Attenuation and recovery of an avoidance response to a chemical antipredator cue in an invasive fish: implications for use as a repellent in conservation

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The detection of predation risk without direct engagement with a predator is an important driver of prey movement strategies. Consequently, the application of alarm cues may prove an effective tool in guiding the movements of fishes targeted for control or conservation. However, failure to contemplate the sensory, physiological and cognitive outcomes of repeated or persistent exposure to the cue will likely lead to poor performance of management practices. Using a series of behavioural tests and physiological recordings from the olfactory organ, we examined the timing of onset and recovery of the alarm response in sea lamprey (Petromyzon marinus L.) when exposed continuously or sporadically to its alarm cue. In the laboratory, sea lamprey exhibited short-term, reversible attenuation of the alarm response over 2–4 h with continuous exposure. The alarm response spontaneously recovered after 30–60 min of removal from the cue. In long-duration free-swimming tests, where the animals were allowed to move into and out of the odour plume volitionally, repeated but sporadic encounter with the alarm cue over 5 h did not alter the alarm response. Electro-olfactogram recordings from the main olfactory epithelium indicated that olfactory sensory neurons quickly adapt to alarm cue and recovered within 15 min. Our findings strongly implicate habituation as the mechanism that induces reduction in the alarm response and provide insight into the design of effective management practices that seek to use fish alarm cues as repellents.

Key words: Habituation, Alarm Cue, Repellent, Lamprey, Invasive Species

Introduction

The ability to influence the movement decisions of targeted fishes would improve a number of pervasive conservation problems in aquatic ecosystems. These include increasing passage through barriers to migration (Castro-Santos and Haro, 2010; Moser et al., 2019), preventing entrainment and impingement at water intake structures (Noatch and Suski, 2012), the capture and removal of nuisance species (Sorensen, 2015; Butler et al., 2019) and the protection of endangered
species (Farnsley et al., 2018). Individual movement decisions are informed by sensory systems that have evolved to detect cues explicitly associated with fitness-impacting circumstances (i.e. the detection of food, predators and mates). In aquatic ecosystems, this information often takes the form of semiochemicals: substances that either directly communicate (signals) or inadvertently advertise (cues) the immediacy of opportunities or risks. These substances function as potent attractants or repellents and have great appeal for use in conservation, as they typically are taxon specific, and have few unintended negative consequences (Sorensen and Johnson, 2016).

The use of aversive odours associated with the detection and management of predation risk may prove particularly useful in guiding the movements of animals. The risk of predation is rarely absent, yet varies considerably over space and time (Sih et al., 2000). Consequently, the perception of predation risk broadly influences movement decisions, and prey continuously gather sensory information to estimate the immediacy of predator threat (Lima and Dill, 1990; Preisser and Bolnick, 2008; Gaynor et al., 2019; Sabal et al., 2021). Fish alarm cues, substances released from damaged tissue that elicit antipredator behaviour in conspecifics (= schreckstoff per von Frisch, 1938, 1941), have been extensively studied (for recent reviews, see Ferrari et al., 2010; Wisenden, 2015). Alarm cues are suspected to be mixtures of compounds, as cross-reactivity is limited to conspecific species, and behavioural responses tend to diminish with increasing phylogenetic distance between the emitter and receiver species (Hazlett and McLay, 2005; Dalesman et al., 2007; Schoepfner and Relyea, 2009; Mitchell et al., 2012; Hume and Wagner, 2018). The compounds that compose the mixture are principally detected by ciliated olfactory sensory neurons (OSNs) that stimulate the medial olfactory tract, inducing avoidance or other antipredator responses (Hamdan and Dovin, 2002, 2007; Dovin and Lastein, 2009). Perception of the cues can result in persistent avoidance of areas activated by the odour (Wisenden et al., 2009; Chivers et al., 2013).

Introducing an alarm cue to ensure avoidance of an area, or to ‘push’ an animal towards a target, may require sustained application (hours, days or months). For example, guiding migrating fish towards the entrance of a fish passage device may entail temporary exposure of a few hours for each individual, whereas the need to block resident fishes from a water intake would be continuous. However, continuous or frequent exposure to an odour often results in reduced reaction to its presence. Reduction or loss of the avoidance response to alarm cue (hereafter referred to as response attenuation) has been noted in cane toad (Bufo marinus; Hagan and Shine, 2009), chub (Leuciscus cephalus; Krause, 1993a), dace (Leuciscus leuciscus; Krause, 1993b) and sea lamprey (Petromyzon marinus; Imre et al., 2017). Diagnosing the sensory and/or cognitive mechanisms underlying the attenuation of the alarm response in a species targeted for manipulation is a necessary step in designing an appropriate application of the cue for conservation activities (Greggor et al., 2014; Blumstein, 2016).

