

PROGRESS TOWARD CHEMICAL IDENTIFICATION OF THE SEA LAMPREY (*Petromyzon marinus*) ALARM
CUE

By

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ABSTRACT

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This thesis examined the chemical constituents of repellent odors in the sea lamprey (*Petromyzon marinus*), an invasive fish in the Great Lakes basin, for use in management and conservation. Odors are powerful tools that guide organism's movement decisions, and repellent odors such as conspecific alarm cues are particularly potent. In chapter one, we investigated the chemistry of the alarm cue through behaviorally guided fractionation. We found substantial avoidance responses to two major fractions: water-soluble and chloroform soluble and a full avoidance response upon recombination of the two fractions. We found no consistent avoidance patterns to subfractions, or individual compounds identified in the water-soluble fraction, and we found no avoidance response to all 32 identified compounds from the water-soluble fraction when combined at observed ratios in the skin. In chapter two, we investigated the role of a potential repellent molecule, putrescine, on sea lamprey activity and avoidance in a small individual behavioral assay and avoidance in a large, multi-animal assay. We found a context-dependent response, where sea lamprey did not increase activity in the small assay but did show a substantial avoidance response to putrescine. No evidence of avoidance to putrescine was observed in the large assay. In sum, these results suggest the alarm cue is likely not contained in the 32 identified compounds in the water-soluble-fraction alone and that the behavioral response to putrescine is context dependent. While elucidating the chemistry of the sea lamprey alarm cue may be more difficult than through behaviorally guided fractionation alone, the continued pursuit is worthwhile because of the utility in invasive species management and conservation of native species.

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GENERAL INTRODUCTION

Aquatic organisms rely heavily on odor cues to complete their lifecycles. Odors that give information about the risk of predation are particularly powerful in informing movement choices through complex systems and during migration (reviewed in Ferrari, Wisenden, & Chivers, 2010). One important class of risk-related odors are alarm cues, which are species-specific chemical mixtures released from conspecific injuries that give migrants reliable information about the presence of an active predator (Chivers & Smith, 1998; Smith, 1992; Wisenden et al., 2004). Adding alarm cues to manipulate landscapes of perceived risk has the potential to aid management and conservation efforts by providing effective species-specific repellents to guide organisms into desired locations (such as traps or fish pass devices). This thesis aims to investigate the chemical identity of the injury released alarm cue from sea lamprey (*Petromyzon marinus*), a species which is invasive in the Laurentian Great Lakes, causing significant economic and ecological damage in the region, and is dually a species of conservation concern in its native range in the basins of the northern Atlantic ocean (Hume et al., 2021). To use the alarm cue in management, its chemistry must be known for synthesis and application, as per U.S. federal regulations (Ferguson & Gray, 1989). Here, we use odor-guided fractionation, a process that partitions odor into fractions and identifies behaviorally active mixtures (subfractions) and individual compounds through testing in behavioral bioassays. We tested the repellent activity of solvent-partitioned fractions of alarm cue extracts in controlled laboratory raceway streams. We built upon past studies to test behavioral reactivity to previously identified behaviorally active fractions, subfractions and individual compounds reported in Dissanayake et al. (2016, 2019).

Previous studies have shown that alarm cue elicits a robust avoidance response in sea lamprey in laboratory (Bals & Wagner, 2012; Byford et al., 2016; Hume & Wagner, 2018; Imre et al., 2014; Imre et al., 2016) and field studies (Hume et al., 2015; Imre et al., 2010; Luhring et al., 2016; Wagner et al., 2011). The alarm cue is contained throughout the sea lamprey's body and is concentrated in

the skin (Bals & Wagner, 2012). Chemically characterizing the crude skin alarm cue extract began through fractionation via solvent partitioning (Dissanayake et al., 2016, 2019). Two distinct fractions were elucidated through this process: the water-soluble fraction and the chloroform-soluble fraction. Initial behavioral screenings found the water-soluble fraction to be the most behaviorally active fraction (Dissanayake et al., 2019), and 32 compounds within the water-soluble fraction were identified (Dissanayake et al., 2019). This thesis investigates the role of these compounds in eliciting anti-predator responses (avoidance and flight) in isolation and combination to build understanding of the chemical makeup of the behaviorally active constituents of the alarm cue extract.

