



Sea Lamprey Alarm Cue Comprises Water- and Chloroform- Soluble Components

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Abstract

A diversity of aquatic organisms manage predation risk by avoiding waters activated with conspecific alarm cues, a chemical mixture released from injuries. The sea lamprey (*Petromyzon marinus*) is a nocturnal migratory species that relies on its alarm cue to navigate around areas of predation risk when moving through river channels. Identification of the cue's chemistry would allow managers to harness this innate behavioral response to guide migrating sea lamprey to traps (invasive population in the Laurentian Great Lakes) or to fish passage devices where dams block migrations in their native range. We pursued isolation of the sea lamprey alarm cue through behaviorally guided fractionation, fractionating the alarm cue into water-soluble and chloroform-soluble fractions, each of which elicited a substantial avoidance response. Recombining the two fractions restored full reactivity, suggesting the alarm cue mixture contains components that exhibit high solubility in water (e.g., nitrogenous compounds), chloroform (e.g., lipids), or perhaps materials that dissolve readily in either solvent. We further screened 13 individual compounds or pure isolates and 6 sub-fractions from the water-soluble fraction and found one of the pure isolates, isoleucine, evoked an avoidance response on its own, but not consistently when found in other mixtures. In a third experiment, we observed no behavioral response after recombining 32 compounds isolated and identified from the water-soluble fraction. These results confirm other suggestions that the process of elucidating alarm cue constituents is challenging. However, we suggest the pursuit is worthwhile given the strong evidence for the utility of alarm cues for use in the conservation and management of fishes and other aquatic organisms.

Keywords Semiochemical · Predation · Risk · Pheromone · Behavioral Manipulation · Alarm Substance

Introduction

Animals perceive and react to predation risk, and they balance the costs of their responses against other needs, including energy acquisition and reproduction (Ferrari et al. 2009; Lima and Bednekoff 1999). Migratory species face a particular challenge, as movement between distant foraging and reproductive habitats requires individuals to navigate through complex risk landscapes where the location and identity of predators are uncertain, and the environmental cues that indicate safety may be misaligned with actual risk

(Gallagher et al. 2017; Moore 2018; Sabal et al. 2021). Consequently, accurate assessments of the immediacy of predation risk in space and time are crucial to migratory success.

The ability to perceive sensory cues associated with predation risk may be innate or acquired. Innate predator recognition is unlearned and exhibited in a variety of prey organisms when they share an eco-evolutionary history with the predator, or a closely related species (Carthey and Blumstein 2018). For example, the Seychelles warbler (*Acrocephalus sechellensis*) will respond to decoy predators whether born in populations isolated from predators or not (Veen et al. 2000), naïve giant pandas (*Ailuropoda melanoleuca*) display defense behaviors when exposed to predator urine (Du et al. 2012), and newly hatched Atlantic salmon (*Salmo salar*) show innate antipredator behaviors to piscivorous pike (*Esox lucius*) (Hawkins et al. 2004). Acquired predator recognition typically involves learning (Ferrari et al. 2007). Examples include blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) that acquire recognition of

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acoustic predator cues through socially mediated learning (Keen et al. 2020), and zebrafish (*Danio rerio*) that can learn to label novel odors as risky when paired with known fear cues (Lucon-Xiccato et al. 2020).

Among aquatic organisms, these so-called “fear” cues include damage-released alarm cues. Alarm cues are public information; substances released from the tissues of injured conspecifics that reliably alert receivers to the presence of an active predator (Chivers and Smith 1998; Smith 1992; Wisenden et al. 2004). Exposure to an alarm cue typically elicits antipredator behaviors including increased shelter use, decreased activity, and area avoidance (Lawrence and Smith 1989; Ferrari et al. 2010; Wisenden 2015). Evidence suggests alarm cues unite innate and acquired risk recognition. Detection of the alarm cue is innate (Lucon-Xiccato et al. 2020; Atherton and McCormick 2015; Poisson et al. 2017), and patterns in the dispersion of the cue in the environment reveal locations of risk for conspecifics and closely related species who may share similar predators (Døving and Lastein 2009; Ferrari et al. 2010; Hume and Wagner 2018). When alarm cues are presented with the odor of an unfamiliar predator, the prey may learn to associate predator odor with danger and avoid it in the future (Brown 2003; Ferrari 2005; Kelley and Magurran 2003). Alarm cue associated learning is important in the life history of settling coral reef fish, by facilitating predator detection during transitional life stages (Holmes & McCormick, 2010), and pairing alarm cue odors with predator odors has been used to condition hatchery-reared fish to promote post-release survival (Griffin 2004; Hawkins et al. 2008; Kopack et al. 2016; Sloychuk et al. 2016). This duality makes alarm cues particularly useful in mitigating uncertain risk landscapes during migration because alarm cue is consistently associated with a direct risk of injury to conspecifics or closely related species.

The sea lamprey (*Petromyzon marinus*) is a semelparous ectoparasitic fish that relies extensively on olfaction to complete its terminal spawning migration from the open waters of oceans or large lakes into streams. Migrants are guided into streams by the odor of conspecific larvae that labels the habitat as suitable for spawning and offspring survival (Sorensen et al. 2004; Sorensen and Vrieze 2003; Vrieze et al. 2011; Wagner et al. 2006, 2009). Transition from deep open waters into narrow shallow streams exposes migrants to a suite of difficult to detect predators that patrol the shorelines (Imre et al. 2014; Scott and Crossman 1998; Boulétreau et al. 2020). Because this migration is nocturnal, and sea lamprey move solitarily (Almeida et al. 2002; Binder and McDonald 2007; McCann et al. 2018), they must rely on chemical public information to assess predation risk. Consequently, it is unsurprising that exposure to their alarm cue elicits immediate antipredator responses in rivers, including

movement away from the shoreline activated with the cue (Hume et al. 2015; Imre et al. 2010; Wagner et al. 2011) and acceleration to pass through the risky area more quickly (Luhring et al. 2016).