Physiologically, response attenuation may occur in the peripheral sensory system, and/or in any neuron along the sensory pathway, via adaptation (Kurahashi and Menini, 1997; Zufall and Leinders-Zufall, 2000). Olfactory adaptation operates at the receptor, resulting in a loss of signal transduction. Alternatively, or in concert with adaptation, habituation may occur, a process occurring in the brain that is a form of non-associative learning (Wilson and Linster, 2008; Rankin et al., 2009). Habituation typically results in the animal adjusting its response threshold to attenuate the behavioural response to a continuous ‘background’ signal from the sensory organ that is uninformative. If either adaptation or habituation, or both, are operating, the continuous application of an odour to manipulate animal movement would rapidly become ineffective. Evaluating the timing of the onset and recovery of response attenuation should prove instructive in resolving the mechanisms in play. Adaptation of vertebrate OSNs may be short (onset within <5 s exposure, recovery in 25 s) or long (onset after 25 s of exposure, recovery in a few minutes to an hour) (Dalton, 2000; Zufall and Leinders-Zufall, 2000). Onset and recovery of longer duration implicates habituation (onset in minutes to hours, recovery in several minutes to >1 h, or a much as a few weeks; Wilson and Linster, 2008).

In the current study, we examine the onset and recovery of response attenuation to an alarm cue in sea lamprey. The invasive population of sea lamprey in the Laurentian Great Lakes continues to be a significant impediment to restoration of the largest freshwater ecosystem in the world (Brant, 2019). A great deal of research has been directed towards understanding the annual reproductive migration into rivers, as these habitat selection decisions regulate the distribution of larvae throughout the basin, and thereby the cost and extent of the pesticide applications directed at reducing larval populations (Marsden and Siefkes, 2019). During the migration, sea lamprey respond strongly to an alarm cue (Wagner et al., 2011; Bals and Wagner, 2012; Di Rocco et al., 2016; Luhring et al., 2016), that may be used to guide the movements of migrants in rivers towards control devices (e.g. traps; Hume et al., 2013; Hume et al., 2020) or tributaries targeted for pesticide application (Imre et al., 2010; Wagner et al., 2011). As the animal’s migratory movements are nocturnal, the ability to sustain such manipulations throughout the nighttime (8–10 h) is necessary for achieving conservation goals. Further, the partial conservation of alarm cue in lampreys (Hume and Wagner, 2018) suggests similar movement manipulations would be useful for guiding migrating lampreys of restoration concern towards fish passage devices that provide entry into spawning habitats (Moser et al., 2002; Moser et al., 2011; Maitland et al., 2015; Byford et al., 2016; Porter et al., 2017; Moser et al., 2019).

The first objective of our study was to characterize the timing of attenuation and spontaneous recovery of the alarm cue.

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response in sea lamprey during continuous exposure to the alarm cue. Second, as animals may also habituate to repeated exposures of olfactory cues, we examined the behavioural responses to alarm cue when the animal was allowed to move freely into and out of the odour plume in a laboratory raceway for evidence of habituation (reduced avoidance) or sensitization (increased avoidance). Finally, we examined the olfactory sensitivity to the alarm cue via electro-olfactogram (EOG) recording to gain insight into the magnitude of sensory adaptation in sea lamprey. We discuss the implications of the findings for use of the alarm cues in behavioural manipulations designed to guide the movements of fishes prioritized for conservation or control.

Materials and methods

Test subjects

For these experiments, we used 1106 adult migratory (immature) sea lamprey captured in traps from four tributaries that discharge into Lake Huron or Lake Michigan: the Cheboygan, Ocqueoc, Manistee and St. Mary’s Rivers. Experiments 1, 2 and 3 used immature male sea lamprey. Experiment 4 used immature male and female sea lamprey. Immature male and female sea lamprey exhibit a similar avoidance response to alarm cue (Bals and Wagner, 2012). After capture, the animals were transported to the U.S. Geological Survey’s Hammond Bay Biological Station near Millersburg, MI, USA, in aerated tanks. Lampreys were held in 1000-l tanks (250 animals per tank) with continuous water flow from Lake Huron at ambient water temperature (range, 13–17°C) and the natural light schedule. As migrating sea lamprey cease feeding prior to entering rivers, the lampreys did not receive food. Each animal was held for a minimum of 48 h prior to use in experiments and was used in a single trial. Lamprey used for EOG recordings were transported to the University Research Containment Facility at Michigan State University, East Lansing, MI, USA, and held in continuous flow-through tanks supplied with aerated, chilled well water (range, 7–9°C). All experimental procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF nos. 02/11-027-00 and 02/18-025-00).