Chapter 1 explores the chemistry of the sea lamprey alarm cue through behaviorally guided fractionation. In a high-replication study in laboratory raceways, avoidance responses were investigated to each fraction (water-soluble and chloroform-soluble) as well as subfractions and individual compounds identified from the water-soluble fraction. In contrast to previous studies, we found that each fraction elicited stark and substantial avoidance responses at 75% of the total alarm cue skin extract. When the two fractions were combined, an avoidance response of equal magnitude to the skin extract was restored. No subfractions nor isolated compounds elicited consistent avoidance responses, except for isoleucine which was repellent on its own but not in combination with other compounds. Finally, a recombination of the 32 compounds identified to date from the water-soluble fraction elicited no avoidance response. These results provide considerable headway in the steps to understanding the chemistry of the alarm cue by eliminating many compounds identified in the water-soluble fraction as being part of the alarm cue alone. This sets up next steps: isolate trace material within the water-soluble fraction and investigate the overlap in compounds between the water-soluble and chloroform-soluble fractions. We show that elucidating this chemistry is likely harder than traditional behaviorally guided fractionation methods alone.

Chapter 2 continues the exploration of behaviorally relevant odors for use in management and conservation by investigating the role of putrescine, a decay compound released from rotting flesh.

This odorant is contained in the sea lamprey skin extract, as described in chapter 1, and human saliva, which is known to elicit strong flight and avoidance behaviors in sea lamprey. Two behavioral assays were used in this experiment: 1) a small arena assay to investigate individual activity rates and avoidance behavior, and 2) a large arena assay which investigated avoidance behavior in group trials. We found no change in activity in animals exposed to putrescine and found repellent behavior in the small assay but no robust avoidance patterns in the larger, multi-animal assay. However, we found a consistent increase in activity and avoidance behaviors in alarm cue treatments in the small assay and consistent avoidance in the large assay. We also investigated the role of petromyzonacil, a unique compound identified in the sea lamprey skin (described in chapter 1) and found no evidence of behavioral effects on its own or in combination with putrescine. These results show limited support for using putrescine as a repellent and give evidence for the efficacy of a small assay design in high-throughput screening studies. Future studies should investigate other decay odors, such as cadaverine, on sea lamprey repellent behavior and research the response to saliva treatments from other mammalian sources.

The work contained in this thesis has furthered our understanding the chemistry of the sea lamprey alarm cue. Here, it was found that the alarm cue is likely not contained within the 32 compounds identified in the water-soluble fraction of the crude skin extract alone and that behavioral responses to the decay odor putrescine were dependent on the context of assay design. Overall, these findings agree with workers seeking the alarm cue in other aquatic species, suggesting isolation and identification of alarm cues is more complex and difficult than reproductive pheromones. However, given that alarm cue repellents have proven more efficacious in guiding the movements of migrating sea lamprey (vs reproductive pheromones), elucidating the chemical structures of behaviorally relevant odors should remain a high priority. Next steps should include investigating minor compounds in the water-soluble fraction and areas of chemical overlap between the water-soluble and chloroform-soluble fractions. More research is also needed to understand the

utility of individual assays for high-throughput screenings in chemical ecology research and to understand the effect of individual variation and personalities on behavioral patterns.