Exploiting the sea lamprey’s behavioral responses to the alarm cue is driving the development of innovative approaches to control this species in the Laurentian Great Lakes where they are invasive, that also could be used to conserve them in locations where they are native (Imre et al. 2010; Wagner et al. 2022). For example, in the Great Lakes, traps are used to capture sea lamprey, and encounter rates with traps determine their effectiveness (Bravener & McLaughlin, 2013; Miehl et al. 2020). Traps cannot be effectively baited, as sea lampreys cease feeding prior to the spawning migration, and attempts to bait with attractant pheromones have proven insufficient (Johnson, Siefkes, et al., 2015; Johnson et al. 2013; Johnson, Tix, et al., 2015). Application of the alarm cue to the opposite side of a river channel substantially increases encounter rates with traps placed near dams and in open river channels (Hume et al. 2015; Hume, Luhring, & Wagner, 2020). Within their native range, migrating sea lampreys are blocked from spawning habitat by dams (Hogg et al. 2013; Kynard and Horgan 2019; Lasne et al. 2015). Here too, conservation outcomes could be improved by using the alarm cue to guide migrants toward fish passage devices (Byford et al. 2016; Hume et al. 2020; Pereira et al. 2017). Consequently, there is substantial interest in isolating and identifying the chemical constituents of the odor to support cost-effective synthesis of the large quantities needed for use of a repellent to achieve conservation goals, and to meet Federal requirements for pesticide registration (Ferguson and Gray 1989).

Describing the chemical messengers that constitute fish alarm cues is a critical gap in our understanding of alarm cues and in being able to employ these for conservation or invasive species management (Døving and Lastein 2009; Ferrari et al. 2010; Wisenden 2000). Yet, few efforts have sought to identify compounds in fish alarm cues, and few commonalities in the compounds that may constitute the odors have arisen. For example, hypoxanthine-3-N-oxide (H3NO) has been hypothesized to be an active molecule in alarm cues from teleost fishes (Pfeiffer et al. 1984). Synthesized H3NO elicits consistent alarm responses in zebrafish (Parra et al. 2009), fathead minnows (Brown, Adrian, & Shih, 2001), and black tetra (Pfeiffer et al. 1984), but exhibited conflicting responses in salmonids and cichlids (Brown et al. 2003). This led to the suggestion that the nitrogen oxide functional group is important in initiating antipredator behavior, but is anchored to purine rings that differ in structure across taxa, allowing for species specificity in the cue (Brown et al. 2003). Another compound, chondroitin sulfate, elicits alarm responses in zebrafish (Mathuru et al.

2012) and fathead minnows (Faulkner et al. 2017), but the activity is less than the cue produced by injured tissue from the same species, suggesting the alarm cue is a mixture. One common pattern that has arisen is the response to alarm cues obtained from closely related taxa, where the magnitude of the alarm response declines with increasing phylogenetic distance between the cue donor and the responding species (Mirza and Chivers 2001; Mitchell, Cowman, & McCormick, 2012; Schoeppner and Relyea 2009; Mathis and Smith 1993). In previous studies, sea lamprey exhibit the phylogenetic relatedness pattern when responding to the cues from other lampreys, but did not respond to alarm cues extracted from bluegill sunfish (*Lepomis macrochirus*) or white sucker (*Catostomus commersoni*), suggesting little or no overlap between lampreys of the Petromyzontiformes and the distantly related clades in the Teleostei (Bals and Wagner 2012; Hume and Wagner 2018).

The most common method used to isolate olfactory cues in aquatic organisms is behaviorally guided fractionation, a stepwise iterative process that partitions an odor into fractions, typically by molecular weight, and uses a behavioral bioassay to identify the reactive fractions (Scott et al. 2018). This process has been successful in the identification of key components used in chemical communication of aquatic and marine systems, including chemical defenses in common seaweed (*Lobophora variegata*; Kubanek et al. 2003) and reef sponges (*Erylus formosus*; Kubanek et al. 2000), and sex pheromones in a variety of species (Algranati and Perlmutter 1981; Yambe et al. 2006; Zielinski et al. 2004) including sea lamprey (Li et al. 2002; Scott et al. 2018). The chemistry of alarm cues has proven more enigmatic, with some species exhibiting reactivity to individual fractions, and others requiring all fractions from a crude odor extract in combination to elicit any antipredator response (Mirza et al. 2013). The aim of this study was to pursue the chemical constituents of the sea lamprey alarm cue using behaviorally guided fractionation. We examined the reactivity to two major subfractions of the full alarm cue extract (chloroform- and water-soluble) and examined responses to 32 compounds that have been previously identified from the highly reactive water-soluble fraction (Dissanayake et al. 2016, 2019), alone and in combination, in a standard laboratory assay.

Methods and Materials

Study Design. To begin isolation of the alarm cue, we fractionated crude skin extract and tested the activity of individual sub-fractions and isolated compounds in a behavioral assay through a series of three experiments. The assay involves independent replicates, each consisting of a group

of ten unique migratory sea lamprey used in only a single trial in a large raceway with a standard two-choice test where an odor is pumped into one side of flow and animals are free move throughout the arena. Avoidance or preference is ascertained from the distribution of fish on the two sides of the raceway (with or without the test odor), and the side receiving the odor (left or right) is alternated across replicates. A fish is used only once in a single replicate. The first experiment evaluated the sea lamprey's behavioral response to a solvent control (N=20), crude alarm cue extracts derived from the whole body (N=20) or the skin (N=20), a water-soluble (WS) fraction derived from the skin alarm cue (N=20), a chloroform-soluble (CS) fraction derived from the skin extract (N=20), and the WS and CS extracts combined (N=20). Prior reports have indicated that the WS fraction from skin exhibited full behavioral reactivity when compared to whole-body extracts, with indications of partial and highly variable reactivity to the CS fraction (Dissanayake et al. 2019). The second experiment screened (sample sizes listed below) a series of six sub-fractions, 13 isolated compounds, and one compound mixture derived from the WS fraction to ascertain whether the behavioral reactivity was contained within one or a few sub-fractions. Another compound, chondroitin-sulfate, was not isolated from the WS extract, but was also screened, as previous studies found it played a role in the teleost fish alarm response (Farnsley et al. 2016; Faulkner et al. 2017; Mathuru et al. 2012). Because screening failed to identify a clear set of highly reactive candidate sub-fractions, the third experiment sought to determine whether partial or full reactivity was contained in the set of individual compounds that had been isolated and identified from these sub-fractions to date. We created a mixture of the 32 identified compounds that represented 98% (dry weight) of the material contained in the WS fraction and compared the behavioral reactivity of the mixture (N=20) to the crude WS extract (N=20) and solvent (N=20).

Odor Collection.

