Di Rocco, R.T., Brown, G.E., & Johnson, N.S. (2016). Habituation of adult sea lamprey repeatedly exposed to damage-released alarm and predator cues– Environ. Biol. Fishes 99: 613–620.

King, L.E., Douglas-Hamilton, I., & Vollrath, F. (2007). African elephants run from the sound of disturbed bees– Curr. Biol. 17: R832–R833.

Larsen, L.O. (1980). Physiology of Adult Lampreys, with Special Regard to Natural Starvation, Reproduction, and Death after Spawning– Can. J. Fish. Aquat. Sci. 37: 1762–1779.

Li, W., Scott, A., Siefkes, M., Yan, H., Liu, Q., Yun, S.-S., & Gage, D. (2002). Bile acid secreted by male sea lamprey that acts as a sex pheromone.

Lima, S.L. & Bednekoff, P.A. (1999). Temporal Variation in Danger Drives Antipredator Behavior: The Predation Risk Allocation Hypothesis.– Am. Nat. 153: 649–659.

Luhring, T.M., Meckley, T.D., Johnson, N.S., Siefkes, M.J., Hume, J.B., & Wagner, C.M. (2016). A semelparous fish continues upstream migration when exposed to alarm cue, but adjusts movement speed and timing– Anim. Behav. 121: 41–51.

Mcdiarmid, T.A., Yu, A.J., & Rankin, C.H. (2019). Habituation Is More Than Learning to Ignore : Multiple Mechanisms Serve to Facilitate Shifts in Behavioral Strategy 1900077: 1–10.

Potter, I.C. (1980). Ecology of Larval and Metamorphosing Lampreys– Can. J. Fish. Aquat. Sci. 37: 1641–1657.

Purvis, H.A. (1980). Effects of Temperature on Metamorphosis and the Age and Length at Metamorphosis in Sea Lamprey (*Petromyzon marinus*) in the Great Lakes– Can. J. Fish. Aquat. Sci. 37: 1827–1834.

Rankin, C.H., Abrams, T., Barry, R.J., Bhatnagar, S., Clayton, D.F., Colombo, J., Coppola, G., Geyer, M.A., Glanzman, D.L., Marsland, S., McSweeney, F.K., Wilson, D.A., Wu, C.F., & Thompson, R.F. (2009). Habituation revisited: An updated and revised description of the behavioral characteristics of habituation– Neurobiol. Learn. Mem. 92: 135–138.

Renaud, C. (2011). Lampreys of the World: an Annotated and Illustrated Catalogue of Lamprey Species Known To Date– FAO Species Cat. Fish. Purp., Vol. 5.

Sakamoto, K., Dew, W.A., Hecnar, S.J., & Pyle, G.G. (2016). Effects of Lampricide on Olfaction and Behavior in Young-of-the-Year Lake Sturgeon (*Acipenser fulvescens*)– Environ. Sci. Technol. 50: 3462–3468.

Siefkes, M.J. (2017). Use of physiological knowledge to control the invasive sea lamprey (*Petromyzon marinus*) in the Laurentian Great Lakes– Conserv. Physiol. 5.

Sorensen, P.W., Fine, J.M., Dvornikovs, V., Jeffrey, C.S., Shao, F., Wang, J., Vrieze, L.A., Anderson, K.R., & Hoye, T.R. (2005). Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey– Nat. Chem. Biol. 2005 16 1: 324–328.

CHAPTER 1: Effects of pulsed and continuous application of alarm cue on movement behavior of migratory sea lamprey (*Petromyzon marinus*)

Abstract

Animals continuously detect, learn from, and respond to sensory information in their environments to survive. This sensory information can be utilized as a behavioral tool by both managers and conservationists to guide reactions and movements for species of concern. This technique has the potential to be used with sea lamprey, *Petromyzon marinus,* a species of management concern in the Laurentian Great Lakes with the repellant, alarm cue. Alarm cues are a semiochemical, released from damaged tissues when an animal is injured, such as an attack, warning nearby conspecifics of a predation event. Sea lamprey respond to their alarm cue with spatial avoidance and increased swimming speed, moving out of the affected area. However, previous work has indicated that sea lamprey habituate to their alarm cue within four hours of continuous exposure. If alarm cues are to be used as a management technique, we must understand the behavioral response in its entirety, including the habituation response and how to prevent it. This study examined two application techniques to prevent habituation: pulsing the alarm cue (45/15 min. pulse rate) and modulating the concentration (1 ppm or 10 ppm AC). These application techniques were compared against three controls: a negative control (conditioning sea lamprey with water, followed by an application of water in behavioral assay), a positive control (conditioning with water and followed by alarm cue in behavioral assay), and a habituation control (conditioning with alarm cue and followed by alarm cue in behavioral assay). Sea lamprey were conditioned to their treatments for up to 4h before they were released into the behavioral assay, a Y-maze, where we examined responses to the alarm cue, which was pumped into the assay at the upstream end. In the Y-maze, sea lamprey were released in the downstream end and allowed 5 min to swim upstream to an event line. We found that all conditioning treatments with alarm cue and the positive control had greater upstream movement compared to the negative control.

Compared to the negative control, these conditioning treatments had an odor to orient to, alarm cue. Additionally, greater upstream movement by these treatment groups indicates detection and response to the alarm cue as sea lamprey may respond to the alarm by increasing their swimming speed. Across the alarm cue conditioning treatments, we found differences in arrival times to the upstream event line, suggestive of varying anti-predation strategies. Overall, the alarm cue side was avoided by all conditioning treatments who had slower upstream movement, suggesting the more time spent gathering information about the alarm cue, the better sea lamprey were able to avoid the predation risk.

Introduction

An emerging management and conservation technique is the use of species-specific behavioral responses to sensory information. Animals detect sensory information in their environment to find food, mates, and avoid predation. This sensory information may be in the form of an audio, visual, olfactory, tactile, or taste stimulus (to name a few) (Nielsen et al., 2015). These stimuli either attract or repel animals to a specific area and thus, can be utilized as a management tool (Greggor, Berger-Tal, & Blumstein, 2020). The use of attractants and repellants as species specific behavioral tools provides opportunities to manage and conserve species (Blumstein, 2016; Greggor, Berger-Tal, & Blumstein, 2020). For example, the invasive cane toad tadpole has been successfully trapped using the attractant, bufadienolide toxin, an odor native toads use to avert predators (Crossland et al., 2012). Repellants have also been shown to be successful in species management, and are often used in marine ecology as a non-lethal technique to mitigate human-wildlife conflict in the fisheries industry (Schakner & Blumstein, 2013). One example of this was demonstrated in a study done with *Salmo salar*, marine salmon and *phocid*, seals. With a pulsed acoustic stimulus, seal predation on the farmed salmon decreased by 97% compared to the controls over a 19-month period (Gotz & Janik, 2016).

In cases where a stimulus will be used to manage a species, it is important to make sure the stimulus is being reinforced and habituation is not occurring with repeated exposure (Blumstein, 2016). Habituation is a decremented response due to repeated exposure to a stimulus (Rankin et al., 2009; Blumstein, 2016). If a stimulus is used repeatedly as a behavioral management tool, response attenuation or habituation may occur, weakening the effectiveness of the management tool (Blumstein, 2016; Greggor, Berger-Tal, & Blumstein, 2020). While habituation has been coined, "one of the simplest forms of learning", recent studies have shown this may not be the case (Mcdiarmid, Yu, & Rankin, 2019). Before they are fully habituated, animals may begin to alter or shift their behavioral response strategy (Ardiel et al., 2017; Mcdiarmid, Yu, & Rankin, 2019). Habituation can also occur to a stimulus when there is no perceived benefit to the receiver in responding (Blumstein, 2016). An example of this phenomenon may be shown in sea lamprey.

Sea lamprey habituate to their alarm cue, an odor that induces anti-predation behavior (Bals, 2012; Imre et al., 2016). Alarm cues are a chemical mixture that is released from damaged tissues when an animal is injured, such as by an attack from a predator, warning nearby conspecifics of a predation risk. Many species, including sea lamprey respond to their alarm cue with antipredation behaviors (Ferrari, Wisenden, & Chivers, 2010; Wagner, Stroud, & Meckley, 2011). Animals avoid areas activated with alarm cues in space and time, but durations vary dependent upon the species (Wisenden, 2015). Additionally, alarm cues can be utilized by animals to learn and recognize novel predators through association (Chivers & Smith, 1998; Brown, 2003). With continuous exposure to alarm cue, a sea lamprey's response attenuates after 2h and is eliminated after 4h of continuous exposure (Bals, 2012; Imre et al., 2016). Sea lamprey migrate upstream in their sub-adult phase to spawn. They are semelparous fish and their life fitness culminates in this single spawning event. Allocating time and energy to spawning may provide greater fitness benefit than responding to a repetitive, background predation cue, especially if there is not additional information indicating predation (Lima & Bednekoff, 1999; Ferrari et al., 2010; Luhring et al., 2016).

Alarm cues have the potential to be utilized as a behavioral management tool. In the Laurentian Great Lakes, Sea lamprey are an invasive species, feeding on the blood and tissues of their hosts. Sea lamprey have caused significant decline in native fish populations, especially Lake Trout (*Salvelinus namaycush*), which were nearly extirpated in the Great Lakes (GLFC, n.d.). Currently, sea lamprey are managed with a lampricide application TFM, but could be additionally managed with alarm cue, which has repellant-like effects as an anti-predation cue. As sea lamprey avoid areas activated with alarm cue, it may be used to guide them to a trap, or a designated area for TFM application (Hume et al., 2015; Hume, Luhring, & Wagner, 2020).

If we are to use species-specific repellants such as alarm cues, to aid in the management of species such as sea lamprey, we must understand their response to the repellant, including whether response attenuation will occur and how to prevent it. Sea lamprey's response to alarm cue includes spatial avoidance and increased swimming speed to move out of the area activated with alarm cue (Hume et al., 2015; Di Rocco et al., 2016a; Luhring et al., 2016). Sea lamprey habituate to alarm cue after 4h of continuous exposure (Bals, 2012; Imre et al., 2016). To prevent habituation from occurring, application of a stronger stimulus may reduce or eliminate habituation (Rankin et al., 2009). A stronger stimulus may in fact, cause an opposite effect of habituation over time, sensitization, in which an animal increases response intensity after repeated exposure (Bejder et al., 2009). Recovery from habituation can occur when the stimulus is withheld (partially or wholly), this is defined as spontaneous recovery (Rankin et al., 2009; Blumstein, 2016). For example, spontaneous recovery to alarm cue has been demonstrated in habituated responses after 1h of stimulus removal (Bals, 2012). In management applications, habituation prevention techniques may also consider an animal's umwelt, or their perception of the stimulus in their environment (Dyck, 2012). Fish, including sea lamprey, may encounter a range of alarm cue pulses and concentrations in the field. Sea lamprey maintained their response to alarm cue with 4h of continuous exposure when they were in a raceway and could swim in and out of the plume like a pulse (Bals, 2012). In a habituation context, the stimulation interval can determine the rate of both response attenuation and recovery (Rankin 2009).

The objective of this study was to examine the ability of two application practices designed to prevent habituation to the alarm cue from occurring in migratory sea lamprey: pulsing the odor and increasing its concentration. We conditioned lamprey with a pulse application and continuous application of alarm cue at two dilutions: 1 ppm and 10 ppm, by volume, representing the concentration that elicits full behavioral reactivity in the lab, and 10X that concentration (Wagner, Stroud, & Meckley, 2011). This study was conducted in behavioral assays built in raceways at the USGS Hammond Bay Biological Station, Roger City Michigan.

Methods

Study Design

The objective of this study was to examine the effectiveness of three applications of alarm cue designed to prevent habituation to the cue (Table 2, hereafter referred to as *conditioning treatments*). These were: (1) alarm cue applied continuously at a dilution of 10 ppm (*Continuous AC 10 PPM*), (2) alarm cue pulsed On/Off at a dilution of 1 ppm (*Pulse AC 1 PPM*), and (3) alarm cue pulsed On/Off at a dilution of 10 ppm (*Pulse AC 10 PPM*). For the pulsing treatments, the alarm cue was applied at a pulsing rate of 45 minutes ON/15 minutes OFF. We contrasted the conditioning treatments with three control conditions:

(a) a negative control in which animals were conditioned with water and tested in the behavioral assay with water (hereafter referred to as *Water/Water*), (b) a positive control in which animals were conditioned to water and tested in the behavioral assay with alarm cue at a dilution of 1 ppm (*Water/AC 1 PPM*), and (c) a habituation control, where animals were conditioned with alarm cue, pumping continuously at a dilution of 1 ppm and then tested in the behavioral assay with alarm cue at a dilution of 1 ppm (*Continuous AC 1 PPM*, per Bals 2012). These conditions represent animals in a non-fear state (Water/Water), animals with unhabituated responses to alarm cue (Water/AC 1 PPM), and individuals that should exhibit habituated responses to the cue beginning after 2 h of conditioning treatment (Continuous AC 1 PPM).

Individual lamprey were conditioned to a treatment or control for durations up to 4h in increments of 12 min (i.e. 0, 12, 24, 48…240 min of a conditioning treatment prior to testing, referred to as *Conditioning Durations*). After conditioning, we released each subject into a 5 X 1.5 m section of a laboratory maze with flowing water and observed its movement for up to 5 min. During the observation period, one side of the maze was activated with alarm cue (hereafter referred to as the s*timulus side*), except during negative control trials, which received water. Alarm cue dilution for the behavioral observation period was equal to the dilution the subject experienced during the conditioning period. We recorded whether the animal moved upstream (*Upstream Movement)* 3.5 m to a line marked on the bottom of the maze (event line in Fig. 1), and the side of the maze (*Stimulus Avoidance)* it occupied when crossing the line (stimulus or non-stimulus). For animals that crossed the event line, we recorded the time of crossing (*Time to Crossing Event Line*) and removed the subject. Animals that did not proceed to the event line were removed after an observation period of 5 min. If a conditioning treatment (Continuous AC 10 PPM, Pulse AC 1 PPM, and Pulse AC 10 PPM) prevented habituation to the alarm cue, we expected: (Prediction #1) greater upstream movement vs the negative control for all conditioning durations, consistent with results of Luhring et al. 2016, demonstrating sea lamprey swim upstream regardless of alarm cue presence, but adjust their timing and speed to avoid the predation cue; (Prediction #2): greater avoidance of the stimulus side vs the negative control for all conditioning durations (i.e., evidence of an alarm response); (Prediction #3) upstream movement and avoidance of the stimulus side similar to that observed in the positive control for all conditioning durations (i.e., equal response to the unhabituated control); and (Prediction #4) upstream movement and avoidance of the stimulus side greater than observed in the habituation control after 120 min of exposure (i.e., greater response compared to habituation control).