REFERENCES

REFERENCES

- Bals, J. D., & Wagner, C. M. (2012). Behavioral responses of sea lamprey (*Petromyzon marinus*) to a putative alarm cue derived from conspecific and heterospecific sources. *Behaviour*, 149(9), 901–923.
- 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. *N. Am. J. Fish. Manag.*, 36(5), 1090–1096.
- Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signaling in aquatic predator-prey systems: A review and prospectus. *Ecoscience*, 5(3), 338–352.
- Dissanayake, A. A., Wagner, C. M., & Nair, M. G. (2016). Chemical characterization of lipophilic constituents in the skin of migratory adult sea lamprey from the Great Lakes Region. *PLoS One*, 11(12), 1–17.
- Dissanayake, A. A., Wagner, C. M., & Nair, M. G. (2019). Nitrogenous compounds characterized in the deterrent skin extract of migratory adult sea lamprey from the Great Lakes region. *PLoS ONE*, 11(12), 1–19.
- Ferguson, S., & Gray, E. (1989). 1988 FIFRA Amendments: A Major Step in Pesticide Regulation. *Envtl. L. Rep. News & Analysis*, 19, 10070.
- Ferrari, M. C. O., Wisenden, B. D., & Chivers, D. P. (2010). Chemical ecology of predator – prey interactions in aquatic ecosystems: a review and prospectus. *Can. J. Zool.*, 88, 698–724.
- Hume, J. B., Almeida, P. R., Buckley, C. M., Criger, L. A., Madenjian, C. P., Robinson, K. F., Wang, C. J., & Muir, A. M. (2021). Managing native and non-native sea lamprey (*Petromyzon marinus*) through anthropogenic change: A prospective assessment of key threats and uncertainties. *J. Great Lakes Res.*, 47, S704–S722.
- 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(12), 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(7), 3751–3762.
- Imre, I., Rocco, R. T. Di, Belanger, C. F., Brown, G. E., & Johnson, N. S. (2014). The behavioural response of adult *Petromyzon marinus* to damage-released alarm and predator cues. *J. Fish Biol.*, 84, 1490–1502.
- Imre, I., Rocco, R. T. Di, 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(8), 613–620.
- Imre, I., Brown, G. E., Bergstedt, R. A., & McDonald, R. (2010). Use of chemosensory cues as repellents for sea lamprey: Potential directions for population management. *J. Great Lakes Res.*, 36(4), 790–793.

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.

Smith, R. J. F. (1992). Alarm signals in fishes. *Rev. Fish Biol. Fish.*, 2(1), 33–63.

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(7), 1157–1160.

Wisenden, Brian D., Vollbrecht, K. A., & Brown, J. L. (2004). Is there a fish alarm cue? Affirming evidence from a wild study. *Anim. Behav.*, 67(1), 59-67.

CHAPTER 1: Behavioral responses of sea lamprey (*Petromyzon marinus*) to fractions, sub-fractions, and individual compounds identified from the alarm cue

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*), a destructive invasive species in the Laurentian Great Lakes that is targeted for conservation in its native range, relies on its alarm cue to navigate around areas of predation risk when migrating. Identification of the cue constituents would allow managers to harness this innate behavioral response to guide migrants towards control or conservation measures. We pursued the chemical make-up of the sea lamprey alarm cue through behaviorally guided fractionation, using solvents to fractionate the alarm cue extract into water-soluble and chloroform-soluble fractions, each of which elicited 75% of the avoidance response observed from crude skin extract. Recombining the two fractions restored full reactivity, suggesting alarm cue components may include lipids in addition to water-soluble compounds. We further screened 13 individual compounds and 6 sub-fractions from the water-soluble fraction and found one individual compound, 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 identified from the water-soluble fraction. These results confirm other suggestions that the process of elucidating alarm cue constituents will prove more challenging than isolating reproductive pheromones. However, we suggest the pursuit is worthwhile given the strong evidence for the utility of alarm cue for use in conservation and management of the sea lamprey.

Introduction

Animals need to perceive and react to predation risk, and they must balance the costs of their responses against other needs, including energy acquisition and reproduction (Ferrari, Sih, & Chivers, 2009; Lima & 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 is 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 is 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. For example, the Seychelle 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 anti-predator behaviors to piscivorous pike (*Esox lucius*) (Hawkins, Magurran, & Armstrong, 2004). Acquired predator recognition typically involves learning (Ferrari, Gonzalo, & Chivers, 2007). Examples include blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) that acquire recognition of 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 & Smith, 1998; Smith, 1992; Brian D.

Wisenden, Vollbrecht, & Brown, 2004). Exposure to an alarm cue typically elicits anti-predator behaviors including increased shelter use, decreased activity, and area avoidance (Lawrence & 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 & 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 & Lastein, 2009; Ferrari, Wisenden, & Chivers, 2010; Hume & Wagner, 2018). When alarm cues are presented with the odor of an unfamiliar predator, the prey learns to associate predator odor with danger and avoids it in the future (Brown, 2003; Ferrari, 2005; Kelley & 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, Magurran, & Armstrong, 2008; Kopack, Broder, Fetherman, Lepak, & Angeloni, 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.