Whole body extract- Alarm cue was obtained from Soxhlet extraction of sea lamprey carcasses that naturally senesced in captivity per the method of Wagner et al. 2011. Odor extracted from recently deceased animals elicit alarm responses equivalent to those from live donors (Bals and Wagner 2012). In short, odor was derived through Soxhlet extraction from nine male and female sea lamprey weighing 1,496.5 g total. All carcasses were kept at -20° C before being used in extractions. Soxhlet extractors (2.08 m, Ace Glass Inc., Vineland, New Jersey, USA) were mounted to six-bulb water-cooled Allihn condensers. Solvent reservoirs (12 L capacity) were loaded with 50:50 solution of 200 proof ethanol and deionized water and heated to 75–80° C with a hemispherical mantle for a minimum of three cycles

Table 1 List of the identified compounds in the water-soluble fraction and the percent of the whole fraction each compound constitutes. The water-soluble fraction is 48.70% of the entire crude skin extract and the chloroform-soluble fraction is 51.30%

Compound	%	Compound	%	Compound	%	Compound	%
Creatine	36.95%	Histidine	0.18%	Serine	0.02%	Putrescine	0.04%
Arginine	1.92%	Tryptophan	0.53%	Aspartic acid	0.19%	Spermine	0.02%
Valine	0.24%	Threonine	1.25%	Inosine	0.22%	3-Phenyllactic acid	0.10%
Leucine	0.51%	Asparagine	1.17%	Adenine	0.17%	Pyruvic acid	0.03%
Tyrosine	0.08%	Methionine	0.41%	Xanthine	0.36%	β -Hydroxybutyric acid	0.06%
Isoleucine	0.17%	Glycine	0.24%	Hypoxanthine	0.85%	α -Ketobutyric acid	0.07%
Phenylalanine	1.29%	Cysteine	0.84%	Adenosine	0.48%	α -Ketoisovaleric acid	0.06%
Glutamic acid	0.09%	Proline	0.07%	Petromyzonacil	0.04%	α -Ketovaleric acid	0.01%

(approximately six hours), creating ~10.2 L of alarm cue extract. Extractions were cooled overnight before being decanted and filtered through muslin and were kept in a -20 °C freezer until use in behavioral assays.

Crude skin extract, fractionation, and identification of individual compounds- Experimental details of collection of alarm cue from sea lamprey skin, solvent-solvent partitioning of aqueous ethanolic skin extract in to chloroform-soluble and water-soluble fractions, and MPLC fractionation (normal and reverse phase) of chloroform-soluble and water-soluble fractions are fully described in Dissanayake et al. (2016, 2019, 2021). Purification of the MPLC subfractions of chloroform-soluble fraction was accomplished by preparative thin layer chromatography (Dissanayake et al. 2016). Purification of the MPLC subfractions of the water-soluble fraction was carried out by preparative HPLC (Dissanayake et al. 2019, 2021), respectively. The chemical identity of all pure isolates from the chloroform-soluble and water-soluble fractions was determined by NMR (1D and 2D) and HRESIMS experiments as described in Dissanayake et al. (2016, 2019, 2021). Chondroitin sulfate used in experiment 2 screening was sourced from shark cartilage (Sigma-Aldrich, CAS-No. 9082-07-9).

Mixture of known compounds in the water-soluble fraction- We mixed the 32 previously identified compounds from the water-soluble fraction (Dissanayake et al. 2019) at observed ratios and concentrations found in the water-soluble fraction, based on mass (Table 1). Each compound (dry material) was weighed and then dissolved in 10 mL stock solvent solution (50:50 DI H₂O: EtOH). Solutions were combined and brought up to the final volume with solvent. The mixture was refrigerated until use, within 48 h.

Test Subjects. All sea lamprey used in experiments were migratory sub-adults obtained via the U.S. Fish and Wildlife Service's trapping operations in the Cheboygan and Ocqueoc Rivers (tributaries to Lake Huron in Michigan, USA), and the St. Mary's River connecting channel between Lakes Superior and Huron. Actively migrating sea lamprey were collected in large traps arrayed near dams and transported to the Hammond Bay Biological Station (HBBS) in tanks receiving continuous aeration. Fish were sorted by sex

and held in 1385 L round tanks that received a continuous flow of Lake Huron water (100% exchange every 4 h) with supplemental aeration until use. Fish were held under natural day-night light cycles. Only males were used in the study as female lamprey decrease their reactivity to alarm cue during sexual maturation, whereas males do not (Bals and Wagner 2012). Prior studies with sexually immature migrants indicated no difference in response to alarm cue between sexes (Bals and Wagner 2012). All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee via permits AUF 02/16-015-00 and PROTO201900060.

Behavioral Assay. Experimental trials were conducted in two laboratory raceways at HBBS (Fig. 1). Each raceway measured 1.44 m x 12.2 m, with a 3.1 m long reach isolated with block nets to form the experimental arena. The experimental arenas were lined with white plastic paneling (1/16in PLAS-TEX, Parkland Plastics, Inc., Middlebury, Indiana, USA) to increase visual contrast between lampreys and their background. Experiments took place in full darkness and were recorded with overhead infrared sensitive video cameras (Axis Communications, Q1604 Network Camera), each illuminated by an array of six infrared lights (Wildlife Engineering; Model IRLamp6). Water flowed into flumes from a head tank supplied directly from Lake Huron. Water temperature ranged from 6 to 18 °C over the course of trials, in accordance with seasonal changes in lake temperature. Discharge was maintained at 0.02–0.03 m³ sec⁻¹ in each channel.