Test odours

We used two test odours: a filtered Soxhlet extract from the whole body that contains a damage-released alarm cue that invokes a strong avoidance response in sea lamprey in laboratory and field environments (Wagner et al., 2011; Bals and Wagner, 2012; Hume et al., 2015) and the Soxhlet extraction solvent (50:50, 200 proof ethanol and deionized water, by volume) as a negative control. We obtained the Soxhlet extract from one male (255 g) and one female (192 g) adult migratory sea lamprey, each extracted separately with a 71/60 Soxhlet body connected to a 3-bulb Allihn condenser and a round bottom flask that contained 1 l of extraction solvent. Each apparatus was heated to 75–80°C using a hemi-spherical mantle and cycled six times (~6 h). After extraction, the material retained in the flask was allowed to cool to room temperature, combined, filtered under light vacuum (GE Healthcare Whatman™ Qualitative filter paper, Grade 4) and stored at −20°C until use.

Experiment 1. Attenuation of the avoidance response

To assess the behavioural reaction to the alarm cue after varying periods of continuous pre-exposure, we examined space use in a laboratory two-choice test. Subjects were randomly assigned to one of 60 groups of 10 individuals each. Each of 50 groups was pre-exposed to the alarm cue via continuous immersion in a dilute solution of the Soxhlet extract (1 μl l−1 in Lake Huron water) for one of five time periods: 0, 60, 120, 240 or 480 min (0 pre-exposure equates to no prior experience with the extraction solvent or the alarm cue extract; N = 10 for each time period). Pre-exposure involved continuously pumping the odour into the flow-through holding water to assure the cue did not breakdown during this stage of the experiment. Ten additional groups were tested for response to the extraction solvent without prior exposure (negative control). The behavioural test was performed in two laboratory raceways, each equipped with a collimator to smooth the flow (stacked PVC pipe, 60 cm l, 2.54 cm inner diameter), and block nets to retain the animals in a 5.0 × 1.84 m experimental reach. Each raceway received a continuous flow of fresh Lake Huron water (13–17°C); water depth was maintained at 30 cm with a mean velocity of 1.8 cm sec−1. The source water into the raceway is the same as experienced during holding and pre-exposure, ensuring the animals did not experience any sudden change in water condition at any point in the experiment.

All trials were conducted after 22:00 h, as sea lamprey are nocturnal. At the start of a trial, a group of ten lampreys was released into the experimental reach and allowed to acclimate to the raceway conditions for 10 min. After the acclimation period, a stimulus odour was pumped into one-half of the channel width via peristaltic pump (MasterFlex model 7533-20) for 20 min. The stimulus was formulated by mixing 4 ml of a test odour (alarm cue extract or extraction solvent) into 400 ml of Lake Huron water collected from the raceway in a 500 ml Erlenmeyer flask that was continuously stirred with a 2-cm magnetic stir bar. This mixture was pumped into the raceway at a rate of 15 ml min−1 to create a 1 μl l−1 dilution in the target half of the channel (odour distribution confirmed via dye tests prior to the start of the experiment). The side of the channel receiving the stimulus odour was alternated between replicates for each pre-treatment time (left or right). The raceways were illuminated with infrared light, and separate infrared-sensitive video cameras recorded the position and movements of each animal during the 30 min trial against two lines painted onto the raceway floors that separated each arena into four equal-sized rectangles. At the
conclusion of a trial, the subjects were removed from the raceway and returned to a separate holding tank.

**Experiment 2. Spontaneous recovery of the avoidance response**

Results from Experiment 1 suggested 240 min of pre-exposure to the alarm cue fully attenuated the response. To establish the spontaneous recovery threshold (timing of reemergence of the avoidance response), we submitted an additional 400 lamprey (40 groups of 10 individuals each) to an attenuation-recovery experiment. Each group was pre-exposed to the dilute alarm cue extract (1 μl l⁻¹) for 240 min. After pre-exposure, groups were either moved immediately into the raceway (recovery time = 0 min), or into recovery tanks receiving continuous ‘clean’ Lake Huron water for 30, 60 or 120 min (N = 10 for each time period). After the recovery period, each group was subjected to the two-choice test procedure described in Experiment 1 to ascertain whether and when the animals would again avoid the alarm cue. We expected a recovery time of 0 min would result in no avoidance of the alarm cue during the two-choice test.

**Experiment 3. Behavioural effects of protracted activation of an area with the alarm cue**

To test whether frequent, sporadic exposure to the alarm cue in the same location would alter the use of space via learning (predict greater avoidance) or response attenuation (predict lesser avoidance), we conducted a protracted (300 min) two-choice test with lampreys that were not pre-exposed to the alarm cue. Lampreys were randomly assigned into 17 groups of 10 individuals each, and subjected to the two-choice test described in Experiment 1 (sans the pre-exposure protocol). During the 5-h trials, either the alarm cue extract (N = 7) or the extraction solvent (N = 10) was continuously pumped into one side of the raceway and the animal’s movements were recorded onto digital video. At the conclusion of the experiment, we examined the use of space to ascertain whether the tendency of lamprey to occupy the side of the raceway containing the stimulus changed over time and/or as a function of the odour (solvent control vs. alarm cue).