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, Vrieze, & Fine, 2004; Sorensen & Vrieze, 2003; Vrieze, Bergstedt, & Sorensen, 2011; Wagner et al., 2006; Wagner, Twohey, & Fine, 2009). Transition from deep open waters into narrow shallow streams exposes migrants to a suite of difficult to detect predators that patrol the shorelines (e.g. raccoon, otters; Imre et al., 2014; Scott & Crossman 1998). Because this migration is nocturnal, and sea lamprey move solitarily (Almeida, Quintella, & Dias, 2002; Binder & 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 anti-

predator 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, and 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, Coghlan Jr, & Zydlewski 2013; Kynard & 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 control a pest species, and to meet Federal requirements for pesticide registration (Ferguson & Gray, 1989).

Describing the chemical messengers that constitute fish alarm cues is one of the most important gaps in the field of chemical ecology (Døving & Lastein, 2009; Hüttel 1941; 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, Adrian, & Gerlai, 2009), fathead minnows (Brown, Adrian, & Shih, 2000) 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 anti-predator 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. A common pattern that has arisen is reduced reactivity to alarm cues obtained from related taxa, where the magnitude of the alarm response declines with increasing phylogenetic distance between the cue donor and the responding species (Mirza & Chivers, 2001; Mitchell, Cowman, & McCormick, 2012; Schoeppner & Relyea, 2009; Mathis & Smith 1993). In two previous studies, sea lamprey 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, Li, & Li, 2018). This process has been successful in the identification of components of sex pheromones in a variety of species (Algranati & Perlmutter, 1981; Yambe et al., 2006; Zielinski et al., 2004), including sea lamprey (Li et al., 2002; Scott et al., 2018). Alarm cues have 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 an anti-predator response (Mirza, Laraby, & Marcellus, 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, Wagner, & Nair, 2016, 2019), alone and in combination, in a standard laboratory assay.

Materials and Methods

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 first experiment evaluated the sea lamprey's behavioral response to a solvent control, crude alarm cue extracts derived from the whole body or the skin, a water-soluble (WS) fraction derived from the skin alarm cue, a chloroform-soluble (CS) fraction derived from the skin extract, and the WS and CS extracts combined. Prior reports had indicated the alarm cue is fully contained within the WS fraction from skin (i.e., exhibited full behavioral reactivity when compared to whole-body extract), with indications of partial reactivity in the CS fraction in a high variance, low sample size screening (Dissanayake et al. 2019). The second experiment screened (low replication) 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 (Table 1). Another compound, chondroitin-sulfate, was not isolated from the WS extract, but was also screened (Table 1), as previous studies which 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 (Table 2) that represented 98% (dry weight) of the material contained in the WS fraction and compared the behavioral reactivity of the mixture to the crude extracts from whole body and skin.

Table 1. List of individual compounds and subfractions tested in screenings. 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

Table 2. 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%
						β -Hydroxybutyric acid	0.06%
Tyrosine	0.08%	Methionine	0.41%	Xanthine	0.36%	α -Ketobutyric acid	0.07%
Isoleucine	0.17%	Glycine	0.24%	Hypoxanthine	0.85%	α -Ketoisovaleric acid	0.06%
Phenylalanine	1.29%	Cysteine	0.84%	Adenosine	0.48%	α -Ketovaleric acid	0.01%
Glutamic acid	0.09%	Proline	0.07%	Petromyzonacil	0.04%		