Experimental Subjects

Actively migrating sea lamprey were collected by the U.S. Fish and Wildlife Service (USFWS) during the annual monitoring program in 2019 from the Ocqueoc, Cheboygan and Carp Lake Rivers, Michigan. Lamprey were also collected from St. Mary's River by the USFWS and Fisheries and Oceans Canada. As response to alarm cue diminishes with maturity in females (Bals & Wagner, 2012), only male experimental subjects who did not exhibit external signs of sexual maturation (e.g. lack of well-developed dorsal rope, blue coloration) nor external injuries were used in this study, n=503 (total length range: 29 – 55 cm, total length mean ± sd: 46 cm ± 4/weight range: 61- 343 g, weight mean ± sd: 196 ± 50 g). Sea lamprey were housed in 1385 L flow through tanks at the US Geological Survey's (USGS) Hammond Bay Biological Station (HBBS). Animal handling and experimentation procedures were approved by the Michigan State University Institutional Animal Care and Use Committee under permit number AUF 03/18-039-00.

Alarm Cue Preparation

For this experiment, alarm cue was combined from Soxhlet extracts made in 2017. This alarm cue was Soxhlet extracted from 35 adult sea lamprey per the procedures reported in Bals and Wagner 2012. Euthanized or freshly deceased sea lamprey were kept at -20°C until whole-body alarm cue extraction could take place. Before alarm cue extraction, sea lamprey were thawed, weighed, and rinsed with DI water. The extraction was set up as follows (top to bottom): an Allihn condenser was attached to a 1-L 71/60 Soxhlet Extractor Body (Ace Glass Inc., Vineland, New Jersey), this contained the lamprey carcasses, which were attached to a 1-L round bottom flask, which contained the solvent (50:50 weight/weight of 200-proof ethyl alcohol and deionized water). The round bottom flask was heated by a hemispherical mantle to 75-80 °C. Each extraction cycled three times through the Soxhlet extractor body. Following extraction, the solvent ethanol was removed with a rotary evaporator and the extracts were combined into a single well-mixed batch. The final concentration of the extract was equivalent to 175 mg of tissue per ml of alarm cue extract. The alarm cue extraction was stored at -20°C until use.

Behavioral Assay

All trials took place from approximately 09:00 – 16:00 from June 28th – July 19th, 2019. Prior to trials, lamprey were photo reversed for a minimum of 3 days. Photo-reversing lamprey does not affect the response to alarm cue in sea lamprey (Barnett et al., 2016). Trials took place in a Y-maze embedded in a concrete raceway (Fig. 1). Water was supplied to the raceways for this behavioral maze from Lake Huron, with the flow maintained at 0.01 m^3/s. The water temperature ranged from 10.3°C to 21.9°C during the study period. The maze portion of the raceway was 5 m in length by 1.5 m wide. Upstream of the maze, baffles were installed in a honeycomb shape to stabilize the flow. Alarm cue (or water, negative control) was pumped into the maze upstream of the baffles via peristaltic pumps (MasterFlex model 7533-20) at a rate of 20 ml/min. The alarm cue (or water) was kept on ice and continually mixed with a stir plate as it pumped into the assay. The alarm cue dilution was calculated so the addition of the alarm cue to the maze targeted the conditioning treatment dilution (1 ppm or 10 ppm) with the raceways discharge. Downstream of the maze, fish were conditioned to their assigned treatment for up to 4h in individual holding baskets (15 x 30.5 x 30.5 cm) before being released into the behavioral assay. For each conditioning treatment, lamprey were released every 12 min, for up to 4h to examine response attenuation across time and treatments. Six treatment groups examined this effect (Table 2). The behavioral assay lasted for 5 min. After a fish was released into the maze, they were allowed 5 min to swim upstream to the event line. Similar to a Y-maze design, the event line is where the assay bifurcates into two segments, dividing the stimulus and non-stimulus side. We recorded Upstream Movement to the event line (yes/no), Stimulus Avoidance (alarm cue side avoidance), and Time to Crossing Event Line. If the sea lamprey did not swim upstream within 5 min to the event line, they were removed from the assay.

Table 2. Experimental Treatments.

Treatment Name	Conditioning Treatment	Behavioral Assay Observation Treatment
Water/Water	Continuous water	Continuous water
Water/AC 1 PPM	Continuous water	Continuous alarm cue, 1 PPM
Continuous AC 1 PPM	Continuous alarm cue at 1 PPM	Continuous alarm cue, 1 PPM
Continuous AC 10 PPM	Continuous alarm cue at 10 PPM	Continuous alarm cue, 10 PPM
Pulse AC 1 PPM	Alarm cue, 1 PPM, applied at a pulse - 45 minutes ON and 15 minutes OFF.	Continuous alarm cue, 1 PPM
Pulse AC 10 PPM	Alarm cue, 10 PPM, applied at a pulse - 45 minutes ON and 15 minutes OFF.	Continuous alarm cue, 10 PPM

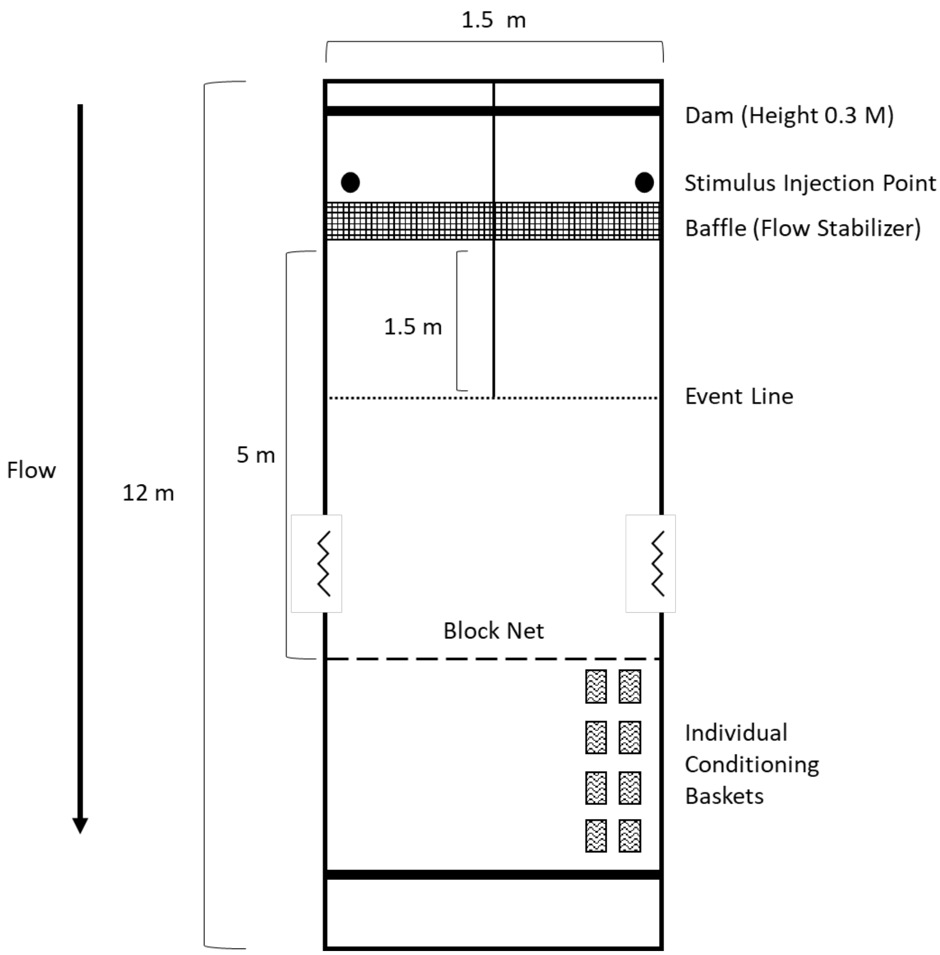


Figure 1. Schematic of behavioral Y-maze used in experiment (not to scale). Lamprey were conditioned to assigned treatment for up to 4h in individual holding baskets in downstream end before being released into behavioral assay. Assay examined both upstream movement and avoidance of stimulus.

Statistical Analysis

Initially, 503 subjects were included in this study. Eighty-nine subjects were dropped from the analysis as the light came on during their trial, which could be considered a dishabituating stimulus. Dishabituation can occur if an animal is habituated to a stimulus such as alarm cue, and another stimulus is presented, like a flash of light, increasing or recovering the response to the original stimulus (Rankin et al., 2009; Blumstein, 2016). Of these eighty-nine subjects, twenty-seven were Water/AC 1 PPM, forty-two were Pulse AC 1 PPM, and twenty were Pulse 10 PPM. Two subjects were dropped from analysis (Water/AC 1 PPM and Continuous AC 1 PPM) as after the behavioral trial as they were discovered to be female, which doesn't fit the criteria of our experimental design. Sexually immature female sea lamprey do not release compounds that may affect the behavior of sexually immature male sea lamprey or induce physiological changes (Fissette et al., 2021). Thus, male sea lamprey conditioned with the female sea lamprey were kept for analysis. The total number of subjects included in this study were 412. All data analysis was performed using R (R Core Team, 2020).

To test the effects of the Conditioning Treatments on Upstream Movement, we constructed five a priori candidate models (logistic, generalized linear models). The candidate models for Upstream Movement included an intercept model, single factor models for each predictor variable (Conditioning Treatment and Conditioning Duration), and global models, with and without interactions. To test the effects of Conditioning Treatments on Stimulus Avoidance, we constructed twelve a priori candidate models. These models included: an intercept model, single factor models for each predictor variable (Conditioning Treatment, Conditioning Duration, and Time to Crossing Event Line), all two-factor models (N=6), and global models, with and without interactions.

Models were ranked and compared according to their Akaike Information Criterion corrected for sample size (AIC_C) and lowest ΔAIC_C values. Models were given support according to their ΔAIC_C values, those with values less than 2 were considered to have substantial support (Fabozzi et al., 2014). The supported models from the AIC_C comparison were then analyzed with the function "Anova" in the "car" package to assess model parameters. Type II Sum of Squares were used for models without interactions and Type III for those with interactions. In models with significant treatment effects, a post-hoc Tukey test was used to analyze our model and predictions 1, 2, and 3. We used the function "glht" in "multcomp" package (Hothorn, Bretz, & Westfall, 2008).

Results

Upstream Movement Models

Presence of alarm cue during the behavioral observation period was associated with a greater tendency to move upstream. The model that best explained upstream movement was the single term Conditioning Treatment model (Table 3). This model was 1.3 AIC units away from the next model and accounted for 64.4% of the AIC weight. The second model accounted for 34.3% of the AIC weight and included the terms Conditioning Treatment and Conditioning Duration. Conditioning Treatment was a commonality across the top three models, which accounted for 99.9% of the AIC weight. Significant differences between upstream movement were observed across the levels of Conditioning Treatment (ANOVA: $F_{5, 406}$= 4.93, p=0.0002; Fig. 2). Consistent with our predictions, all treatments had significantly greater upstream movement relative to the negative control (Tukey Test, p<0.05) except Water/AC 1 PPM which differed from the negative control at p=0.06 (Tukey Test; Table 4). Within each conditioning treatment, sea lamprey varied in time (s) to upstream movement (Fig. 4). Fifty-two percent of the negative control animals moved upstream taking 206 ± 110 seconds (mean ± 1 sd) to cross the Event Line. Compared to the negative control, greater upstream movement and earlier arrival times were observed for the positive control (Water/AC 1 PPM, 76% upstream, 128 ± 120 seconds), the habituation control (Continuous AC 1 PPM, 82% upstream, 126 ± 108 seconds), and the three conditioning treatments designed to prevent habituation (Continuous AC 10 PPM, 79% upstream, 115 ± 118 seconds; Pulse AC 1 PPM, 81% upstream, 134 ± 108; Pulse AC 10 PPM, 82% upstream, 118 ± 111 seconds).

Stimulus Avoidance Models

There was not strong evidence for alarm cue avoidance across Conditioning Treatments when measured by the position of the animal as it crossed the Event Line. Across Conditioning Treatments, the alarm cue side was avoided by Water/AC 1 PPM 57%, Continuous AC 1 PPM 60%, Continuous AC 10 PPM 45%, Pulse AC 1 PPM 47% and Pulse 10 PPM 63%. Stimulus Avoidance was best explained by models which included Time to Crossing Event Line (Table 3). Increased Time to Crossing Event Line was associated with greater alarm cue avoidance. The top model included both Conditioning Duration and Time to Crossing Event Line and accounted for 30.25% of the AIC_C weight. The next model was 0.2 AIC_C units away and was a single term model with Time to Crossing Event Line. This model accounted for 27.55% of the AIC_C weight. In the top model, Time to Crossing Event Line was a significant term (ANOVA: $F_{1, 303}$= 5.03, p=0.02), but Conditioning Duration was not (ANOVA: $F_{1, 303}$= 2.20, p=0.14; Fig. 3).

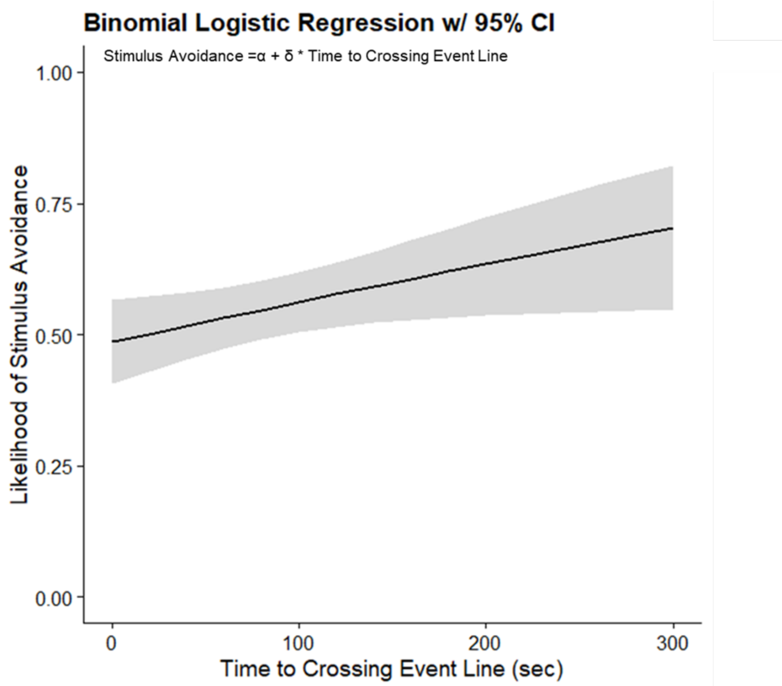
Table 3. AIC values obtained from logistic regression models on upstream movement and stimulus avoidance. Global models marked with (G).