**Experiment 4. EOG recordings**

To determine if continuous exposure to dilute alarm cue resulted in olfactory adaption, we followed previously established EOG recording procedures (Buchinger et al., 2020; Siefkes and Li, 2004) (N = 6). Briefly, sea lampreys were anaesthetized with 3-aminobenzoic acid ethyl ester (100 mg l⁻¹, MS222, Sigma-Aldrich) and immobilized with an injection of gallamine triethiodide (30 mg kg⁻¹ of body weight, Sigma-Aldrich). Gills were continuously irrigated with aerated water containing 50 mg l⁻¹ MS222 throughout the experiment. The olfactory lamellae were surgically exposed, and the differential EOG response magnitude was recorded using glass capillary borosilicate electrodes filled with 0.4% agar in 0.9% saline and connected to solid state electrodes with Ag/AgCl pellets (Warner Instruments LLC, model ESP-M15N) in 3 M KCl. EOG signals were amplified (NeuroLog system, Digitimer Ltd, model NL102), filtered (low-pass 60 Hz, Digitimer Ltd, model NL125), digitized (Molecular Devices LLC, Digidata 1440A) and recorded on a computer running AxoScope 10.4 software (Molecular Devices LLC).

Stock solutions of L-arginine (10⁻³ M) in deionized water, a sea lamprey sex pheromone 3-keto petromyzonol sulphate (3kPZS; 10⁻⁵ M; Bridge Organics Co.) (Li et al., 2002) in deionized water/methanol (1:1, v:v), and filtered whole body alarm cue extract (Whatman, Puradisc syringe filter 2.0 μm) were prepared, stored at −20°C and then diluted with filtered water to yield working solutions. A baseline EOG response to the blank control, 10⁻³ M L-arginine standard, and test stimuli (1 μl⁻¹ alarm cue, 10⁻⁷ M L-arginine, 10⁻⁹ M 3kPZS; before unadapted responses) were recorded. The test stimuli were used at concentrations that elicited approximately equal magnitude EOG responses. Then, the olfactory epithelium was continuously exposed to 1 μl⁻¹ alarm cue for 15 min. Next, the responses to mixtures of 1 μl⁻¹ alarm cue and 1 μl⁻¹ alarm cue, 1 μl⁻¹ alarm cue and 10⁻⁷ M L-arginine, and 1 μl⁻¹ alarm cue and 10⁻⁹ M 3kPZS were recorded (during adapted responses). The olfactory epithelium was flushed with charcoal filtered water for 15 min, and responses to the blank control, 10⁻³ M L-arginine standard and test stimuli (1 μl⁻¹ alarm cue, 10⁻⁷ M L-arginine, 10⁻⁹ M 3kPZS; after unadapted responses) were recorded to determine if the olfactory responses recovered after exposure to alarm cue.

**Statistical analyses**

All trials in Experiments 1–3 were video-recorded and the videos were analysed during replay. For Experiments 1 and 2, we assigned the position of each animal to the stimulus or non-stimulus side of the raceway every 30 s after the onset of odour introduction for the full 20-min period. To generate the measurement of the proportion of animals on the stimulus side, we summed the counts from each measurement taken during the final 10 min of the trial (20 measures) and calculated the proportion on the stimulus side. For Experiment 3, we analysed every other 10-min period of video beginning 20 min after the onset of odour introduction, using 30-s intervals as previous, similarly generating the proportion on the stimulus side (observation times: 30, 50, 70, 90, 110, 130, 150, 170, 190, 210, 230, 250, 270 and 290 min after the onset of odour introduction). The 10-min intervals between measurement windows were meant to decrease the likelihood of spatial autocorrelation in the measurements during the protracted trials. The data from each experiment were analysed as the proportion of fish on the stimulus side of the raceway, comparing mean proportions across the treatments. Untransformed proportions were examined as the data met the assumption of normality per Shapiro Wilk’s Test
Data for Experiment 1 were analysed via one-way ANOVA ($\alpha =0.05$) with pre-exposure time as the main effect (factor, six levels: solvent control and 0, 60, 120, 240, 480 min pre-exposure times). A post-hoc Tukey’s HSD test was used to make pairwise comparisons across treatments. In addition, we compared the solvent control result to a nominal 50:50 distribution via one-way $t$-test ($\alpha =0.05$). Similarly, data from Experiment 2 were examined with a one-way ANOVA ($\alpha =0.05$) with recovery time as the main effect (factor, four levels: 0, 30, 60, 120 min recovery times). A post-hoc Tukey’s HSD test was again used to make pairwise comparisons across recovery times. Due to limited animals, we did not repeat the extraction solvent control during this experiment. Rather, the data for each recovery time was also subjected to a one-way $t$-test to compare the mean observed proportion vs. a nominal 50:50 distribution. All analyses for Experiments 1 and 2 were conducted in STATA ver. 14.2 (StataCorp LLC).