Because the sea lamprey is a nocturnal migrant, all trials were conducted between 18:00 and 02:00 h during the spring migratory season. Two hours before experimental trials began subjects were visually inspected to ensure immature status and transferred to holding baskets with ten animals per basket, constituting trial groups. Each trial group represented a single independent sample, and each fish was only used once in a single trial. As such, each independent trial group represented a single replicate, with 1,200 fish total used for experiment 1, 1,800 fish total used for experiment 2, and 600 fish used for experiment 3. Each trial began by carefully releasing the ten animals from their

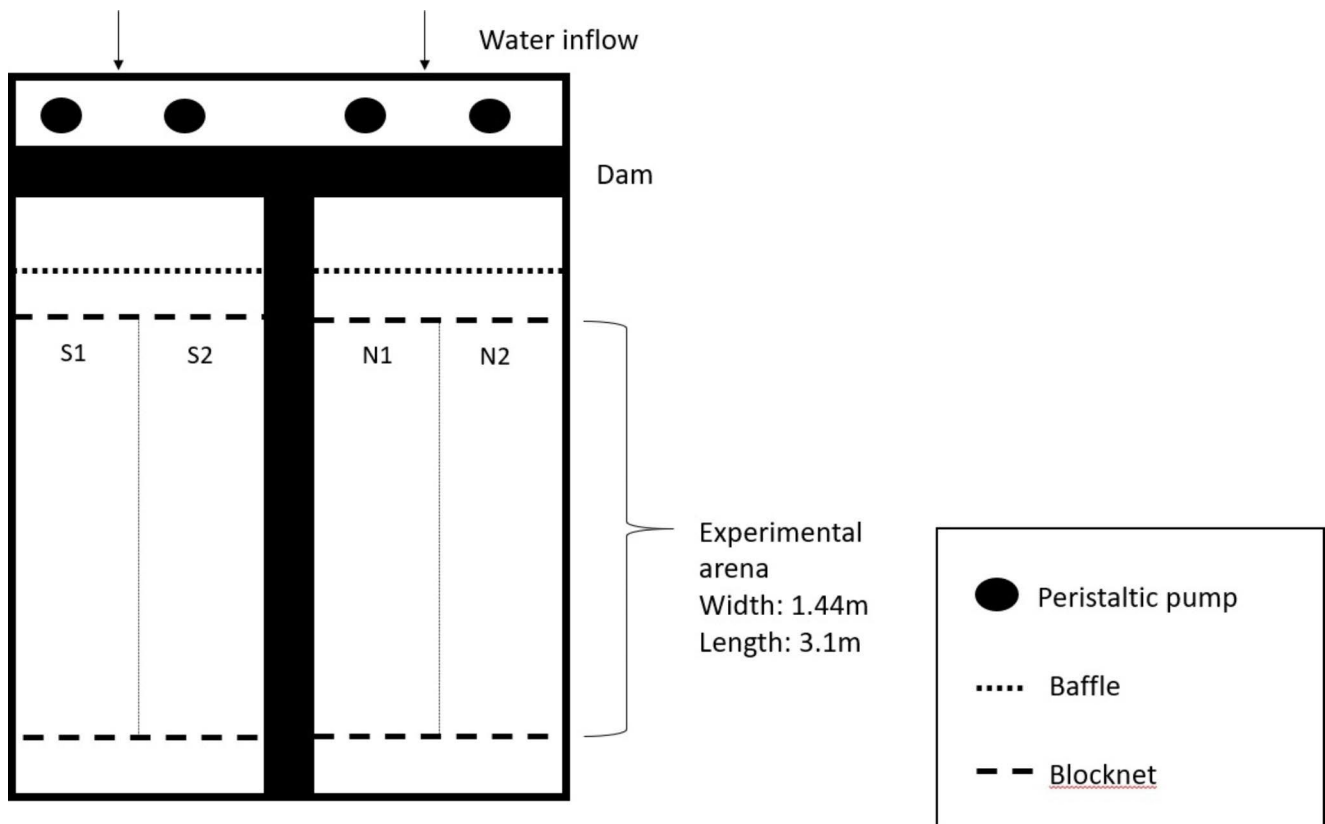


Fig. 1 Schematic of laboratory raceway. Fish were introduced into middle of either south (S1 and S2) or north (N1 and N2) raceways at beginning of trial. Odor was introduced via one peristaltic pump

during the “stimulus” period of the trial, and pump sides were switched between each trial to account for side bias

holding basket into the middle of the experimental arena. Trials lasted 30 min including a 10 min acclimation period and a 20 min observation period, during which test odors were introduced. During a trial, test odors were introduced into one-half of the experimental arena (left or right side), with the side receiving the odor alternating on subsequent replicates. All odors were pumped into the channels from a beaker at the rate necessary to achieve a 1 000 000:2 DI water:odor extract dilution. To achieve this dilution, 88mL of odor extract was placed in a beaker and brought up to a total 524mL odor solution by adding 436mL of DI water (calculations of odor extract dilutions were done based on activated channel width, depth, and velocity). The odor solution was then transferred to a 1 L Nalgene bottle and continuously stirred with a 2 cm magnetic stir bar to ensure a homogenous mixture. Odors were pumped into the system at a fixed rate of 20mL min^{-1} with peristaltic pumps (MasterFlex model 7533-20) through PVC tubing. A separate set of tubing was used for each odor or odorant to ensure no cross contamination occurred. Visual rhodamine dye tests were conducted to confirm the odor plume was confined to the target half of the experimental arena. At the conclusion of each trial, subjects were removed from the arenas, and

total length (TL, cm) and wet weight (g) were recorded for each individual.

Analyses.

Video analysis- Behaviors were quantified for each trial, representing independent samples, during the final 10 min of the observation period (post-stimulus period) to ensure the odor reached the end of the raceway and allow for the distribution of animals to stabilize after the addition of the odor (approximately 5 min per Wagner et al. 2011). Each video recording was examined by pausing each 30s and tallying the number of fish on each side of the channel (stimulus side or non-stimulus side), based on position of the head, as an indication of channel preference. Distribution (proportion on the stimulus side) was calculated as follows:

$$\frac{\Sigma(\text{number of fish on the stimulus side})}{\Sigma(\text{number of fish on the stimulus side}) + \Sigma(\text{number of fish on the non-stimulus side})}$$

The distribution for each treatment was computed by taking the mean of all trials in each treatment group. A proportion of fish significantly greater than 50% on the stimulus side indicated attraction, a proportion not significantly different from 50% indicated no preference, and a proportion significantly less than 50% indicated avoidance.