Model	AIC_C	ΔAIC_C	W_i
Upstream Movement			
Conditioning Treatment	457.0	0.0	0.644
Conditioning Treatment + Conditioning Duration (G)	458.3	1.3	0.343
Conditioning Treatment * Conditioning Duration (G)	464.9	7.9	0.012
Intercept	471.8	14.8	<0.001
Conditioning Duration	472.8	15.7	<0.001
Stimulus Avoidance			
Conditioning Duration + Time to Crossing Event Line	420.6	0.0	0.3025
Time to Crossing Event Line	420.8	0.2	0.2755
Conditioning Duration * Time to Crossing Event Line	421.3	0.7	0.2143
Intercept	423.3	2.6	0.0815
Conditioning Duration	423.7	3.0	0.0660
Conditioning Treatment + Time to Crossing Event Line	425.6	4.9	0.0261
Conditioning Treatment + Conditioning Duration + Time to Crossing Event Line (G)	426.1	5.4	0.0201
Conditioning Treatment	427.9	7.2	0.0081
Conditioning Treatment + Conditioning Duration	428.9	8.2	0.0049
Conditioning Treatment * Time to Crossing Event Line	432.1	1.5	<0.001
Conditioning Treatment * Conditioning Duration	437.3	16.7	<0.001
Conditioning Treatment * Conditioning Duration * Time to Crossing Event Line (G)	452.0	31.4	<0.001

Table 4. Summary of Post-Hoc Tukey Comparisons across Conditioning Treatments for Upstream Movement.

Prediction Tested	Conditioning Treatment Comparison		Estimated Difference in Means	Std. Error	*P*
Prediction #1	Water/AC 1 PPM	Water/Water	1.05	0.38	0.070
Prediction #1	Continuous AC 1 PPM	Water/Water	1.41	0.36	0.0011
Prediction #1	Continuous AC 10 PPM	Water/Water	1.21	0.34	0.0060
Prediction #1	Pulse AC 1 PPM	Water/Water	1.35	0.45	0.030
Prediction #1	Pulse AC 10 PPM	Water/Water	1.34	0.39	0.0071
Prediction #3	Continuous AC 1 PPM	Water/AC 1 PPM	0.367	0.43	0.95
Prediction #3	Continuous AC 10 PPM	Water/AC 1 PPM	0.156	0.42	0.99
Prediction #3	Pulse AC 1 PPM	Water/AC 1 PPM	0.304	0.51	0.99
Prediction #3	Pulse AC 10 PPM	Water/AC 1 PPM	0.293	0.45	0.98

(a)



(b)

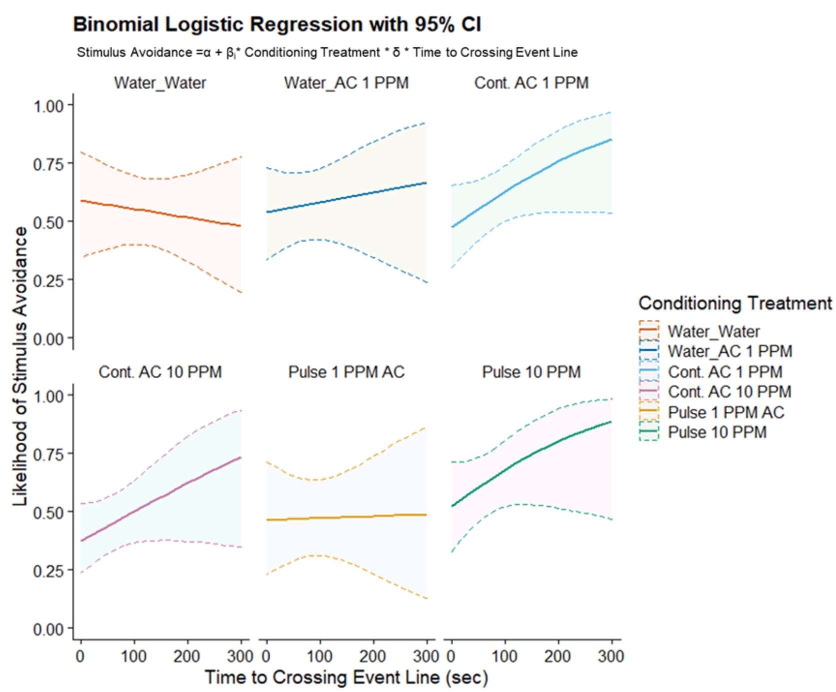
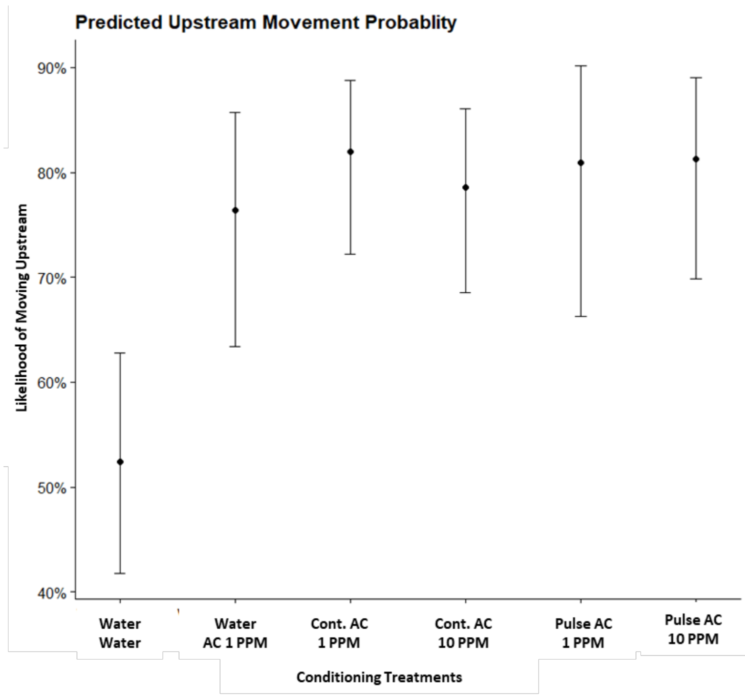


Figure 2. (a) Predicted movement from the top Upstream Movement model (mean ± 95% CI). Upstream movement differed across conditioning treatments (ANOVA: $F_{5, 406}$= 4.93, p=0.0002). All conditioning treatments differed from Water/Water at p=0.05, except for Water/AC 1 PPM which p=0.06. (b) Predicted movement for second top Upstream Movement model across Conditioning Durations.

(a)



(b)

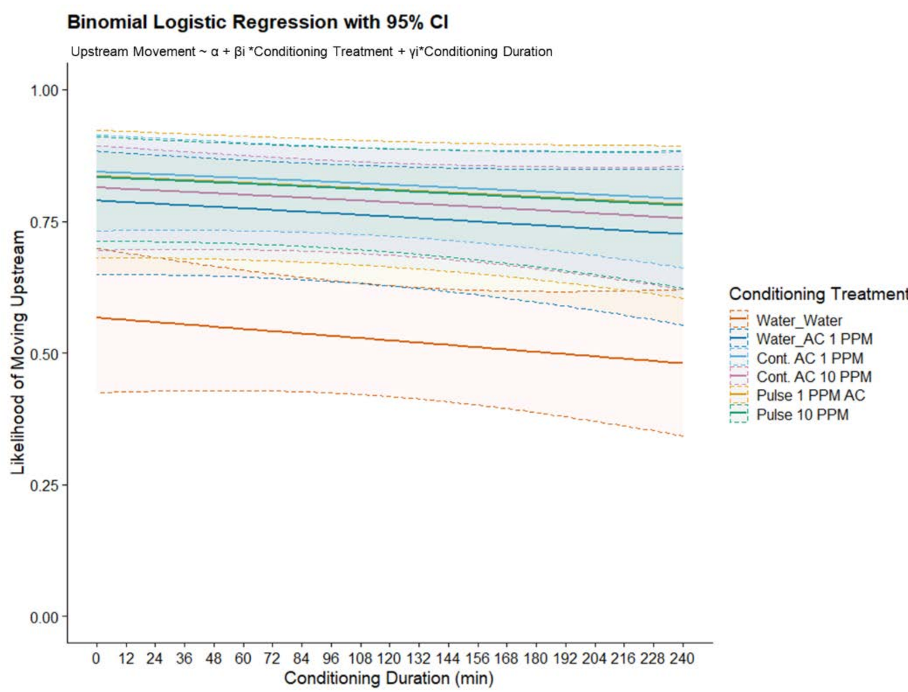


Figure 3. Illustrating the effects of Time to Crossing Event Line and Stimulus (alarm cue) avoidance. (a) Predicted effects of Time to Crossing Event Line on top model for Stimulus Avoidance. (b) Predicted effects of Time to Crossing Event Line across Conditioning Treatments. Conditioning Treatment was not a significant term in the Stimulus Avoidance models, shown to illustrate trends across treatments

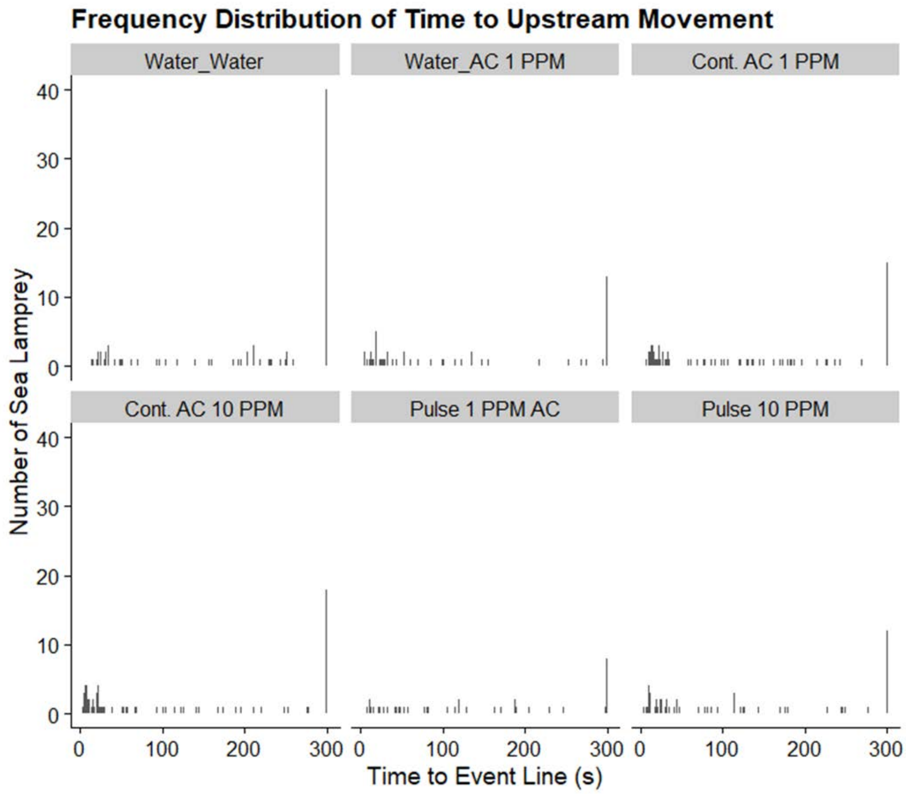


Figure 4. Examining individual differences in upstream movement arrival time across Conditioning Treatments. After 300 s, sea lamprey were removed from the maze and their upstream movement outcome was marked as *no* (i.e., no upstream movement).

Discussion

The results of this study suggest that sea lamprey continued to respond to the alarm cue for each conditioning treatment, including the habituation control across all conditioning durations. Overall, the alarm cue conditioning treatments (Pulse 1 PPM, Pulse 10 PPM, and Continuous AC 10 PPM) arrived at the event line faster compared to the negative control, suggesting the detection of alarm cue induced more rapid upstream movement. Surprisingly, the habituation control, Continuous AC 1 PPM also had increased upstream movement over all conditioning durations, indicating a reinforced, non-attenuated response. In accordance with the study done by Bals 2012, we predicted the sea lamprey in the habituated control treatment to decrease or stop responding to the alarm cue after 2h of continuous exposure. In the case of the habituation control, we may have observed a shift in the sea lamprey's behavioral response strategy adapting to their perceived sensory information and environment (Mcdiarmid, Yu, & Rankin, 2019). Overall, the alarm cue application increased upstream movement, reinforcing antipredation responses in all conditioning treatments.

The sensory information of a predation risk, alarm cue, increased upstream movement and overall activity compared to the negative control. Consistent with Luhring et al. (2016), sea lamprey moved upstream regardless of alarm cue concentration. Increased activity is also consistent with sea lamprey's behavioral response to alarm cue as sea lamprey increase their swimming speed to move out of the area (Hume et al., 2015; Luhring et al., 2016). While we did observe differences in upstream arrival time across conditioning treatment groups and individuals (Fig. 4), the magnitude observed in this laboratory study (seconds to minutes) may have been too small for biological relevance with related field studies (hours – days), which demonstrate sea lamprey adjust their response to alarm cue in a threat sensitive manner (Luhring et al., 2016). In the Luhring study, sea lamprey increased their swimming speed as a temporal tactic in a high-risk environment (a river fully activated with alarm cue) to decrease their time spent in an area of risk. In our study, sea lamprey may have also adjusted their response, varying their time coming upstream, thus changing their perception of risk (i.e., certainty of location of risk). For example, some sea lamprey exhibit a flight response, trying to escape upstream (low certainty of risk) while others may respond by avoiding the stimulus, or (in context of this assay) 'shelter' and use their oral disk to attach to the floor to cease movement, gathering more information (higher certainty of risk) (Wisenden et al., 2010). Future tests with a longer assay duration or field studies to better understand how time to upstream movement varies across individuals and conditioning treatments would be informative.

Greater upstream movement and activity in the presence of a predation risk is also consistent with risk assessment for a semelparous fish, such as sea lamprey in their sub-adult stage (Ferrari et al., 2010; Luhring et al., 2016). Risk assessment and allocation of time to other activities is often considered a trade-off: respond to a predator or spend energy on foraging or reproduction (Lima & Bednekoff, 1999; Ferrari et al., 2010). However, in the case of semelparous fish migrating upstream to spawn, they can do both: increase their swimming speed to avoid the predation event while continuing their migration path. This is energetically efficient for a fish whose lifetime biological fitness accrues from a single spawning event. As a response to the alarm cue, sea lamprey will spatially avoid it and increase their swimming speed to move out of the area activated with alarm cue (Hume et al., 2015; Di Rocco et al., 2016b; Luhring et al., 2016).