For Experiment 4, the normalized EOG response was calculated for each stimulus before, during, and after alarm cue exposure as Normalized EOG Amplitude = $[(Rt - Rb)/(Ra - Rb)]/[(R_{unadapt} - Rb)/(Ra - Rb)] \times 100$. $Rb$ is the response magnitude to the blank, and $Ra$ is the response magnitude to $10^{-5}$ M L-arginine. For example, to calculate the change in olfactory response of 3kPZS, $R_{adapt}$ was the response to $10^{-5}$ M 3kPZS during alarm cue exposure relative to the average of the response to $10^{-5}$ M 3kPZS recorded before and after exposure ($R_{unadapt}$). Then, the percentage of unadapted response data were analysed via one-way ANOVA ($\alpha =0.05$) with test stimuli as the main effect (factor, three levels: alarm cue, L-arginine, and 3kPZS). A post-hoc Tukey’s HSD test was conducted to make pairwise comparisons across treatments. The percentage of unadapted response data met the assumption of normality per Shapiro Wilk’s Test (Alarm cue, $W=0.80$, $P=0.06$; 1-arginine, $W=0.89$, $P=0.33$; 3kPZS, $W=0.94$, $P=0.68$) and the assumption of equal variances per Bartlett’s Test ($\chi^2(2) =3.25$, $P=0.20$).

Results

Experiment 1. Attenuation of the avoidance response

The aim of the first experiment was to determine the timing of the onset of response attenuation during continuous exposure to a fixed dilution of the alarm cue. ANOVA revealed a significant effect of pre-exposure time on the avoidance response to the alarm cue ($F(4,59)=11.12$, $P<0.001$; Fig. 1). Avoidance of the alarm cue began to attenuate after 120 min of pre-exposure, and was fully attenuated after 240 min of pre-exposure. Per Tukey’s HSD pairwise comparisons, the avoidance of the alarm cue was significantly greater than the control only for the 0 min ($t=5.03$, $P<0.001$) and 60 min ($t=5.47$, $P<0.001$) pre-exposure times. At 240 and 480 min the response did not differ from control (240 min, $t=2.22$, $P=0.025$; 480 min, $t=1.09$, $P=0.88$) and was significantly less than the avoidance observed after 0 min (240 min, $t=4.62$, $P<0.001$; 480 min, $t=3.94$, $P=0.003$) and 60 min (240 min, $t=5.06$, $P<0.001$; 480 min, $t=4.38$, $P=0.001$) of pre-exposure. Avoidance after 120 min, the pre-exposure time intermediate to the fully responding (0 and 60 min) and fully attenuated (240 and 480 min) periods, was significantly less than the 60-min treatment ($t=3.26$, $P=0.023$), but differed from no other treatment or the control (Con, $t=2.22$, $P=0.025$; 0 min, $t=2.82$, $P=0.07$; 240 min, $t=1.80$, $P=0.047$; 480 min, $t=1.12$, $P=0.87$).

Experiment 2. Spontaneous recovery of the avoidance response

The aim of the second experiment was to determine the timing of spontaneous recovery of the avoidance response to alarm cue. ANOVA revealed a significant effect of recovery time on avoidance of the alarm cue ($F(3,39)=14.55$, $P<0.001$; Fig. 2). Post-hoc comparisons (Tukey’s HSD) revealed significant differences in avoidance behaviour after 60 min ($P<0.001$; 0 vs. 30 min, $t=0.62$, $P=0.93$; 0 vs. 60 min, $t=-4.30$, $P=0.001$; 0 vs.
The aim of the third experiment was to ascertain whether the avoidance of an area activated with the alarm cue would change over a 5-h time period (Fig. 3). Type II ANOVA on the mixed-effects GLM coefficients revealed a persistent avoidance of the alarm cue vs. ethanol control: \( \chi^2 = 31.82, P < 0.001 \), with no effect of observation time \( \chi^2 = 1.52, P = 0.22 \), nor evidence of a time-by-odour interaction \( \chi^2 = 1.42, P = 0.23 \).