Table 2 List of individual compounds and subfractions tested in screenings of individual compounds and subfractions. P-values are derived from one-way two-sided T-tests comparing proportion of sea lamprey on the stimulus side after introduction of odorant to a null-hypothesis of 50:50 proportion of sea lamprey on the stimulus side (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Treatment	P-value	Mean	N
Solvent	0.725	0.517	20
Whole Body Alarm Cue	3.6e-07 ***	0.323	37
Arginine	0.417	0.408	5
Valine	0.731	0.537	5
Isoleucine	0.036 *	0.371	10
Leucine	0.456	0.582	5
Hypoxanthine	0.062	0.381	10
Tyrosine	0.222	0.441	10
Phenylalanine	0.785	0.431	5
Inosine	0.121	0.582	5
Tryptophan	0.090	0.651	5
Glutamic acid	0.374	0.610	5
Histidine	0.528	0.465	5
Creatine	0.933	0.483	5
Isoleucine + Tyrosine + Hypoxanthine	0.886	0.487	5
Pure Compound 1 (Petromyzonicil)	0.700	0.46	5
Chondroitin Sulfate	0.351	0.578	5
Subfraction SL-3 (Creatine + Arginine)	0.336	0.546	10
Subfraction SL-4 (Creatine + Arginine + Valine + Leucine + Isoleucine)	0.034*	0.319	5
Subfraction SL-5 (Hypoxanthine + Inosine)	0.333	0.439	10
Subfraction SL-6 (Adenine + Tyrosine + Xanthine)	0.869	0.491	10
Subfraction SL-7 (Histidine + Phenylalanine + Glutamic Acid + Tryptophan + Threonine)	0.860	0.514	10
Subfraction SL-8 (Asparagine + Methionine + Cysteine + Adenosine + Glycine)	0.430	0.566	5

Statistical analysis- All analyses were conducted in R (Version 1.4.1103). A one-way ANOVA was performed for each experiment with the response variable as proportion of animals on the stimulus side and stimulus (odor) type as fixed effect. In experiment 1, data were transformed using log transformations and normality was confirmed with a Shapiro-Wilk's test ($\alpha = 0.05$). Tukey's Honestly Significant Difference (HSD) ($\alpha = 0.05$) was completed as a post-hoc means comparisons for each treatment. The mean proportion of fish on the stimulus side for the water-soluble and chloroform-soluble fractions were compared to the whole body and crude skin alarm cue extracts, and the solvent control, to determine whether the alarm cue was partially, completely, or not significantly contained in either fraction. In experiment 2, data were transformed with an arcsine (square root) transformation, and normality was confirmed with a Shapiro-Wilk's test ($\alpha = 0.05$). The means of a whole-body extract treatment, solvent treatment, 14 individual compounds, one mixture of individual compounds, and 6 subfractions (Table 2) were compared to a null hypothesis of 50:50 proportion of fish on the stimulus side with separate paired t-tests for each odor (two-tailed, $\alpha = 0.05$) to screen for any attractant or repulsive response. In experiment 3, data were log-transformed and normality was confirmed with Shapiro-Wilk's test ($\alpha = 0.05$). Tukey's Honestly Significant Difference (HSD) ($\alpha = 0.05$) was used for post-hoc

means comparisons of each treatment. Here, the mean of the mixture of the identified compounds from the water-soluble fraction was compared to the whole-body alarm cue extract, the water-soluble fraction, and the solvent control to determine whether the alarm cue was contained within these identified components.

Results

Experiment 1: Comparison of Water- and Chloroform-Soluble Fractions. Model results (ANOVA, $F_{5,114} = 12.76$, $p < 0.001$) showed clear evidence that the type of odor introduced into the raceway significantly influenced the sea lamprey's use of space. Both alarm cue extracts (skin, whole-body) showed significant avoidance when compared to the solvent control (Tukey HSD, all solvent comparisons $p < 0.05$; Fig. 2). The response to the whole-body extract was not significantly different from that of the crude skin extract (Tukey HSD, $p = 0.99$; Fig. 2). Avoidance of the water-soluble fraction was 33% less than the whole-body extract (Tukey HSD, $p < 0.01$; Figs. 2) and 28% lower than the crude skin extract (Tukey HSD, $p < 0.05$; Fig. 2), and was not significantly different from the chloroform-soluble fraction (Tukey HSD, $p = 0.98$; Fig. 2). Avoidance of the chloroform-soluble fraction was not significantly different

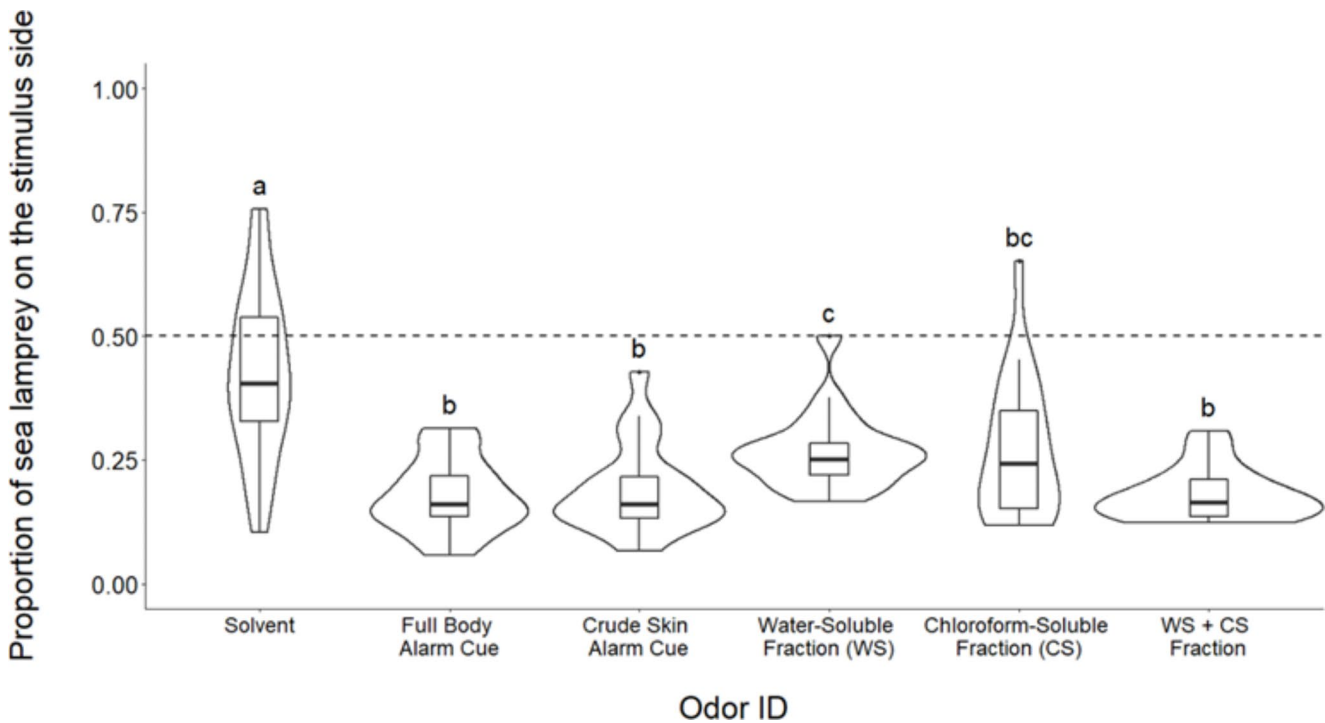


Fig. 2 Boxplots representing the proportion of sea lamprey on the stimulus side after the addition of odorants. The middle quartile of boxes represents treatment median, and upper and lower quartiles are the 75th and 25th percentile of the range, respectively. Upper and lower whiskers represent the minimum and maximum spread of the data.