Interestingly, spatial avoidance of the alarm cue was not evident within conditioning treatments but there was a consistent tendency toward greater avoidance of the alarm cue with slower upstream movement. The more time a sea lamprey spent gathering information about the alarm cue, the more likely they were to avoid a predation risk. Several conditioning treatments had a positive trend with increased time in the assay, indicating sea lamprey were able to gather information with time, and make a more informed decision to avoid predation risk: Water AC 1 PPM, Continuous AC 1 PPM, Continuous AC 10 PPM, and Pulse 10 PPM. The negative control, Water/Water had a slight negative trend, decreasing to a 0.50 stimulus avoidance probability with time. We would expect a zero slope and 0.50 stimulus avoidance probability for the negative control, indicating no side preference. The trend for Pulse 1 PPM AC was a zero slope with a 50-50 avoidance, which may indicate habituation as there was equal side choice over time, without change. If repeated habituation-spontaneous recovery cycles occur (which may have been happening with our pulse treatment) at low stimulus concentration (i.e., 1 ppm compared to 10 ppm), quicker more evident habituation can occur – this is called the "potentiation of habituation" (Rankin et al., 2009; Blumstein, 2016; Mcdiarmid, Yu, & Rankin, 2019). If the Pulse 1 PPM Alarm Cue treatment caused the potentiation of habituation, sea lamprey may have repeatedly habited to the alarm cue during the 45 minute alarm cue application period and spontaneously recovered to the alarm cue during the 15 minute OFF period. However, with each pulsing cycle, quicker, more evident habituation would have occurred. We did not however observe this effect in the upstream movement models as the Pulse 1 PPM Alarm Cue treatment was significantly greater than the negative control across all conditioning durations, indicating the alarm cue response was maintained. Although within-treatment time effects are interesting, it is important to note they are trends (i.e., not significant). To better understand these effects, sea lamprey may need more time to perceive and assess the risk before making an informed decision. Previous studies have demonstrated that in a behavioral raceway assay, sea lamprey take 5-10 min for the response distribution to stabilize (Wagner, Stroud, & Meckley, 2011). This assay allowed up to 5 min for sea lamprey to cross the event line but removed the fish at the 5-min mark. When sea lamprey stayed in the behavioral assay the full 5-min, their avoidance of the alarm cue was greater and closer to that of an expected avoidance: 75%.

Alarm cue provided information on predation risk; this information has value and with time, sea lamprey became better informed (McNamara & Dall, 2010). Theoretically, the value of this information is either zero or positive, but cannot be negative (McNamara & Dall, 2010). Even a repetitive, habituating stimulus, may represent positive information as animals may start to shift or adjust their response as an adaptive behavioral strategy (Mcdiarmid, Yu, & Rankin, 2019). A stimulus that is reinforced, and provides new information will have positive information value (McNamara & Dall, 2010; Blumstein, 2016). In this study, alarm cue conditioning treatments that had higher concentrations (Continuous 10 PPM AC and Pulse 10 PPM AC) had greater upstream movement compared to the negative control and results indicated these conditioning treatments avoided the stimulus side with longer time in the assay, however further data on spatial use will need to be collected to confirm. For greater efficacy in management, both conditioning treatments should be considered. While, Pulse 1 PPM AC did have greater upstream movement, this conditioning treatment tended to not avoid the stimulus with time and is recommended to not be considered for management applications as it may not be able to be reinforced in the field.

In consideration of these major findings, there are a few improvements that could be made to this behavioral maze for improved hypothesis testing. Overall, sea lamprey needed more time in the behavioral maze. We found that sea lamprey that took longer were more likely to avoid the stimulus. Going forward, behavioral assay times of 5-10 min are recommended to allow the response distribution to stabilize (Wagner, Stroud, & Meckley, 2011). There is a desire for a high through-put behavioral assay for sea lamprey as researchers generally have a small window to work with them. Sea lamprey are generally studied during their sub-adult life stage after they are trapped as part of the control program. As sea lamprey are semelparous, this sub-adult stage lasts from about May through July and is dependent on seasonal variables (temperature, river discharge, date) (Binder & McDonald, 2008;

Luhring et al., 2016). In this experiment, we designed a higher-through put behavioral assay, to address some of these time constraints we have with our organism. However, we found that an event-type analysis (event line crossing to record Upstream Movement and Stimulus Avoidance) was insufficient to assess data on stimulus avoidance. To incorporate high through put, increasing the amount of data collected (i.e., space use), and eliminating any potential observer bias in a future assay may include the use of an automated behavior system (Jutfelt et al., 2017). This automated system would have to work under infrared light conditions in the dark with our nocturnal organism.

Future studies with alarm cue and sea lamprey should include applying these conditioning treatment applications in the field. With these applications, do sea lamprey detect alarm cue in a threat sensitive manner? For example, if they detect alarm cue downstream at too low of a concentration, they may react to the predation risk too soon – time may be just as important a factor as space. Additionally, a dishabituation experiment should be explored. After habituating sea lamprey to alarm cue (as Bals 2012), does the original response recover or increase with a dishabituating stimulus (Rankin et al., 2009; Blumstein, 2016)? Dishabituating stimuli may be a light flash or another odor that the animal detects. Further understanding of behavioral responses to alarm cue would aid in the implementation of alarm cue as an alternative management technique.

REFERENCES

REFERENCES

Ardiel, E.L., Yu, A.J., Giles, A.C., & Rankin, C.H. (2017). Habituation as an adaptive shift in response strategy mediated by neuropeptides– npj Sci. Learn. 2: 1–10.

Bals, J. (2012). The sea lamprey alarm response: field and laboratory investigations. MS Thesis. Michigan State University.

Bals, J. & Wagner, C.M. (2012). Behavioral responses of sea lamprey (*Petromyzon marinus*) to a putative alarm cue derived from conspecific and heterospecific sources– Behaviour 149: 901–923.

Barnett, M., Imre, I., Wagner, C.M., Di Rocco, R.T., Johnson, N.S., & Brown, G.E. (2016). Evaluating potential artefacts or phot-reversal on behavioural studies with nocturnal invasive sea lamprey– Can. J. Zool. 94: 405–410.

Bejder, L., Samuels, A., Whitehead, H., Finn, H., & Allen, S. (2009). Impact assessment research: use and misuse of habituation, sensitisation and tolerance in describing wildlife responses to anthropogenic stimuli– Mar. Ecol. Prog. Ser. 395: 177–185.

Binder, T.R. & McDonald, D.G. (2008). The role of dermal photoreceptors during the sea lamprey (Petromyzon marinus) spawning migration– J. Comp. Physiol. A 2008 19411 194: 921–928.

Blumstein, D.T. (2016). Habituation and sensitization: new thoughts about old ideas– Anim. Behav. 120: 255–262.

Brown, G.E. (2003). Learning about danger: chemical alarm cues and local risk assessment in prey fishes– Fish Fish. 4: 227–234.

Chivers, D.P. & Smith, R.J.F. (1998). Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus– http://dx.doi.org.proxy1.cl.msu.edu/10.1080/11956860.1998.11682471 5: 338–352.

Crossland, M.R., Haramura, T., Salim, A.A., Capon, R.J., & Shine, R. (2012). Exploiting intraspecific competitive mechanisms to control invasive cane toads (Rhinella marina)– Proc. R. Soc. B Biol. Sci. 279: 3436–3442.

Di Rocco, R.T., Johnson, N.S., Brege, L., Imre, I., & Brown, G.E. (2016). Sea lamprey avoid areas scented with conspecific tissue extract in Michigan streams– Fish. Manag. Ecol. 23: 548–560.

Dyck, H. Van (2012). Changing organisms in rapidly changing anthropogenic landscapes: the significance of the ‘Umwelt’-concept and functional habitat for animal conservation– Evol. Appl. 5: 144–153.

Fabozzi, F.J., Focardi, S.M., Rachev, S.T., & Arshanapalli, B.G. (2014). Appendix E: Model Selection Criterion: AIC and BIC– Basics Financ. Econom. 399–403.

Ferrari, M.C.O., Elvidge, C.K., Jackson, C.D., Chivers, D.P., & Brown, G.E. (2010). The responses of prey fish to temporal variation in predation risk: Sensory habituation or risk assessment?– Behav. Ecol. 21: 532–536.

Ferrari, M.C.O., Wisenden, B.D., & Chivers, D.P. (2010). Chemical ecology of predator–prey interactions in aquatic ecosystems: a review and prospectusThe present review is one in the special series of reviews on animal–plant interactions.– Can. J. Zool. 88: 698–724.

Fissette, S.D., Buchinger, T.J., Wagner, C.M., Johnson, N.S., Scott, A.M., & Li, W. (2021). Progress towards integrating an understanding of chemical ecology into sea lamprey control– J. Great Lakes Res.

GLFC (n.d.). Great Lakes Fishery Commission - What is at Risk? http://www.glfc.org/what-is-at-risk.php.

Gotz, T. & Janik, V. (2016). Non-lethal management of carnivore predation: long-term tests with a startle reflex-based deterrence system on a fish farm– Anim. Conserv. 212–221.

Greggor, A.L., Berger-Tal, O., & Blumstein, D.T. (2020). The Rules of Attraction: The Necessary Role of Animal Cognition in Explaining Conservation Failures and Successes– Annu. Rev. Ecol. Evol. Syst. 51: 483–503.

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models– Biometrical J. 50: 346–363.

Hume, J.B., Luhring, T.M., & Wagner, C.M. (2020). Push, pull, or push–pull? An alarm cue better guides sea lamprey towards capture devices than a mating pheromone during the reproductive migration– Biol. Invasions 22: 2129–2142.

Hume, J.B., Meckley, T.D., Johnson, N.S., Luhring, T.M., Siefkes, M.J., & Wagner, C.M. (2015). Application of a putative alarm cue hastens the arrival of invasive sea lamprey (Petromyzon marinus) at a trapping location– Can. J. Fish. Aquat. Sci. 72: 1799–1806.

Imre, I., Di Rocco, R.T., Brown, G.E., & Johnson, N.S. (2016). Habituation of adult sea lamprey repeatedly exposed to damage-released alarm and predator cues– Environ. Biol. Fishes 99: 613–620.

Jutfelt, F., Sundin, J., Raby, G.D., Krång, A.S., & Clark, T.D. (2017). Two-current choice flumes for testing avoidance and preference in aquatic animals– Methods Ecol. Evol. 8: 379–390.

Lima, S.L. & Bednekoff, P.A. (1999). Temporal Variation in Danger Drives Antipredator Behavior: The Predation Risk Allocation Hypothesis.– Am. Nat. 153: 649–659.

Luhring, T.M., Meckley, T.D., Johnson, N.S., Siefkes, M.J., Hume, J.B., & Wagner, C.M. (2016). A semelparous fish continues upstream migration when exposed to alarm cue, but adjusts movement speed and timing– Anim. Behav. 121: 41–51.

Mcdiarmid, T.A., Yu, A.J., & Rankin, C.H. (2019). Habituation Is More Than Learning to Ignore : Multiple Mechanisms Serve to Facilitate Shifts in Behavioral Strategy 1900077: 1–10.

McNamara, J.M. & Dall, S.R.X. (2010). Information is a fitness enhancing resource– Oikos 119: 231–236.

Nielsen, B.L., Rampin, O., Meunier, N., & Bombail, V. (2015). Behavioral responses to odors from other species: Introducing a complementary model of allelochemics involving vertebrates– Front. Neurosci. 9: 1–11.

R Core Team (2020). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Rankin, C.H., Abrams, T., Barry, R.J., Bhatnagar, S., Clayton, D.F., Colombo, J., Coppola, G., Geyer, M.A., Glanzman, D.L., Marsland, S., McSweeney, F.K., Wilson, D.A., Wu, C.F., & Thompson, R.F. (2009). Habituation revisited: An updated and revised description of the behavioral characteristics of habituation– Neurobiol. Learn. Mem. 92: 135–138.

Schakner, Z.A. & Blumstein, D.T. (2013). Behavioral biology of marine mammal deterrents: A review and prospectus– Biol. Conserv. 167: 380–389.

Wagner, C.M., Stroud, E.M., & Meckley, T.D. (2011). A deathly odor suggests a new sustainable tool for controlling a costly invasive species– Can. J. Fish. Aquat. Sci. 68: 1157–1160.

Wisenden, B.D. (2015). Fish Pheromones and Related Cues– In: (Sorensen, P.W. & Wisenden, B.D., eds). John Wiley & Sons, Ltd, p. 131–148.

Wisenden, B.D., Binstock, C.L., Knoll, K.E., Linke, A.J., & Demuth, B.S. (2010). Risk-sensitive information gathering by cyprinids following release of chemical alarm cues– Anim. Behav. 79: 1101–1107.

CHAPTER 2: Activity level changes with pulsed and continuous alarm cue application in migratory sea lamprey (*Petromyzon marinus*)

Abstract

Habituation is a decreased response to a stimulus with repeated exposure. In an ecological context, organisms can learn from a repeated stimulus and save time and energy from not responding, allocating time to other resources that may increase their biological fitness such as foraging or reproduction. Sea lamprey (*Petromyzon marinus*) demonstrate a habituation to alarm cue after 4h of continuous exposure at a fixed concentration. Alarm cues indicate predation risk, as they are released from damaged tissues when an animal is injured, for example, from an attack by a predator. A typical alarm response involves spatial avoidance and increased swimming speed to swim upstream out of areas labeled with alarm cue. For a semelparous fish like a sea lamprey, who is migrating upstream to spawn, increasing their speed to avoid the predation event while continuing their migration path may be energetically efficient at their sub-adult life stage than spatially avoiding the risk. Sea lamprey are an invasive species of management concern in the Laurentian Great Lakes. Currently they are managed with lampricides and dams. Alarm cues, provide a new opportunity to manage species of concern via behavioral tools as they are species specific. However, if we are to use these tools, we must fully understand the spectrum of the animal's response to the odors. This study further examined the use of alarm cue as behavioral management tool and habituation prevention techniques. In this experiment, we examined two pulsing applications to prevent habituation: Pulse On/Off Alarm Cue, applied at 1 ppm and Pulse Low/High Alarm Cue, alternating between 1 ppm (low) and 10 ppm (high) with pulsing rates of 15 minutes. We compared these pulsing applications to a positive control, Water/Alarm Cue 1 ppm, and a habituation control, Continuous Alarm Cue 1 ppm. Each treatment was conditioned for 4h before individuals were tested in the behavioral assay. The behavioral assay was a small (1.22 x 1.22 m) testing arena in which we examined response to the alarm cue (1 ppm) as an activity metric for each treatment. As activity was a

stronger behavioral metric in the data collection in 2019, we switched to a smaller activity-based behavioral assay. We measured both activity and spatial avoidance before (Pre-Observation Period) and after (Post-Observation Period) the addition of alarm cue to the testing arena. We found activity differed across treatments before the alarm cue was applied in the Pre-Observation Period. The habituation control, Continuous Alarm Cue 1 ppm had the lowest activity in the Pre-Observation Period compared to the other conditioning treatments, which may have been indicative of a state difference based on their perception of the threat. Within a conditioning treatment, three out of four treatments had significantly increased activity from the Pre-Observation Period to the Post-Observation Period, suggesting detection and response to the predation risk. These conditioning treatments included: Water/Alarm Cue 1 ppm, Continuous Alarm Cue 1 ppm, and Pulse Low/High Alarm Cue 10 ppm. The conditioning treatment Pulse On/Off did not increase activity from the Pre-Observation Period to the Post-Observation Period, which may indicate response attenuation to the predation risk.