Experiment 4. EOG recordings

The aim of the fourth experiment was to compare the olfactory response to each test stimulus before, during and after a 15-min continuous exposure of the olfactory epithelium to alarm cue. A repeated measures one-way ANOVA revealed the normalized EOG responses to each alarm cue \( (1 \mu l^{-1}) \), L-arginine \( (10^{-7} M) \) and 3kPZS \( (10^{-9} M) \) were different across the three time points within a test stimulus (Alarm cue, \( F_{(2,10)} = 44.21, P < 0.001 \); L-arginine, \( F_{(2,10)} = 31.87, P < 0.001 \); 3kPZS, \( F_{(2,10)} = 22.58, P < 0.001 \); Fig. 4A). Post-hoc comparisons (paired \( t \)-test with Bonferroni adjustments) used to make pairwise comparisons across time points within a stimulus showed the olfactory response to alarm cue during adaptation was less than the unadapted responses (before vs. during, \( t_{(5)} = -9.8 \), adj. \( P < 0.001 \); during vs. after, \( t_{(5)} = 6.27 \), adj. \( P = 0.005 \)). However, the olfactory response to alarm cue after adaptation was not different than the response to alarm cue before (before vs. after, \( t_{(5)} = 0.658 \), adj. \( P = 1.0 \)), indicating the response
to alarm cue was recovered within 15 min or less after flushing the olfactory epithelium with charcoal filtered water. Similar trends were observed when comparing the adapted versus unadapted responses of L-arginine (before vs. during, \( t(5) = -8.72 \), adj. \( P < 0.001 \); during vs. after, \( t(5) = 9.36 \), adj. \( P < 0.001 \); before vs. after, \( t(5) = -0.525 \), adj. \( P = 1.0 \) ) and 3kPZS (before vs. during, \( t(5) = -5.34 \), adj. \( P = 0.009 \); during vs. after, \( t(5) = 5.64 \), adj. \( P = 0.007 \); before vs. after, \( t(5) = -0.963 \), adj. \( P = 1.0 \) ) with reduced responses recorded to both L-arginine and 3kPZS during exposure to alarm cue. Although exposure to alarm cue reduced olfactory responses to all three stimuli tested, the response to alarm cue was reduced more than responses to L-arginine or 3kPZS. A one-way ANOVA was conducted and revealed the percent of unadapted responses were different across test stimuli (\( F(2,19) = 29.54, P < 0.001 \); Fig. 4B). Further, exposure to alarm cue reduced the response to alarm cue (95.7 ± 2.1\%), mean ± SE) more than it reduced the response to L-arginine (70.5 ± 4.1\%, \( q = 6.29, P = 0.001 \) ) or 3kPZS (52.3 ± 5.1\%, \( q = 10.82, P < 0.001 \) ) according to post-hoc comparisons (Tukey’s HSD test).

**Discussion**

The present study suggests sea lamprey exhibit short-term, reversible habituation to a conspecific alarm cue, typified by a response declination involving reduced spatial avoidance of the cue, when exposed continuously for several hours. With continuous immersion in a fixed concentration of the alarm cue, the alarm response was partially attenuated after 2 h and absent after 4 h, consistent with a previous report for sea lamprey (Imre et al., 2017) and notably similar to cane toad (Bufo marinus; Hagman and Shine, 2009). Newly observed, the alarm response spontaneously recovered after the cue was eliminated from the environment for 30–60 min. The recorded times of onset and recovery from response attenuation were substantially longer than is typical for the three forms of neuronal adaptation described in vertebrate olfactory systems: short- and long-form sensory adaptation and desensitization (Zufall and Leinders-Zufall, 2000). In EOG studies, direct application of alarm cue to the olfactory epithelium resulted in rapid onset of adaptation, with recovery in 15 min. This was in contrast to the sporadic, repeated exposure that occurred when the animals were allowed to move freely over 5 h. Here, despite frequently encountering and avoiding the alarm cue, we observed no evidence of any change in the avoidance response that would be associated with adaptation, habituation, sensitization or spatial learning. These characteristics implicate habituation as the mechanism of the observed response declination to alarm cue in sea lamprey (per Rankin et al., 2009).

Rankin et al., 2009 (see also McDiarmid et al., 2019) recognize two forms of habituation. Short-term habituation, typically lasting from a few seconds to hours, arises with rapidly repeated stimulation [i.e. a short interstimulus interval (ISI)], whereas long-term habituation may persist for hours to weeks and is more likely to result when ISIs are of relatively long duration. Short-term habituation to persistent cues is considered adaptive, as it allows animals to avoid reacting to uninformative or harmless stimuli. However, long-term habituation to various stimuli that induce avoidance is a well-known problem in management and conservation of animal species (Blumstein, 2016; Greggor et al., 2020). Two features of the application of alarm cue in our study likely resulted in the rapid onset of short-term habituation for animals continuously exposed to the odour. First, the magnitude of the response declination is an inverse function of the ISI, defined as the time between sequential exposures to the stimulus (Rankin and Broster, 1992). In other words, high frequency stimulation of OSNs should lead to more rapid and complete response declination. We applied the cue continuously at a fixed concentration, an ISI = 0. Second, the rapidity of response declination is related to the strength (concentration) of the stimulus. Low concentrations result in more rapid habituation than higher concentrations, and a very strong stimulus may fail to induce habituation. The dilution of alarm cue extract we applied was the minimum necessary to achieve full repellency based on prior laboratory dose-response tests (CM Wagner, unpublished data).