from the whole-body extract (Tukey HSD, $p=0.05$; Fig. 2), the crude skin extract (Tukey HSD, $p=0.18$; Fig. 2), or the water-soluble fraction (Tukey HSD, $p=0.98$; Fig. 2). The behavioral response to the water-soluble fraction appeared more consistent (variance=0.006; Fig. 2) than the response to the chloroform-soluble fraction (variance=0.02; Fig. 2). Avoidance of a mixture of the water-soluble and chloroform-soluble fractions was not significantly different than observed for the whole-body extract (Tukey HSD, $p=0.99$; Fig. 2) or the crude skin extract (Tukey HSD, $p=0.99$; Fig. 2).

Experiment 2: Screening of Sub-Fractions and Compounds in the Water-Soluble Fraction. Odor introduced into the channel significantly influenced sea lamprey behavior in the screenings of individual compounds and subfractions (ANOVA, $F_{22,173} = 2.12$, $p < 0.01$). As predicted, the observed response to the solvent control was not significantly different from the null expectation of a 50:50 distribution ($t_{19} = 0.36$, $p = 0.73$, Table 2), and subjects significantly avoided the whole-body alarm cue ($t_{36} = -6.21$, $p < 0.001$, Table 2). Only one of the 13 individual compounds screened from the water-soluble fraction elicited significant avoidance, isoleucine ($t_9 = -2.47$, $p = 0.04$, Table 2). Another compound, hypoxanthine, exhibited a marginally non-significant

Odor ID

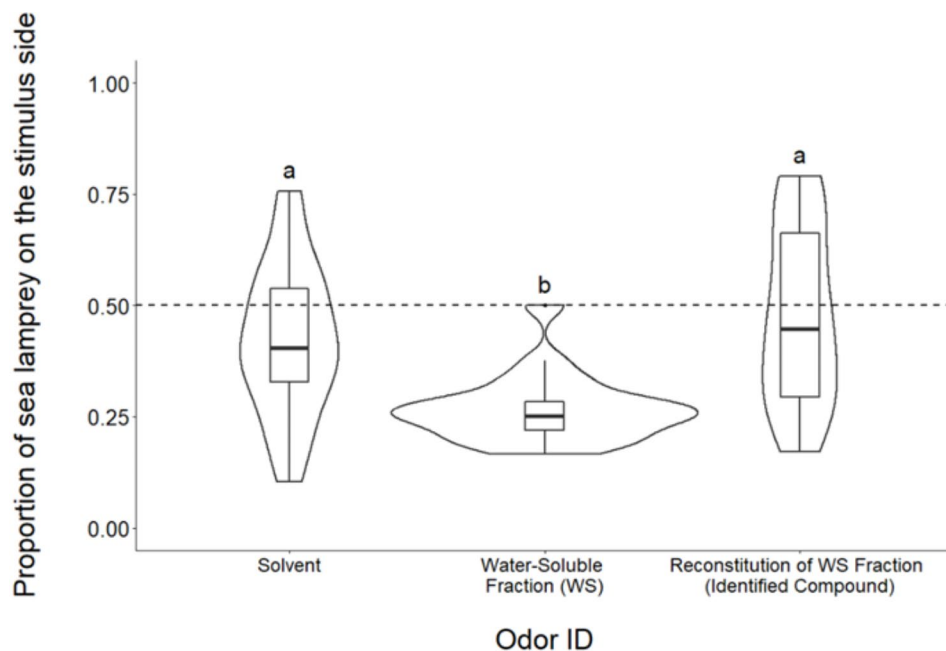
Violin plots demonstrate the frequency of proportion values for each treatment. WS=water-soluble and CS=chloroform soluble. Dashed line at 0.50 represents the null hypothesis of a true neutral response to introduced stimulus. Treatments with different letters are significantly different from one another based on Tukey HSD ($\alpha=0.05$). $N=20$ for each bar

avoidance response ($t_9 = -2.13$, $p = 0.06$, Table 2). Similarly, only one of the six screened subfractions, SL-4, elicited avoidance ($t_4 = -3.15$, $p = 0.03$, Table 2), and contained creatine, arginine, valine, and isoleucine. Other than isoleucine, none of these compounds showed an avoidance response when tested alone (creatine, $t_4 = -0.09$, $p = 0.93$; arginine, $t_4 = -0.90$, $p = 0.42$; valine, $t_4 = 0.37$, $p = 0.73$; Table 2). Additionally, a mixture of isoleucine, tyrosine, and hypoxanthine ($t_4 = -0.15$, $p = 0.89$, Table 2) showed no evidence of behavioral reactivity. Chondroitin-sulfate also showed no evidence of behavioral reactivity ($t_4 = 1.06$, $p = 0.35$, Table 2).

Experiment 3: Testing the Mixture of Identified Compounds from the Water-Soluble Fraction. Odorant type significantly influenced sea lamprey avoidance behavior (ANOVA, $F_{2,58} = 8.99$, $p < 0.001$).

Here, a mixture of the 32 identified compounds from the water-soluble fraction exhibited no avoidance response, indicated by no significant difference in response when compared to the negative solvent control (Tukey HSD, $p = 0.59$; Fig. 3), and a significantly lower avoidance response compared to the water-soluble fraction (Tukey HSD, $p < 0.001$; Fig. 3).