Introduction

Broadly, habituation is a decreased response to a stimulus with repeated exposure and in an ecological context it saves a prey species time and energy as a learned behavioral process. In a study done with the Honey Bee *(Tetragonisca angustula)*, habituation to the alarm pheromone varied based on the level of threat and type of bee within the colony: guards habituated to the alarm pheromone most rapidly at 2h, followed by foragers at 12h (Jernigan et al., 2018). Differences in habituation rates may be of ecological and energetic value as forgers maintain their antipredation response and guards do not expend too much energy (Jernigan et al., 2018). Male sea lamprey habituate to alarm cue after 4h of continuous exposure at a fixed concentration (Bals, 2012; Imre et al., 2016). Like the Honey Bee, sea lamprey may habituate to alarm cue for an ecological energetic value. Sea lamprey are semelparous, migrating upstream in their sub-adult phase to spawn. Habituating to a background risk may be more beneficial in terms of their biological fitness and risk assessment as upstream migration to the spawning ground is more profitable than responding to a predation cue , especially for a semelparous fish (Lima & Bednekoff, 1999; Ferrari et al., 2010; Luhring et al., 2016). Sea lamprey also vary their response to the alarm cue with sexual dimorphism, similar to the type of honey bee within the colony. Both male and female sub-adult migratory sea lamprey maintain their response to alarm cue with 20 minutes of exposure (Bals & Wagner, 2012). However, when females are sexually matured, their response to alarm cue attenuates, while sexually matured males response to alarm cue is maintained with 20 minutes of exposure (Bals & Wagner, 2012).

Alarm pheromones signal risk and elicit escape responses in social insects (Wyatt, 2014). In fish, alarm cues are a chemical mixture that are released from damaged tissues when an animal is injured, such as an attack by a predator, warning nearby conspecific species of predation risk. Many species, including sea lamprey, respond to their alarm cue with antipredation behaviors (Ferrari, Wisenden, & Chivers, 2010; Wagner, Stroud, & Meckley, 2011). Sea lamprey behaviorally respond to alarm cue with spatial avoidance and increased swimming speed to move out of the area activated by alarm cue (Hume et al., 2015; Di Rocco et al., 2016; Luhring et al., 2016).

Sea Lamprey are an important species of both management and conservation concern. Sea lamprey are an invasive species in the Great Lakes, feeding on the blood and tissues of native fish populations. Pacific lamprey are an imperiled species on the west coast. In the case of an invasive or imperiled species, behavioral manipulation through the application of semiochemicals may be an excellent management tool. Semiochemicals is a general term to describe chemicals used for communication between organisms (Wyatt, 2014). Lamprey heavily rely on semiochemicals, especially during their migration. During migration in the sub-adult phase, lamprey use semiochemicals to select a stream and find a mate

(Li et al., 2002; Sorensen et al., 2005; Wagner et al., 2006; Johnson et al., 2009). Additionally, they use semiochemicals, like alarm cues, during migration to avoid predation. As sea lamprey avoid areas activated with alarm cue, the cue can be used to guide lamprey towards a specified target (Hume et al., 2015; Hume, Luhring, & Wagner, 2020). This target may include a trap, or a stream targeted for TFM application. Alarm cue may also be used to keep sea lamprey away from a selective fish passage device or specific branch of a stream. Alternatively, alarm cue could be used to guide Pacific lamprey towards a fish passage device or an ideal spawning habitat.

As sea lamprey migrate during the nocturnal period, the ability to maintain the effect of the alarm cue response for 8-9 continuous hours will be necessary to maximize the number of individuals directed towards a targeted area. Response to the stimulus can be maintained by both the interstimulus interval (ISI) and intensity of the stimulus: the weaker the stimulus and more rapid the interval of the stimulus, the more pronounced and probable habituation is to occur (Mcdiarmid, Yu, & Rankin, 2019). The opposite is also true, the stronger the stimulus, and slower interval, less likely habituation is to occur (Mcdiarmid, Yu, & Rankin, 2019). Lamprey habituate to the alarm cue after 4 h of continuous exposure but do spontaneously recover after 1h of stimulus removal (Bals, 2012). Additionally, these studies showed when lamprey swam in and out of the stimulus over 4h (like a pulse), their response to alarm cue was maintained (Bals 2012).

The objective of this study was to examine pulse applications designed to prevent habituation to alarm cue from occurring in sea lamprey. We tested two alarm cue pulsing application schemes: *Pulse On/Off Alarm Cue* applied at 1 ppm and *Pulse Low/High Alarm Cue*, applied between 1 ppm (Low) and 10 ppm (High). Both pulsing applications were pulsed at a rate of 15 min over 4h. These pulsing applications were tested against a positive control, *Water/Alarm Cue 1 PPM* and a habituation control*, Continuous*

Alarm Cue 1 PPM, over 4h. After 4h of conditioning sea lamprey to their assigned treatment, their response to alarm cue was quantified in an activity-based behavioral assay at the USGS Hammond Bay Biological Station, Roger City Michigan.

Methods

Study Design

This study examined two pulsing applications designed to prevent habituation: Pulse On/Off Alarm Cue applied at 1 ppm and Pulse Low/High Alarm Cue, alternating between 1 ppm (Low) and 10 ppm (High) at a pulsing rate of 15 min over 4h. The pulsing applications were compared to a positive control, Water/Alarm Cue 1 PPM, which conditioned sea lamprey to water and a habituation control, Continuous Alarm Cue 1 PPM, conditioning sea lamprey to alarm cue, 1 ppm. Sea lamprey were conditioned to their treatment in individual holding baskets for 4h before they were released into a

1.2 x 1.2 m section of the behavioral assay, the testing arena. The behavioral assay consisted of four periods: 1) Acclimation, 2) Pre-Stimulus Observation (Pre), 3) Alarm Cue On, and 4) Post-Stimulus Observation (Post). In the first two periods, the fish was allowed to swim and explore the arena without any cue present. In the Alarm Cue on period, Alarm Cue, 1 ppm, was pumped down the *stimulus side* of the testing arena and continued pumping through the Post-Stimulus Observation period for behavioral testing. For each trial, the Pre and Post periods were quantified with an ethogram in BORIS. The exported state behavioral data from BORIS was converted to a composite activity level score, 1 having the lowest activity (resting) to 3 having the highest activity (frequent darting). Other behaviors were also quantified, such as event behaviors, measured by frequency of occurrence, and overall space use in the assay to assess stimulus avoidance. Event behaviors included sharp turns, a movement at 90° or greater and breaching, in which the sea lamprey's head emerges out of the water column.

If a *pulsing treatment* were to prevent habituation from occurring, we would predict:

Pre-Observation Period

(Prediction #1): Similar activity vs the Positive Control (Water) (composite score, # of sharp turns, # of breaches)

(Prediction #2): Increased activity vs the Habituation Control

Post Observation Period

(Prediction #3): Increased activity in response to alarm cue

(Prediction #4): Stimulus side avoidance of the alarm cue

Experimental Subjects

Lamprey were collected through a collaborative effort by the U.S. Geological Service and U.S. Fish and Wildlife Service during the annual sea lamprey monitoring program in July of 2020 from the Ocqueoc River, Cheboygan River and Carp Lake River, Michigan. Male sea lamprey that did not exhibit signs of sexual maturity (e.g. lack of well-developed dorsal rope, blue coloration) nor external injuries were used in this study (n=100) as female lampreys start to diminish their response to alarm cue with maturation (Bals & Wagner, 2012) (total length range: 34-54 cm, total length mean ± sd: 46 cm± 4/ weight range: 72- 327 g, weight mean ± sd: 194± 49 g). Animal handling and experimentation procedures were approved by the Michigan State University Institutional Animal Care and Use Committee under permit number AUF 03/18-039-00.

Alarm Cue Preparation

Due to constraints from COVID-19 pandemic, alarm cue was used from batches extracted in 2015. To have enough alarm cue for the experiments in this study, we combined nine batches of alarm cue made in 2015, which was Soxhlet extracted from 81 lamprey (whole-body) following procedures as described by Bals and Wagner 2012. Euthanized or freshly deceased sea lamprey were kept at -20°C until whole-body alarm cue extraction could take place. Before alarm cue extraction, sea lamprey were thawed, weighed, and rinsed with DI water. To summarize, the extraction was set up as follows (top to bottom): an Allihn condenser was attached to a 1-L 71/60 Soxhlet Extractor Body (Ace Glass Inc., Vineland, New Jersey), this contained the lamprey skin, which was attached to a 1-L round bottom flask, which contained the solvent (50:50 weight/weight of 200-proof ethyl alcohol and deionized water). The round bottom flask was heated by a hemispherical mantle at 75-80 °C. Each extraction cycled three times through the Soxhlet extractor body. The final concentration of the extract was equivalent to 221 mg of tissue per ml of alarm cue extract. The alarm cue extraction was stored at -20°C until use.

Behavioral Assay

All trials occurred from approximately 19:00 to 02:00 as sea lamprey migrate nocturnally, from July 1st-10th, 2020. As activity was a stronger behavioral metric than spatial avoidance in the Y-maze in 2019, we changed apparatus designs from the Y-maze to a smaller, activity-based behavioral assay. The behavioral assays were prefabricated out of HDPE paneling and installed side-by side into the existing raceways at HBBS (Fig 5). Water was supplied to the raceways from Lake Huron, the flow was maintained at 0.01 m^3/s and the temperature during the study ranged from 11.9°C to 15.6°C. In total, the behavioral arena was 2.43 by 1.22 meters and divided with netting to split the assay into two segments: a mixing zone for the stimulus (1.2 x 1.8 m) and the testing arena (1.2 x 1.8 m) (Fig 5). Infrared lights and a video camera (Lorex 8-Channel 4K UHD NVR with 2TB HDD and 4 5 MP Night Vision

Bullet) were installed above the testing arena of the assay. Odor for the assay was kept in 1000 ml Nalgene bottle aliquots in coolers on ice until use. Odor was continuously mixed with a stir plate and pumped into the assays via peristaltic pumps (MasterFlex model 7533-20) at a rate of 20 ml/min upstream of the mixing zone of the assay. As arenas were adjacent in each raceway, one was used for conditioning the fish (*Conditioning Arena*) and one was used for analyzing their behavior (*Testing Arena*) (Fig 5). To condition the fish to their assigned treatment, individual lamprey were held in mesh rectangular holding baskets (15 x 30.5 x 30.5 cm) in the testing arena of the assay. The treatment pumped over the fish for 4h at a rate of 20ml/min via peristaltic pumps. After the 4-h treatment in the first assay, individual baskets were gently lifted into the behavioral assay, opened and the fish was released into the center of the behavioral assay. Four treatment groups examined pulse applications as a technique to prevent response attenuation from occurring (Table 5).

Table 5. Treatment descriptions for pulsing experiment. Sample size for each treatment is under column N.

Treatment Duration = 4 Hours	**Experimental Predictor**	**Description**	**N**
Continuous Distilled Water	Negative Control	Pumped distilled water over lamprey for four hours.	20
Continuous Alarm Cue [1 PPM]	Positive Control	Pumped 1 PPM alarm cue for four hours over lamprey.	21
Pulse On/Off Alarm Cue Off/ [1 PPM]	Pulse Application 1	Pumped 1 PPM alarm cue for four hours over lamprey, turning alarm cue off and on at 15-minute pulse.	23
Pulse Low/High Alarm Cue [1 PPM]/ [10 PPM]	Pulse Application 2	Pulsed alarm cue between a 1 PPM and 10 PPM dilution for four hours at a 15-minute pulse.	22

(a)

(b)

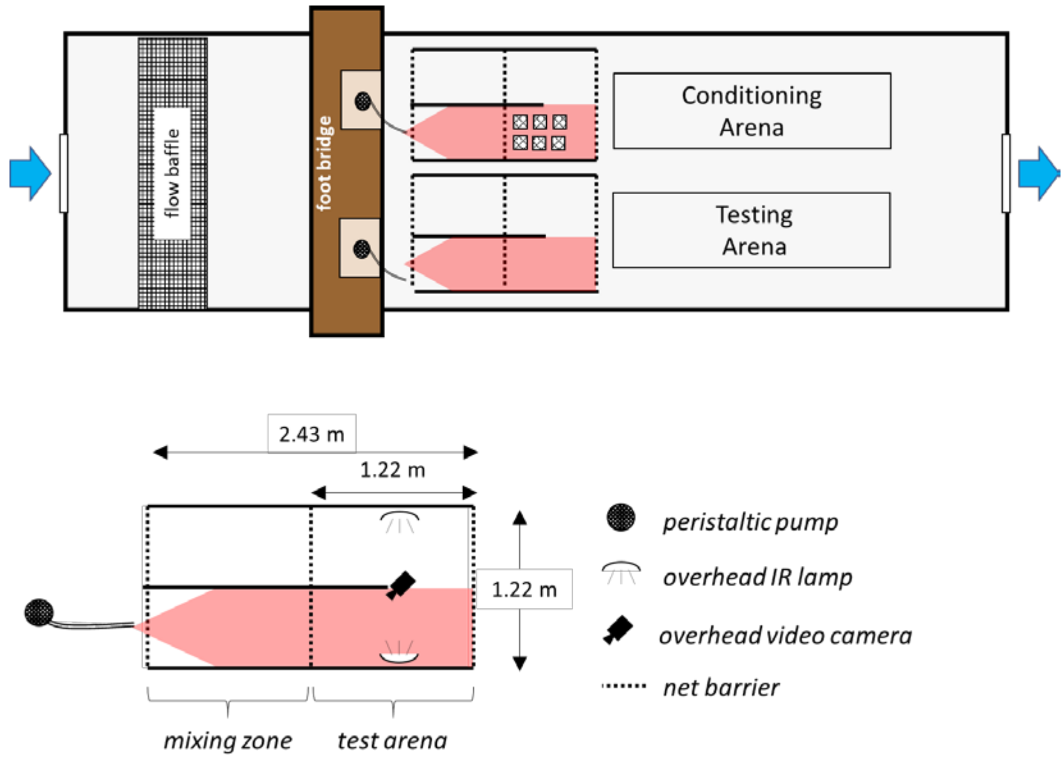


Figure 5. Diagram of pre-treatment and behavioral assays (a). Water flowed in from Lake Huron (left to right). One arena was used to conditioning the fish with one of following treatments for four hours: Water/Alarm Cue 1 PPM, Continuous Alarm Cue 1 PPM, Pulsed On/Off Alarm Cue or Pulsed Low/High Alarm Cue. Following conditioning treatment, individual fish were gently released into the adjacent arena for behavioral testing. A detailed representation of the behavioral assay is shown in b.