Further support for the hypothesis of short-term habituation is found in the timing of spontaneous recovery of the
avoidance behaviour. Habituation is an attentional deficit that dissipates with time. Shorter ISIs are affiliated with more rapid spontaneous recovery of the habituated response (McDiarmid et al., 2019). For example, in a study with Caenorhabditis elegans, an ISI of 2 s resulted in full spontaneous recovery after 20–30 min, whereas an ISI of 60 s resulted in partial recovery (≈50% of the unhabituated response) over the same time period (Rankin and Broster, 1992). Here, sea lamprey exhibited no recovery after 30 min of cessation of the stimulus, and full recovery at 60 min (the next measured time step). Finally, sporadic volitional encountering with the odour plume while freely swimming (vs continuous exposure) failed to elicit a response declination after 5 h, despite the animals being observed within the alarm cue plume an average of 28% of the time. Although we did not attempt to analyse individual tracks within the replicate groups, typically, a sea lamprey would enter the alarm cue plume for brief period of 2–20 s, before undertaking a rapid reversal and accelerating away. These sporadic encounters represent a longer ISI, although intervals would be stochastically distributed. Taken together, these findings strongly support a hypothesis of short-term habituation as the underlying mechanism for the behavioural response declination observed during continuous exposure to the alarm cue (vs sensory adaptation or motor fatigue).

The EOG findings clearly indicate that sensory adaptation may also be operating in sea lamprey continuously exposed to alarm cue. However, the EOG study involved direct application of alarm cue to the olfactory epithelium, likely resulting in a large fraction of the alarm cue sensitive OSNs receiving continuous stimulation with a high concentration of the cue. High cue concentration results in more rapid adaptation in vertebrate olfactory neurons (Dalton, 2000). In our laboratory studies, and in the natural circumstance, turbulent advection fractures odour plumes into swirling filaments and packets of odourants as they disperse in the flow (Moore and Crimaldi, 2004). Consequently, an animal holding station a fixed distance downstream from the odour source is likely to encounter a stochastically varying concentration of the alarm cue. Similarly, if the animal moves in response to the encounter with the odour, variation in the cue concentration perceived by the olfactory organ is maintained. Physiologically, these circumstances may maintain a reservoir of unadapted OSNs capable of signalling alarm cue presence to the brain. Finally, sea lampreys do not solely perceive odourants via the main olfactory organ. Solitary chemosensory cells are distributed in the epidermis in association with cutaneous papillae that exhibit responses to a broad array of chemostimulants including food odours and pheromones and may exhibit different adaptation timing vs OSNs (Daghfous et al., 2020). These circumstances further implicate habituation as the primary cause for the reduced behavioural response observed in the lab.

Development of conservation practices after the discovery of useful repellents should involve creation of application practices designed to mitigate sensory and cognitive processes that inhibit the desired response, accounting for the perceptual constraints imposed by the environment (e.g. background sensory noise; Greggor et al., 2014). The sea lamprey alarm cue repellent is most likely to be useful during the annual spawning migration. The sea lamprey undertakes a solitary, nocturnal, non-homing migration from offshore feeding grounds into rivers to spawn, guided by an attractant odour emitted by larvae that reveals the location of suitable spawning habitat (Sorensen et al., 2005; Wagner et al., 2006; Wagner et al., 2009). Because control of the invasive population in the Great Lakes is achieved by applying pesticides to kill the resulting larvae, these annual decisions made by migrants determine the spatial extent of the management challenge in any given year (i.e. the number, size and locations of the rivers requiring future treatment). Consequently, there is considerable interest in manipulating movement decisions in rivers to either remove migrants prior to spawning (e.g. guide them into fishing devices) or guide them into fewer tributaries to reduce the overall spatial extent of the infestation prior to treatment (Li et al., 2007; Marsden and Siefkes, 2019).

Coincidentally, the riverine migration is also a time of great interest for the conservation of native parasitic lampreys, including the Pacific (Entosphenus tridentatus), European river (Lampetra fluviatilis) and sea lamprey in its native range (Igoe et al., 2004; Clemens et al., 2017). The principal threat to these taxa during migration is the placement of barriers to migration, and conservation managers are interested in the ability to guide migrants into fish passage devices (Maitland et al., 2015; Pereira et al., 2019).