Fig. 3 Boxplots representing the proportion of sea lamprey on the stimulus side after the addition of odorants. The middle quartile of boxes represent treatment median, and upper and lower quartiles are the 75th and 25th percentile of the range, respectively. Upper and lower whiskers represent the minimum and maximum spread of the data. Violin plots demonstrate the frequency of proportion values for each treatment. Dashed line is null hypothesis of 50% of animals on the stimulus side after the introduction of the odor. Treatments with different letters are significantly different from one another based on Tukey HSD ($\alpha = 0.05$). $N = 20$ for each bar



Discussion

Recent research has revealed several potential uses for the sea lamprey alarm cue as a species-specific repellent that can aid in the management of both invasive and threatened populations. We report the behavioral responses of migratory sea lamprey to two major odor fractions derived from Soxhlet extraction of the skin; a tissue known to contain the animal's alarm cue. We found that both the water-soluble and chloroform-soluble fractions elicited substantial avoidance responses, with the water-soluble fraction exhibiting a significantly lower avoidance response than the crude skin extract and the chloroform-soluble fraction exhibiting an avoidance response no different than either the water-soluble or the crude skin. When the two fractions were recombined, the full response was restored. There were six sub-fractions derived from the water-soluble fraction, from which 32 compounds were isolated and identified, representing 98% of the dry mass of extracted material. Only one individual compound, isoleucine, evoked an avoidance response during initial screening; however, this was not consistent across all treatments containing the compound. Finally, to test for synergistic effects, we examined the behavioral response of sea lamprey to a mixture of the 32 identified compounds combined at the ratios and quantities observed in the water-soluble fraction. The mixture failed to evoke an alarm response. Together, these results indicate that the active components of the sea lamprey alarm cue are contained in two chemically dissimilar fractions from skin but were not fully contained in the mixture of compounds identified to date.

Consistent with previous reports (Byford et al. 2016; Hume et al. 2015; Hume and Wagner 2018; Imre et al. 2014, 2016; Luhring et al. 2016; Wagner et al. 2011, 2016), we observed a predator avoidance response to extracts from sea lamprey skin tissue that is consistent with the hypothesis that certain components of the cue exhibit substantial water solubility and are nonvolatile. Specifically, the water-soluble fraction of Soxhlet-extracted skin invoked 72% of the avoidance response observed from the crude extract.

Sea lamprey did not exhibit consistent avoidance responses to the 13 individual compounds, one compound mixture, or six sub-fractions from the water-soluble fraction subset in the screening experiment (Experiment 2, Table 2). While isoleucine exhibited an avoidance response on its own, other treatments where it was mixed with other compounds (namely tyrosine and hypoxanthine), exhibited no avoidance response (Table 2). One possibility is that tyrosine or hypoxanthine could act as an antagonist for isoleucine. However, it has been shown that leucine and valine act antagonistically with isoleucine (amino acids found in the avoidant SL-4 fraction), whereas tyrosine (found in the neutral mixture of isoleucine, tyrosine, and hypoxanthine) does not (Kajikawa et al. 2005). It is also possible that the compounds were run at a sample size too small to accurately detect a significant response. Thus, none of the compounds that were evaluated was sufficient on its own to elicit consistent predator avoidance across all included treatments, but further investigation on the role of isoleucine at higher replication is needed to understand if it elicits a robust avoidance response.

When all 32 identified compounds from the water-soluble fraction (Experiment 3, Table 1) were combined at the observed ratio found in the crude skin extract, a neutral response similar to the solvent treatment was observed (Fig. 3). One plausible explanation for why we saw no antipredator responses to individual compounds, sub-fractions, or recombined identified compounds within the water-soluble fraction is that the alarm cue may consist of a blend of compounds, all of which need to be present in order to elicit a behavioral response. Previous studies have noted singular compounds can be potent in eliciting alarm responses and are hypothesized to contain a component of the active ingredients of alarm cue, such as hypoxanthine 3-N-Oxide in zebrafish (Parra et al. 2009), fathead minnows (Brown et al. 2001), and black tetra (Pfeiffer et al. 1984), and chondroitin sulfate in zebrafish (Mathuru et al. 2012) and fathead minnows (Faulkner et al. 2017). Other studies suggest the full mixture needs to be present (larval grey tree frog *Hyla vesicolor*, Mirza et al. 2013), sea hare *Aplysia californica*, Kieckhefer et al. 2007). Our findings, along with the observed diminishing reactivity with increasing phylogenetic distance between donor and receiver in sea lamprey (Bals and Wagner 2012; Hume and Wagner 2018) align to suggest the sea lamprey alarm cue is a mixture of active components, with some shared compounds across species and species-specific labeling compounds (i.e., the multicomponent pheromone hypothesis). Another possible explanation is that the active compounds of the alarm cue degraded or were otherwise lost during the separation and purification process. Extractions of chemical defense compounds from one vascular plant (*Micranthemum umbrosum*) led to significant degradation and inefficient yields, which may have degraded potentially active compounds below a detection threshold (Parker et al. 2006). In the present study, bioassay guided isolation and purification were conducted under mass balance at every step, and there was no noticeable loss of material during the purification based on mass balance of the original extract or fraction to the isolated compounds. However, given the lack of activity elicited by the major components of the active fractions, the alarm cue components may reside in the 2% of unidentified material remaining in the crude extract. It is also possible that the process of separation and isolation led to degradation of the active molecules after extraction if components in the crude extract functioned as stabilizers for the active material. Future research should focus on identifying the minor compounds of the water-soluble fraction to understand if they complete the cue and play a role in mediating antipredator behavior.

The compounds identified from the water-soluble fraction consisted of 32 amino acids, primarily creatine (Dissanayake et al. 2019; Green et al. 2017) demonstrate that all regions of the sea lamprey olfactory bulb respond to amino

acids, with the lateral bulb responding solely to amino acids and the dorsal and medial regions responding dually to amino acids and steroids, noting that these regions do not act redundantly but rather react to different types of information. Amino acids are associated with feeding behavior in sea lamprey (Kleerekoper and Mogenson, 1963). The concentration of combined amino acids from the water-soluble fraction was 0.023 mol l^{-1} , and the range of individual compounds was between $10^{-5} \text{ mol l}^{-1}$ and 0.536 mol l^{-1} . All but six amino acids in the extract were individually above the probable threshold of detection for sea lamprey ($10^{-3} \text{ mol l}^{-1}$, (Green et al. 2017)). Putrescine ($10^{-4} \text{ mol l}^{-1}$), pyruvic acid ($10^{-4} \text{ mol l}^{-1}$), serine ($10^{-5} \text{ mol l}^{-1}$), adenosine ($10^{-5} \text{ mol l}^{-1}$), spermine ($10^{-5} \text{ mol l}^{-1}$), and α -ketovaleric acid ($10^{-5} \text{ mol l}^{-1}$), were below this threshold. The molarity of the recombined water-soluble fraction should therefore be well above the detection threshold. Migratory sea lamprey are non-feeding, relying on lipids stored during the parasitic life stage to spawn and complete their lifecycle before death (William and Beamish 1979). It would be reasonable for responses to food cues to cease prior to the spawning migration to focus olfactory efforts on avoiding predation and finding mates. Life-stage dependent olfactory sensitivity has been cited in the Pacific lamprey, where reactivity to migratory and sex pheromones remained high and constant throughout the spawning migration before dropping significantly at spawning and maturation, and it is noted that Pacific and sea lampreys share remarkable similarities in odor responses both ecologically and physiologically (Robinson et al. 2009).