Regardless of their conditioning treatment, all fish were tested for behavioral response to the alarm cue (1 ppm) in the testing arena. The behavioral assay consisted of four periods: 1) Acclimation, 2) Pre-Stimulus Observation (Pre), 3) Alarm Cue On, and 4) Post-Stimulus Observation (Post). The first 5 min in the assay was the Acclimation Period, the fish could explore the arena and no behavioral data were collected. The following 5 min were the Pre-Stimulus Observation Period (Pre), in which we started collecting behavioral data to examine nominal behavioral tendencies in the assay. In the third period, Alarm Cue On, alarm cue (1 ppm) was pumped down one-half of the assay and continued pumping until the end of the trial. This period lasted 2 min as this was the time it took for the Rhodamine dye to reach the end of the of the assay in preliminary testing of the arenas. (Note: Rhodamine dye was not used in with any of the conditioning treatments or applications in the testing arena). Alarm cue stimulus sides were switched in between trials to prevent side-favoring bias. In the last period, Post-Stimulus Observation (Post), the behavioral response to alarm cue was examined for 10 min.

Behavior responses were quantified using Behavioral Observation Research Interactive Software (BORIS), version 7.9.8 (Friard & Gamba, 2016). Videos were scored in a random order and the scorer was blind to the trial's treatment and stimulus side. For each trial, the Pre and Post periods were scored. The Post period for each fish began after the fish encountered the stimulus side. The ethogram used to quantify these responses are shown in Table 6. The data exported from BORIS document the lamprey's state and frequency behaviors during the entire trial. State behaviors measured the duration (in seconds) of activity levels (AL1, AL2, or AL3) of the fish. Two event behaviors, measured as number of occurrences were also quantified: sharp turns and breaching. A sharp turn is a swimming movement at 90° or greater. A breaching behavior was recorded when the sea lamprey's head emerged out of the water column.

Table 6. Ethogram used with BORIS to quantify behavioral responses to alarm cue.

Behavior		**Description**
STATE BEHAVIORS		
Activity Level	1	Lamprey at rest. Attached to arena with oral disk or is laying on the bottom of arena unmoving and unattached.
	2	Lamprey is actively swimming in the arena at a consistent nominal speed with infrequent turns, without darting; rarely breaches the surface.
	3	Lamprey increases speed compared to activity level 2. Frequent darting, turns, and breaching of the surface.
FREQUENCY BEHAVIORS		
Sharp Turns		Lamprey turns at 90-degree angle or greater in open water
Breaching		Lamprey's head emerges out of water briefly with body vertical in water column.

Statistical Analysis

Across all treatments, 100 fish were originally included in this data set. If a fish did not swim onto the stimulus side during the Post period, they were removed from the data, as they presumably did not detect the alarm cue. Of the fish that were removed from analysis due to not swimming onto the stimulus side, two were Pulse Low/High Alarm Cue, one was Pulse On/Off Alarm Cue, four were Water/Alarm Cue 1 PPM, and three were Continuous Alarm Cue 1 PPM. Two Low/High Alarm Cue trials were lost due to a power shortage at the end of night and two Water/Alarm Cue 1 PPM trials were lost due to an equipment malfunction. The total number of subjects included in analysis were 86 across all treatments. Data analysis was performed using R (R Core Team 2020).

To examine side use, the assay was divided in half, the stimulus side and non-stimulus side. The amount of time each fish spent on the stimulus side was divided by the period total to create a proportion. A one-tailed t-test and Wilcox's rank test ($\alpha = 0.05$) was used to assess whether the proportion of time spent on the stimulus side was less than 50% for each treatment. If no spatial avoidance of alarm cue was observed, sea lamprey will spend half their time on the stimulus side ($P = 0.5$, H_0). If there is evidence of spatial avoidance of alarm cue, sea lamprey will spend less than half their time on the stimulus side ($P < 0.5$, H_A).

For each trial, the time spent in a behavioral state (AL1, AL2, or AL3) was converted to a proportion for the Pre and Post Periods. The total time a sea lamprey spent in an activity level was divided by the period duration (in seconds). With BORIS, we captured a continuous behavioral response to the alarm cue in three distinct activity levels, from least active to most active (1 to 3). These levels were translated into a composite activity score as shown in Fig 6. Each behavioral activity level was multiplied by an integer, increasing by one as the activity level increased. The frequency behaviors were also converted to a proportion. The total count for each frequency behavior in a period was divided by the period duration (in seconds). This was then converted to an event per minute rate by multiplying the proportion by 60 seconds. The effect of treatment on activity and events per minute was assessed using a Kruskal-Wallis test ($\alpha = 0.05$). When applicable, it was followed up with a post-hoc Dunn's test for multiple comparisons ($\alpha = 0.05$). To compare differences within treatments, Pre to Post, paired t-tests ($\alpha = 0.05$) and Wilcox's rank test ($\alpha = 0.05$) were used.

$$\boxed{(\propto AL1 \times 1) + (\propto AL2 \times 2) + (\propto AL3 \times 3) = Composite\ Activity\ Score}$$

Figure 6. Equation used to calculate composite activity score for each lamprey in the Pre and Post Periods of the behavioral assay.

Results

The proportion of time spent on the stimulus side did not differ from 50% in the Post Period of the behavioral assay for any conditioning treatment (Fig. 7). On average, the animals in the Water/Alarm Cue condition spent 52% of their time on the stimulus side (t_{19} = 0.53, μ= 0.50, *p*=0.69). The Continuous Alarm Cue condition spent 56% of their time on the stimulus side (Wilcoxon's rank test$_{20}$=143, μ=0.50, *p*=0.83). The Pulse On/Off Alarm Cue condition spent 52% of their time on the stimulus side (t_{22} = 0.53, μ= 0.50, *p*=0.69) and Pulse Low/High Alarm Cue condition spent 50% of their time on the stimulus side (t_{21} = 0.06, μ= 0.50, *p*=0.53). Side use also did not differ from 50% in the Pre-Period of the assay for any conditioning treatment: Water/Alarm Cue (t_{19} = 1.5, μ= 0.50, *p*=0.92), Continuous Alarm Cue (Wilcoxon's rank test$_{20}$=169.5, μ=0.50, *p*=0.97), Pulse On/Off Alarm Cue (Wilcoxon's rank test(22)=138, μ=0.50, *p*=0.51), and Pulse Low/High Alarm Cue (t_{21} = -0.18, μ= 0.50, *p*=0.43).

Conditioning treatment effected the overall activity score in the Pre-Period (H_3 = 9.69, *p*= 0.0214), (Fig. 8). Post-hoc comparisons indicated that, on average, sea lamprey in Water/Alarm Cue were 18% more active than those conditioned with Continuous Alarm Cue (Dunn, *p*= 0.0283). Sea lamprey in Pulse Low/High Alarm Cue were also 21% more active than those in Continuous Alarm Cue (Dunn, *p*= 0.0031) (Fig. 4).

Within treatment groups, change in activity was observed between the Pre and Post Periods of the assay (Fig. 9). The sea lamprey in the Water/Alarm Cue conditioning treatment increased their activity (Pre to Post) by 11% (t_{19} = 3.2703, *p*=0.004). Sea lamprey conditioned with Continuous Alarm Cue also increased their activity by 14% (t_{20} = 3.8226, *p*= 0.001). No change was observed for the conditioning treatment Pulse On/Off Alarm Cue (t_{22} = 1.5, *p*= 0.13). Sea lamprey conditioned with Pulse Low/High Alarm Cue increased their activity by 7% (Wilcoxon's rank test $_{21}$ = 182, *z*=0.4891, *p*=0.02179). This behavioral assay measured response with individual fish and individual variation within treatments was observed (Fig. 10).

In the Pre-Period, treatment also effected the number of Breaches per minute (H_3 = 11.8, p = 0.00807), (Fig. 11). Post-hoc comparisons indicated on average, sea lamprey conditioned with Water/Alarm Cue had 0.97 more Breaches per minute compared to sea lamprey conditioned with Continuous Alarm Cue (Dunn, p= 0.0401). Sea lamprey conditioned with Pulse Low/High Alarm Cue had 1.5 more Breaches per minute compared to the Continuous Alarm Cue conditioning treatment (Dunn, p= 0.000763) and 0.66 more breaches compared to the Pulse On/Off Alarm Cue conditioning treatment (Dunn, p=0.045), (Fig. 11). There were no difference in the number of Sharp Turns per minute across treatments in the Pre-Period (H_3=1.56, p=0.668), (Fig. 12).

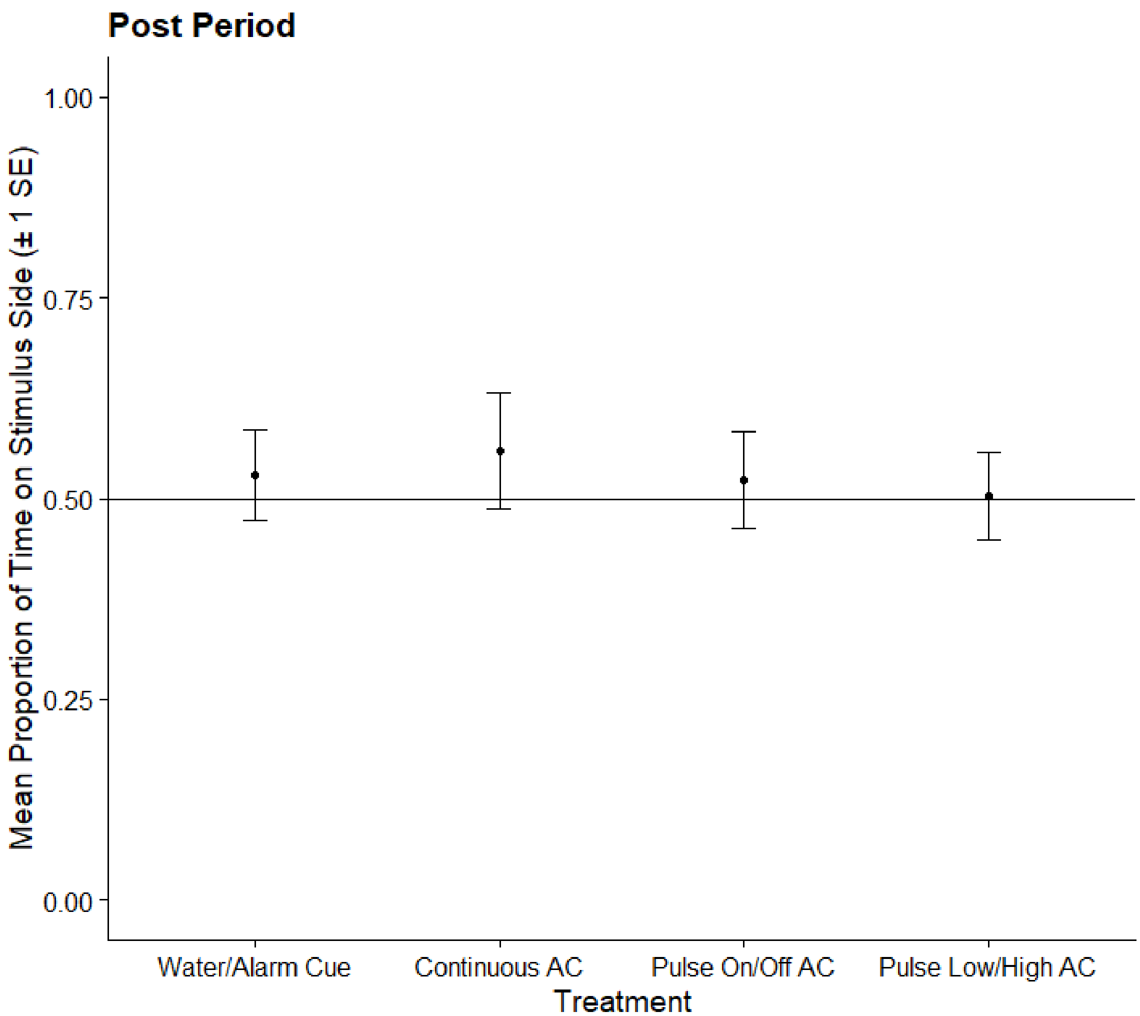


Figure 7. Mean (±1 SE) proportion of time spent on the stimulus side for each conditioning treatment in the Post Period of the behavioral assay. One-tailed t-tests and Wilcox's rank tests were used to assess whether time spent on the stimulus side was less than 0.50.

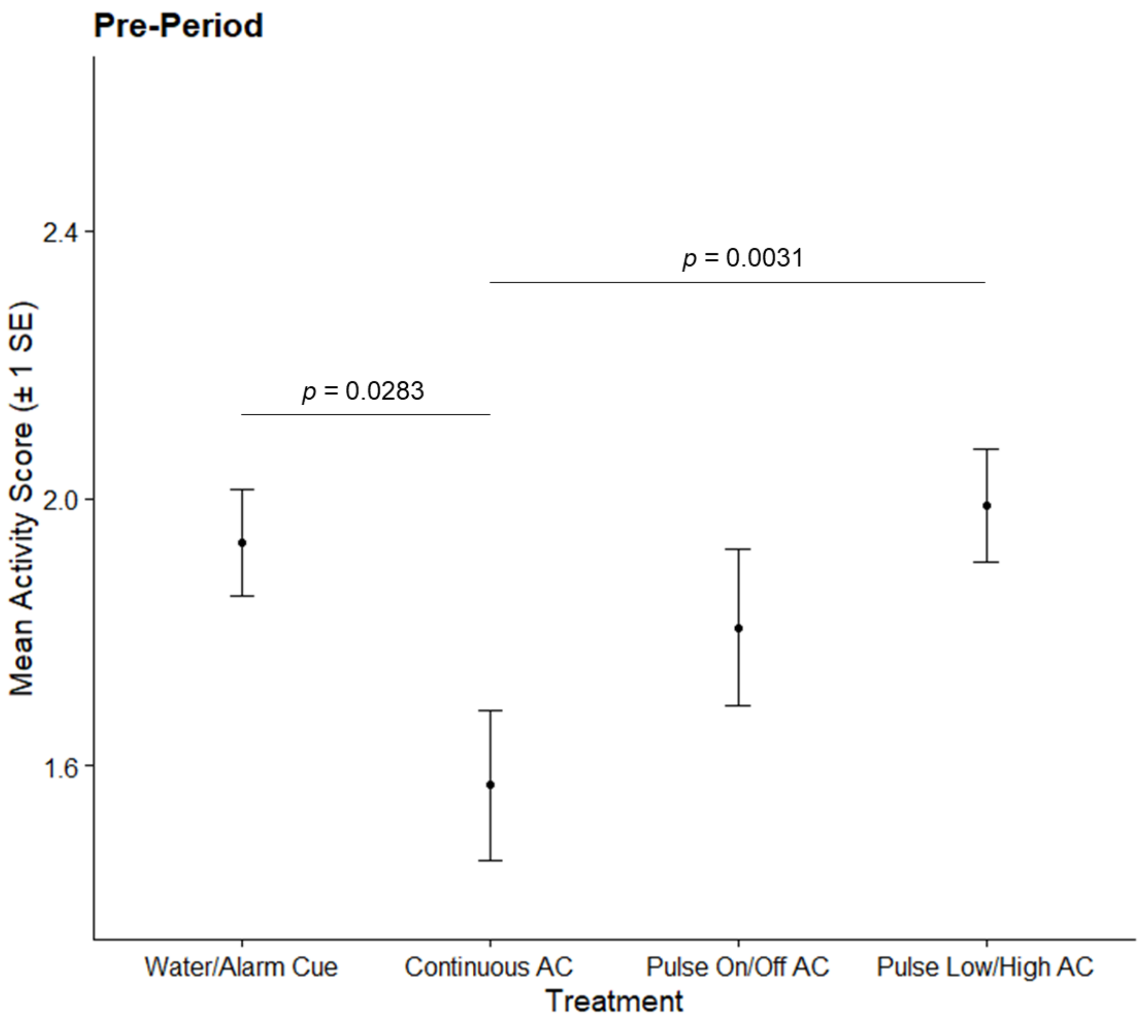


Figure 8. Mean (±1 SE) activity score for each treatment group in the Pre-Period of the behavioral assay. Only significant comparisons are shown, using a Dunn's test for multiple comparisons.