Although the nocturnal movement schedule should allow any short-term habituated or adapted sea lamprey to recover the alarm response on the following day, continuous application of the alarm cue at night is contraindicated. In rivers, reported ground speeds for migrating sea lamprey range from 29.3 to 45.6 body lengths per min (Almeida et al., 2002; Quintella et al., 2009; Castro-Santos et al., 2017), equating to a movement range of 2.1–3.3 km in 4 h for a continuously cruising individual of 300-mm body length (typical of landlocked populations). This is likely an overestimate, as sea lamprey exhibit periods of rest during upstream movement on a given night (Almeida et al., 2002). However, encounter with the alarm cue while migrating upstream may also elicit an increase in ground speed (Luhring et al., 2016). The alarm cue will full mix in the river some distance downstream of the application point and persist until chemical breakdown or dilution from downstream tributaries, a distance we expect to be considerably longer than 5 km in most rivers. Consequently, migrants are likely to habituate prior to encounter with the application site if pumped continuously into the flow.

Our current understanding of habituation in vertebrates offers three potential remedies (per Rankin et al., 2009; McDiarmid et al., 2019): ‘pulsing’ the stimulus input (increasing ISI), increasing the concentration or introducing a dishabituation stimulus. First, increasing the ISI (pulsing the odour on/off) will increase the time until a response decrement
occurs, as evident in the results of Experiment #3. Pulses would have to be modulated such that full mixing is avoided for the desired distance downstream of the application point. One consequence of a relatively long ISI (minutes) would be an increased chance of animals failing to detect the odour in the inter-pulse periods near the application site. Second, the concentration of the odour may be increased to reduce or eliminate the onset of habituation. Little is known about the onset of habituation to high concentration odours in fishes over periods longer than typical laboratory experiments (i.e. minutes to hours) suggesting further research would be needed in the development phase of any alarm cue repellent. Third, unlike sensory adaptation or motor fatigue, habituation involves modification of an animal’s attentional response to a stimulus. Consequently, the presentation of a strong stimulus that differs from the habituated stimulus (e.g. loud noise, light flash) induces spontaneous recovery of the original stimulus response termed dishabituation (e.g. electric fish Gnathonemus petersii, Post and von der Emde, 1999; marine mollusc Aplysia californica, Hawkins et al., 2006; Lepidopteran insect Trichoplusia ni, Akhtar et al., 2003).

Whether long-term exposure to alarm cue that is pulsed (longer ISIs) or pumped continuously at high concentration would bring about long-term habituation is unknown. Imre et al. 2016 failed to observe habituation to an alarm cue in sea lamprey when subjects were exposed four or eight times in the 24 h prior to evaluation of their responses. However, chub (Squalius cephalus; Krause, 1993a) and common dace (Leuciscus leuciscus; Krause, 1993b) exhibited a response declination to their alarm cues when exposed twice daily for 14 (chub) or 10 (dace) consecutive days. These studies did not examine spontaneous recovery of the alarm response. Interestingly, long-term habituation to alarm cue may help explain the observation that fishes in high predation environments exhibit higher response thresholds to alarm cue vs those in low predation environments (e.g. Brown et al., 2006; Mirza et al., 2006). For migratory fishes, brief periods of exposure prior to capture or passage through the application site may prevent serious impacts to conservation and management objectives. Where the goal is to create continuous avoidance of an area for resident fishes (e.g. water intake structures), long-term habituation would pose a problem. The dishabituation phenomenon may imply a solution (e.g. presentation of another strong stimulus to dishabituate resident fishes to the alarm cue(s) in use). However, as the process of identifying the chemical constituents in a fish pheromone can be costly and time-consuming, development of alarm cue based repellents may be recommended only in cases where the benefits to conservation are substantial. Alternatively, an alarm cue may prove effective as a species-specific dishabituating stimulus for non-chemical deterrents that represent conditioned stimuli (e.g. sound barriers for invasive carps; Dennis and Sorensen, 2020), requiring far less cue that may be harvested from animal tissue. In other words, the alarm cue for a species that proves particularly problematic may be used to dishabituate the local population to another more cost-effective deterrent (sound barriers, bubble curtains, light barriers, etc.), extending the useful life of the management practice. Further, fishes and other aquatic organisms can learn to associate novel environmental (unconditioned) stimuli with predation risk, when a deterrent stimulus is periodically paired with the alarm cue, creating more consistent avoidance through reinforcement (Greggor et al., 2020).

The present study supports the hypothesis that short-term habituation underlies the response declination observed in the alarm response in sea lamprey when exposed to a continuous application of alarm cue. Although adaptive, this physiological phenomenon produces substantial challenges for the development of a species-specific repellent. Fortunately, a large body of research into olfactory habituation provides potential remedies in the form of application practices. Further development of this approach should include the following: (i) examination of the effect of longer duration stimulus pulses (e.g. signals that continue for sec to min), (ii) interactions between pulse durations ISIs on the persistence of behavioural responses and (iii) the incorporation of dishabituating stimuli to achieve more effective conservation practices.

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**Data Availability**

The data underlying this article will be shared on reasonable request to the corresponding author.

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