We observed a substantial avoidance response to the chloroform-soluble fraction that was similar in magnitude to the water-soluble fraction, but statistically indistinguishable from the crude extract due to higher variance in the behavioral responses. This finding contrasts somewhat with Dissanayake et al. (2016) who reported a partial (61% of the crude skin extract response) but statistically non-significant avoidance response to a similar fraction. A balanced one-way ANOVA power calculation for the data in Dissanayake et al. (2016) revealed that a sample size greater than or equal to 17 was needed to detect a significant response (power = 0.80, $\alpha = 0.05$), which was surpassed in the current study ($N = 20$), but not in the 2016 screening ($N = 10$). There are at least two plausible explanations for these observations. First, the chloroform-soluble fraction may contain one or more components of the alarm cue that are reactive and not found in the water-soluble fraction at concentrations sufficient to be detected by the olfactory organ. The major components of this fraction were previously identified as four cholesterol esters, five tri- and di-glycerides, a cholesterol, 13 free fatty acids, and two environmental pollutants (Dissanayake et al. 2016), but not tested individually for behavioral responses.

Fatty acids have been shown to be behaviorally relevant in migrating sea lamprey; an active compound, (+)-petromyric acid, of the attractant cue emitted by larvae is a fatty-acid derivative (Li et al. 2018). More broadly, three olfactory sensory neuron (OSN) morphotypes have been identified in teleost fishes (Hamdani and Døving 2007), and the structure of these morphotypes are strikingly similar to those observed in the more primordial sea lamprey (Laframboise et al. 2007). In teleost fishes, the ciliated OSN activates the medial olfactory tract and responds to compounds important in both migration and alarm responses (Hamdani and Døving 2007; Døving and Lastein 2009). The medial bulbar region of the sea lamprey olfactory bulb responds to amino acids, bile salts, and components of the larval cue (Green et al. 2017). If the overlap in sensory pathways among migratory and alarm cues is present in lampreys, further testing of OSN pathway activation may help to discern the identity of the alarm cue component(s) contained in the chloroform-soluble fraction.

Reactivity in the chloroform-soluble fraction could also be attributed to incomplete separation of the mixture, with one or more behaviorally reactive compounds occurring in both major fractions. Interestingly, when the water-soluble and chloroform-soluble fractions were re-combined, the magnitude of the avoidance response increased to that observed from the crude skin extract. Recombination may have restored the correct ratios of alarm cue compounds. Studies on alarm cue phylogenetic patterning in Ostariophysan fishes have suggested that reactivity is dependent on observed ratios of compounds, and that such ratios are species-specific. For example, the purine ratio hypothesis posits the existence of a common set of purine carriers for a nitrogen-oxide alarm trigger in Ostariophysan fishes, with ratios of the carrier molecules differing among related species, and larger differences between more distantly related species (Brown et al. 2001, 2003; Kelly et al. 2006). Alternatively, or in addition, recombination may have restored the full concentration of alarm cue components, eliciting a stronger behavioral reaction. The threat-sensitive response hypothesis predicts that prey who modulate their antipredator behavior in response to the perceived intensity of the threat will have a selective advantage (Helfman 1989). Fishes (Brown et al. 2006; Lönnstedt & McCormick, 2011), amphibians (Ferrari et al. 2009; Fraker 2008), and aquatic insects (Roux and Diabate 2014) are known to respond to varying concentrations of alarm and predator cues in a threat-sensitive manner.

Previous studies have demonstrated that chondroitin fragments play an active role in fish alarm cue chemistry (Faulkner et al. 2017; Mathuru et al. 2012). During screening, sea lamprey failed to respond to chondroitin sulfate derived from shark cartilage (Table 2). An intermediate

alarm response was observed in wild fathead minnows (*Pimephales promelas*) that were introduced to chondroitin sulfate sourced from bovine trachea (Faulkner et al. 2017). Zebrafish exhibited a full suite of alarm behaviors when exposed to chondroitin sulfate sources from shark cartilage and an intermediate response when exposed to chondroitin sulfate from sturgeon notochord, and these differences are likely due to source differences in sulfation which affect signaling properties of chondroitin (Mathuru et al. 2012). Because of the observed differences in fish response to chondroitin sulfate sources, more research into the sea lamprey behavioral response to differently sulfated forms of chondroitin may be warranted. However, as noted above, given the apparent lack of response by sea lamprey to alarm cues of teleost fishes, they may be chemically distinct.

In summary, this study represents the first major steps towards identifying the sea lamprey alarm cue. Our work provides evidence in support of previous studies that hypothesized the sea lamprey alarm cue contains a mixture of stable molecules (Bals and Wagner 2012; Dissanayake et al. 2019; Hume and Wagner 2018), and suggests for the first time that the active constituents are not solely contained in the water-soluble fraction of the crude skin extract. Thus, questions remain regarding the active components of the sea lamprey alarm cue. Future work should include investigating the role of isoleucine and focus on discovering the identity of minor compounds within the water-soluble fraction, testing individual and combined compounds identified from the chloroform-soluble fraction, targeting areas of potential overlap. Additional research is needed to explore the evolution of olfactory roles in predator-prey dynamics, and to understand how alarm cues can be synthesized and thus applied towards conservation goals and the management of aquatic invasive species. We argue that the pursuit of the alarm cue's chemical identity is crucial to answer questions on the evolution of chemosensory cues in predator-prey dynamics and can lead to important information on how wildlife managers and conservation professionals can use such cues for applied work in aquatic systems.

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Statements and Declarations

Competing Interests The authors state that they have no conflict of interests to declare.

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