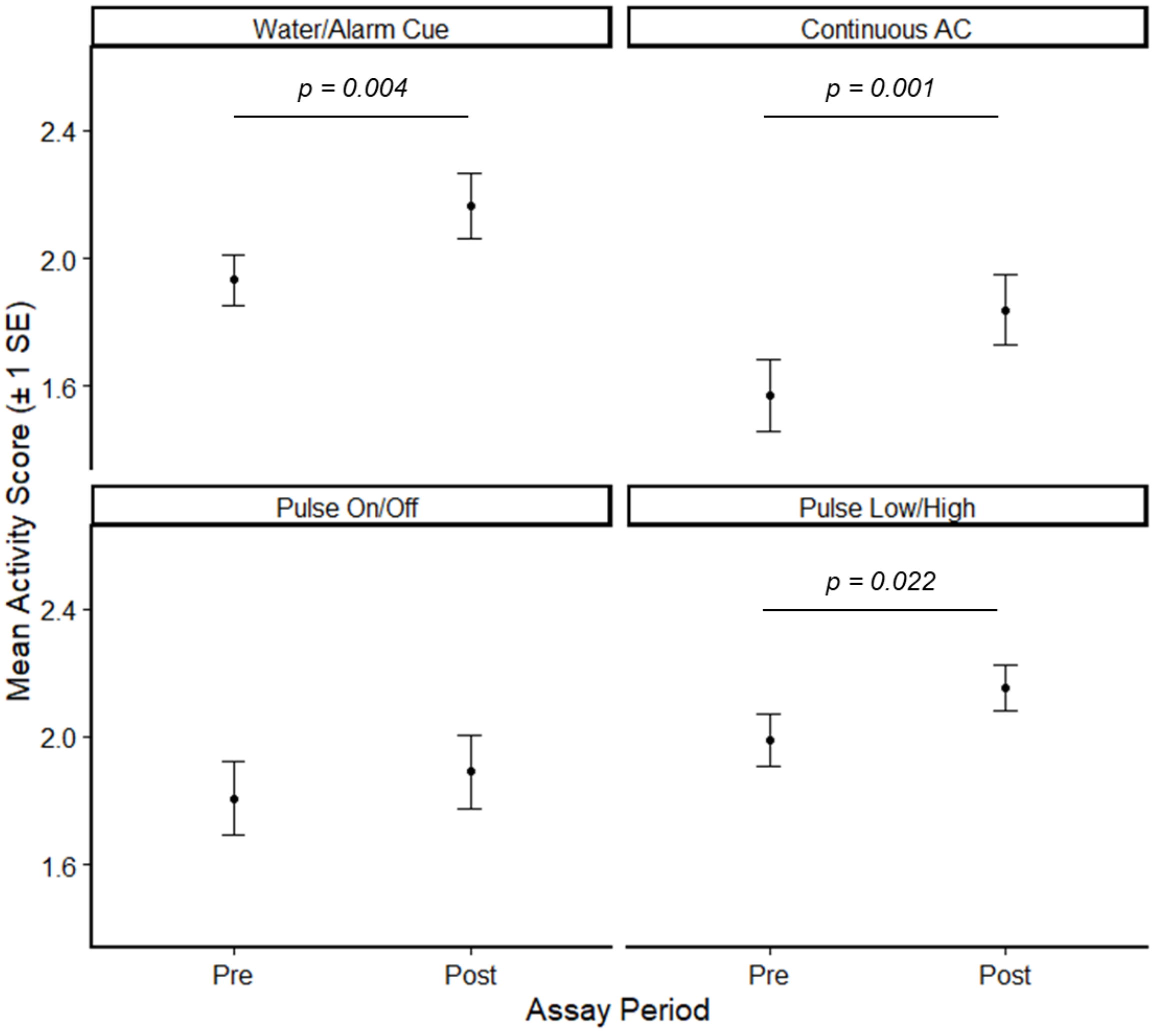


Figure 9. Mean (±1 SE) activity score for each treatment group in the Pre and Post Periods of the behavioral assay. Only significant comparisons are shown, using a paired t-test and Wilcox's rank test (Pulse Low/High AC only).

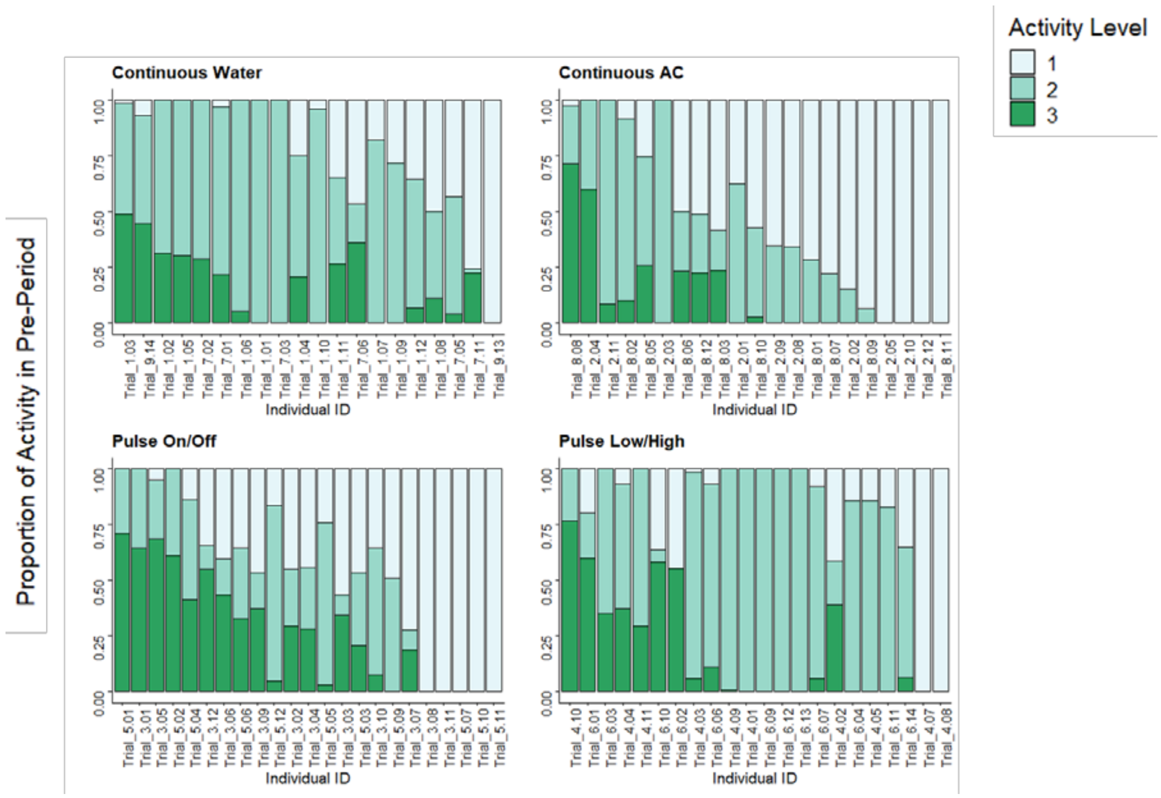


Figure 10. Individual Activity Level proportions during the Pre-Period for each treatment. Activity levels range from 1 (least active) to 3 (most active).

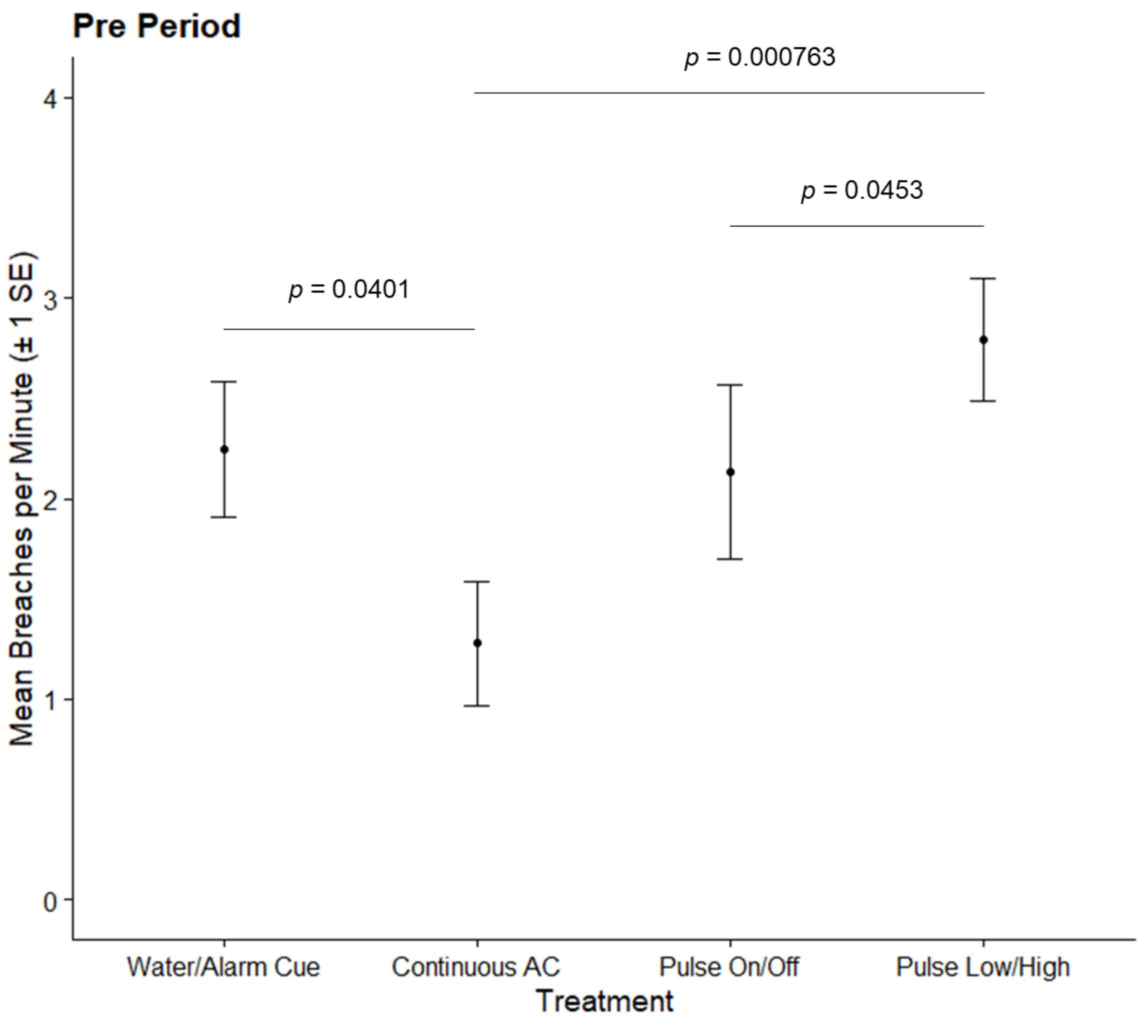


Figure 11. Mean (± 1 SE) Breaches per Minute in Pre-Period across treatments. Only significant comparisons are shown, using a Dunn's test for multiple comparisons

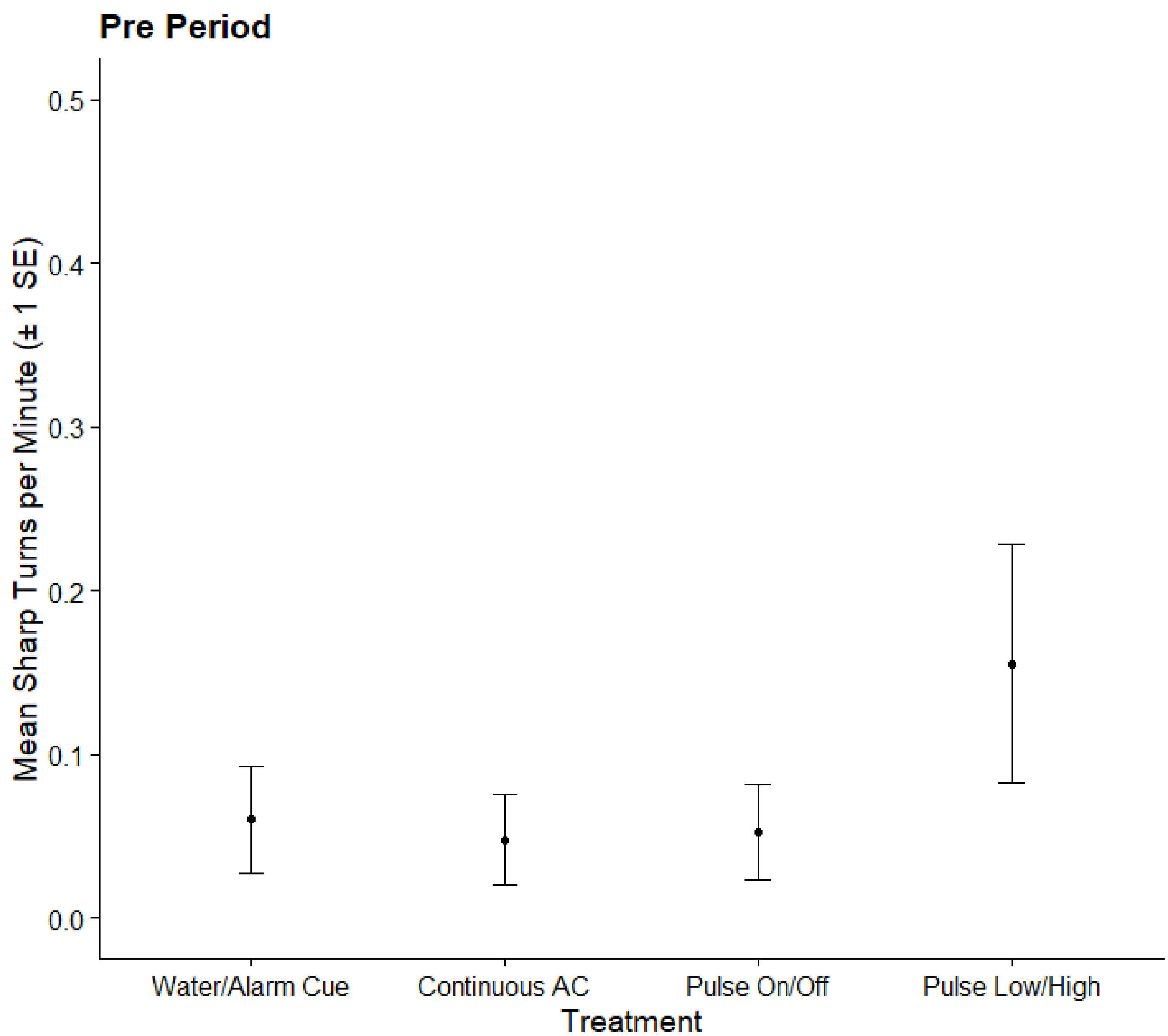


Figure 12. Mean (± 1 SE) Sharp Turns per Minute in Pre-Period across treatments. Only significant comparisons are shown, using a Dunn's test for multiple comparisons.

Discussion

The present study suggests that conditioning to a chemical indicator of predation risk alters baseline activity rates in sea lamprey consistent with reduced movement after exposure to a high-risk circumstance. When exposed to a continuous, fixed concentration of alarm cue for 4 h, sea lamprey exhibited a 18% reduction in activity rate (vs. control). Continuous Alarm Cue had the lowest activity and was significantly different from both Water/Alarm Cue and Pulse Low/High, but not Pulse On/Off. As Water/Alarm Cue received water as a treatment for 4h, we would predict this activity level to be nominal. Water/Alarm Cue and Pulse Low/High had equal activity levels in the pre-period, and Pulse On/Off had intermediate activity levels, indicating a partial response or degradation. The number of Breaches/min also followed a similar pattern as activity levels: Continuous Alarm Cue was lowest, Pulse On/Off was intermediate, and Pulse Low/High and Water/Alarm Cue were the highest, suggesting it may be a good measure of activity. Overall, the low, intermediate, and high activity differences corresponding with treatment (Continuous Alarm Cue, Pulse On/Off, and Water/Alarm Cue and Pulse Low/High), respectively.

The difference in activity in the Pre-Stimulus Observation Period across treatments may be due to a state difference, characterized by a reduction in activity. Many animals adjust their response, and activity based on their perceived threat (Helfman & Winkelman, 1997; Brown et al., 2006). Sea lamprey respond in a threat-sensitive manner to alarm cue, adjusting their timing and speed to evade this predation cue (Luhring et al., 2016). Fish in the Continuous Alarm Cue treatment were conditioned to alarm cue for 4h, the lamprey may have perceived a continual high risk and responded with a reduction in activity. Pulse On/Off also had a reduction in activity in the Pre-Stim. period, but not Pulse Low/High. Pulse Low/High was equivalent to the positive control, Water/Alarm Cue. State differences between the pulsing treatments may be attributed to the interstimulus interval (ISI) effect compounded with the intensity of the stimulus effect: the weaker the concentration and more rapid the interval of the stimulus, the more likely response attenuation will occur (Mcdiarmid, Yu, & Rankin, 2019). The ISI for Continuous Alarm Cue was lesser than either of the pulsing treatments as it was had an ISI of zero compared to 15 min for the pulsing treatments. Additionally, Pulse Low/High had greater concentrations within its intervals than Pulse On/Off, alternating between 1 ppm and 10 ppm alarm cue, rather than 1 ppm and water.

Three out of four treatments increased their activity levels in the Post Observation Period (Post), suggesting sensory detection and response to alarm cue. The increased response in the positive control, Water/Alarm Cue indicates perceived alarm cue detection and response. Surprisingly, the habituation control, Continuous Alarm Cue also had significant increased activity, indicating that animals in this treatment also detected and responded to the alarm cue. We predicted to observe no difference Pre to Post in the habituation control, indicating habituation. Previous studies that observed habituation to alarm cue in sea lamprey after 4h, quantified response to the cue as spatial avoidance (Bals, 2012) rather than activity, as used in this study. In this study, we may still be observing responses to the alarm cue in the habituation control after 4h as our behavioral assay primarily measured activity, not spatial avoidance. While we did not observe the predicted response in the habituation control, we did observe the predicted response in the positive control. Even though we did not observe a fully attenuated response for the habituation control, we may have observed a shift in response as the Continuous Alarm Cue was not equivalent to the positive control, Water/Alarm Cue. Habituation can include multiple mechanisms and animals can start to shift or alter their behaviors before they are fully habituated (Mcdiarmid, Yu, & Rankin, 2019). Pulse On/Off did not have any changes Pre to Post. Pulse Low/High significantly increased their activity Pre to Post, indicating detection and response to the alarm cue. Besides Pulse On/Off, alarm cue increased activity levels in all treatments in the post-period.

Increased activity pre to post alarm cue exposure for three out of four of the treatments may be attributable to changes in sea lamprey's perception of the risk. The conditioning treatment may have caused sea lamprey to adjust their response to the cue in a threat-sensitive manner; behaviorally responding to both changes in their perception and risk of the predation signal (Helfman & Winkelman, 1997; Brown et al., 2006). Sea lamprey in both Water/Alarm Cue and Pulse Low/High responded with a high-risk response as they increased their activity Pre to Post and had a strong response (i.e., flight). In short, these treatments detected the risk (high) and maintained their response to the cue. An intermediate-risk response was demonstrated with sea lamprey in Pulse On/Off, which did not differ from the other treatments. We may consider Pulse On/Off as responding with an intermediate-risk response and partially habituated as they had partial activity in the Pre-Period but did not increase activity Pre to Post. The Continuous Alarm Cue responded to the low risk, but not to the same activity level as Water/Alarm Cue or Pulse Low/High (high-risk), also indicative of a habituated response. In the case of Continuous Alarm Cue, after 4h of continuous exposure, the risk was perceived as low, and resulted in weak response (i.e., vigilance). The conditioning treatment changed the perception of the predation risk and in turn, the sea lamprey shifted their response in a threat-sensitive manner.

Spatial avoidance of alarm cue was not observed for any conditioning treatment. Compared to the assay used in Ch. 1, this behavioral assay was longer in duration, in accordance with previous studies that indicated spatial avoidance arises after 5-7 min in the arena (Wagner, Stroud, & Meckley, 2011). This assay had a 5-min acclimation period, a 5-min pre-observation period, 2 min for the alarm cue plume to stabilize and 10 min for the behavioral observation period. The lack of stimulus avoidance was most likely due to the relatively small size of the arena. In prior studies that examined space use to alarm cue, in sea lamprey the behavioral assay was longer, with a length of 5 m (≈ 17 body lengths), compared to our assay length of 1.2 m (≈ 4 body lengths) (Wagner, Stroud, & Meckley, 2011; Bals & Wagner, 2012;

Byford et al., 2016; Hume & Wagner, 2018). Large fish like sea lamprey require enough space for movement to reduce stress caused by confinement (Jutfelt et al., 2017). Too small of an arena may have led to an increased activity and the animal actively searching for an escape route.

The application, Pulse Low/High, has the best implications for management purposes. Sea lamprey in Pulse Low/High were overall most similar to those conditioned with Water/Alarm Cue, the positive control and differed from sea lamprey conditioned with Continuous Alarm Cue, the habituation control. Additionally, the Pulse Low/High conditioning treatment increased their activity Pre to Post. The Pulse Low/High application also supports results found in Ch. 1, combining two treatments that best prevented response attenuation: Continuous Alarm Cue 10 PPM and Pulse 10 PPM AC. Pulse Low/High, both pulses the alarm cue (ISI effect) and varies the concentration (intensity of the stimulus) (Mcdiarmid, Yu, & Rankin, 2019). Finally, the Pulse Low/High application is practical for field application as there will be continuous alarm cue for sea lamprey to detect and respond to. A pulse application such as Pulse On/Off may be problematic in field applications as there would be periods of time without alarm cue and sea lamprey may miss the targeted area (i.e., a trap) as they didn't have their guiding odor, alarm cue. Future studies applying a pulsed alarm cue between low and high concentrations in the field would be informative to further test the implications for management purposes. Additionally, examining the pulsing rate would be of interest. Does altering the concentration every 15 min maintain alarm response in the field? Adjusting the ISI to longer or varying rates (i.e., random, staying under 2h to prevent habituation) may be informative to further explore habituation prevention techniques. A longer ISI would save on resources and is known to lead to slow or no habituation (Rankin et al., 2009). Additionally, varying the rates of the ISI may maintain the response as there would not be a pattern to the inter-stimulus rate.

REFERENCES

REFERENCES

Bals, J. (2012). The sea lamprey alarm response: field and laboratory investigations. MS Thesis. Michigan State University.

Bals, J. & Wagner, C.M. (2012). Behavioral responses of sea lamprey (*Petromyzon marinus*) to a putative alarm cue derived from conspecific and heterospecific sources– Behaviour 149: 901–923.

Brown, G.E., Rive, A.C., Ferrari, M.C.O., & Chivers, D.P. (2006). The dynamic nature of antipredator behavior: Prey fish integrate threat-sensitive antipredator responses within background levels of predation risk– Behav. Ecol. Sociobiol. 61: 9–16.

Byford, G.J., Wagner, C.M., Hume, J.B., & Moser, M.L. (2016). Do native pacific lamprey and invasive sea lamprey share an alarm cue? Implications for use of a natural repellent to guide imperiled pacific lamprey into fishways– North Am. J. Fish. Manag. 36: 1090–1096.

Di Rocco, R.T., Johnson, N.S., Brege, L., Imre, I., & Brown, G.E. (2016). Sea lamprey avoid areas scented with conspecific tissue extract in Michigan streams– Fish. Manag. Ecol. 23: 548–560.

Ferrari, M.C.O., Elvidge, C.K., Jackson, C.D., Chivers, D.P., & Brown, G.E. (2010). The responses of prey fish to temporal variation in predation risk: Sensory habituation or risk assessment?– Behav. Ecol. 21: 532–536.

Ferrari, M.C.O., Wisenden, B.D., & Chivers, D.P. (2010). Chemical ecology of predator–prey interactions in aquatic ecosystems: a review and prospectusThe present review is one in the special series of reviews on animal–plant interactions.– Can. J. Zool. 88: 698–724.

Friard, O. & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations– Methods Ecol. Evol. 7: 1325–1330.

Helfman, G.S. & Winkelman, D.L. (1997). Threat Sensitivity in Bicolor Damselfish: Effects of Sociality and Body Size– Ethology 103: 369–383.

Hume, J.B., Luhring, T.M., & Wagner, C.M. (2020). Push, pull, or push–pull? An alarm cue better guides sea lamprey towards capture devices than a mating pheromone during the reproductive migration– Biol. Invasions 22: 2129–2142.

Hume, J.B., Meckley, T.D., Johnson, N.S., Luhring, T.M., Siefkes, M.J., & Wagner, C.M. (2015). Application of a putative alarm cue hastens the arrival of invasive sea lamprey (Petromyzon marinus) at a trapping location– Can. J. Fish. Aquat. Sci. 72: 1799–1806.

Hume, J.B. & Wagner, M. (2018). A death in the family: Sea lamprey (Petromyzon marinus) avoidance of confamilial alarm cues diminishes with phylogenetic distance– Ecol. Evol. 8: 3751–3762.

Imre, I., Di Rocco, R.T., Brown, G.E., & Johnson, N.S. (2016). Habituation of adult sea lamprey repeatedly exposed to damage-released alarm and predator cues– Environ. Biol. Fishes 99: 613–620.

Jernigan, C.M., Birgiolas, J., McHugh, C., Roubik, D.W., Wcislo, W.T., & Smith, B.H. (2018). Colony-level non-associative plasticity of alarm responses in the stingless honey bee, *Tetragonisca angustula*– Behav. Ecol. Sociobiol. 72.

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

Jutfelt, F., Sundin, J., Raby, G.D., Krång, A.S., & Clark, T.D. (2017). Two-current choice flumes for testing avoidance and preference in aquatic animals– Methods Ecol. Evol. 8: 379–390.

Li, W., Scott, A., Siefkes, M., Yan, H., Liu, Q., Yun, S.-S., & Gage, D. (2002). Bile acid secreted by male sea lamprey that acts as a sex pheromone.

Lima, S.L. & Bednekoff, P.A. (1999). Temporal Variation in Danger Drives Antipredator Behavior: The Predation Risk Allocation Hypothesis.– Am. Nat. 153: 649–659.

Luhring, T.M., Meckley, T.D., Johnson, N.S., Siefkes, M.J., Hume, J.B., & Wagner, C.M. (2016). A semelparous fish continues upstream migration when exposed to alarm cue, but adjusts movement speed and timing– Anim. Behav. 121: 41–51.

Mcdiarmid, T.A., Yu, A.J., & Rankin, C.H. (2019). Habituation Is More Than Learning to Ignore : Multiple Mechanisms Serve to Facilitate Shifts in Behavioral Strategy 1900077: 1–10.

Rankin, C.H., Abrams, T., Barry, R.J., Bhatnagar, S., Clayton, D.F., Colombo, J., Coppola, G., Geyer, M.A., Glanzman, D.L., Marsland, S., McSweeney, F.K., Wilson, D.A., Wu, C.F., & Thompson, R.F. (2009). Habituation revisited: An updated and revised description of the behavioral characteristics of habituation– Neurobiol. Learn. Mem. 92: 135–138.

Sorensen, P.W., Fine, J.M., Dvornikovs, V., Jeffrey, C.S., Shao, F., Wang, J., Vrieze, L.A., Anderson, K.R., & Hoye, T.R. (2005). Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey– Nat. Chem. Biol. 2005 16 1: 324–328.

Wagner, C.M., Jones, M.L., Twohey, M.B., & Sorensen, P.W. (2006). A field test verifies that pheromones can be useful for sea lamprey (Petromyzon marinus) control in the Great Lakes– Can. J. Fish. Aquat. Sci. 63: 475–479.

Wagner, C.M., Stroud, E.M., & Meckley, T.D. (2011). A deathly odor suggests a new sustainable tool for controlling a costly invasive species– Can. J. Fish. Aquat. Sci. 68: 1157–1160.

Wyatt, T.D. (2014). Phermones and Animal Behavior. Cambridge University Press, Cambridge, United Kingdom, 1st ed.