Odor collection

Whole body extract- Alarm cue was obtained from Soxhlet extraction of sea lamprey carcasses that naturally senesced during captivity per the method of Wagner et al. 2011. In short, odor was derived through Soxhlet extraction from nine male and female sea lamprey weighing 1,496.5g total. All carcasses were kept at -20° C before being used in extractions. Soxhlet extractors (2.08m, Ace Glass Inc., Vineland, New Jersey, USA) were mounted to six-bulb water-cooled Allihn condensers. Solvent reservoirs (12L 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 (approximately six hours), creating ~10.2L 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- Alarm cue was also collected from sea lamprey skin for use in the assays and in the chemical analyses per the method described in Dissanayake, Wagner, & Nair (2016, 2019, 2021). Lamprey skins were extracted through Soxhlet extraction as described above, with solvent (80:20 EtOH:RO water). Extracts underwent rotary evaporation to remove excess ethanol, were lyophilized, and stored at -80° C until experimental use. The skin extract was then fractionated by solvent partitioning (Dissanayake et al., 2019), yielding a chloroform-soluble fraction (21.4 g) and a water-soluble fraction (20.3 g). Subfractions of the water-soluble fraction were elucidated through a CombiFlash MPLC purification system. Individual compounds in the water-soluble fraction were isolated via preparative HPLC to purify the MPLC fractions. The structures of the isolated compounds were elucidated through NMR experiments, and HR-ESITOFMS spectra was recorded via mass spectrometry. Chondroitin sulfate used in experiment 1 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 2). 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 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 & Wagner 2012). Prior studies with sexually immature migrants indicated no difference in response to alarm cue between sexes (Bals & 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 (Figure 1). Each raceway measured 1.44m x 12.2m, 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 6-18 °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 hours 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 began by carefully releasing the ten animals from their holding basket into the middle of the experimental arena. Trials lasted 30 minutes including a 10-minute acclimation period and a 20-minute 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 containing a solution at target 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 1L Nalgene bottle and continuously stirred with a 2cm 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.

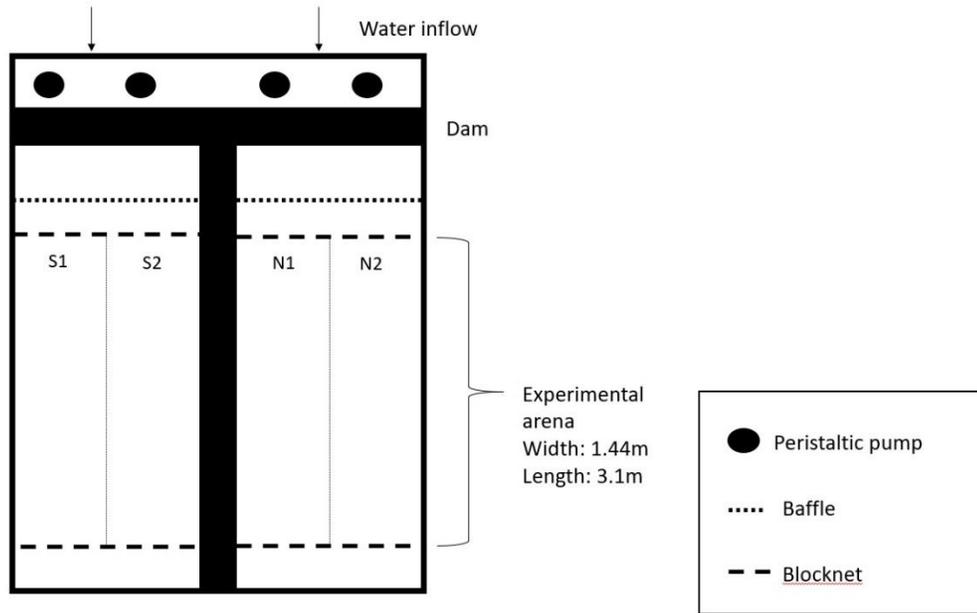


Figure 1. Schematic of laboratory raceway. Fish were introduced into middle of either south (S1 and S2) or north (N1 and N2) raceways at beginning of trial. Odor was introduced via one peristaltic pump during the "stimulus" period of the trial, and pump sides were switched between each trial to account for side bias.

Analyses

Video analysis- Behaviors were quantified during the 10-minute acclimation period (pre-stimulus period) and during the final 10 minutes 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. 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:

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

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

Statistical analysis- All analyses were conducted in R (Version 1.4.1103). A one-way ANOVA was performed for each experiment with response variable as proportion of animals on the stimulus side and stimulus (odor) type as fixed effect. In experiment 1, data was 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 distributions 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 was 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 1) were compared to a null hypothesis 50:50 distribution 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 was 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, $F_{5,114} = 12.76$, $p < 0.001$) demonstrated clear evidence that the type of odor introduced into the raceway significantly influenced the sea lamprey's use of space. All alarm cue treatments demonstrated significant avoidance when compared to the solvent control (Tukey HSD, all solvent comparisons $p < 0.05$; Figure 2). The response to the whole-body extract was not significantly

different from that of the crude skin extract (Tukey HSD, $p = 0.99$; Figure 2). The water-soluble fraction was 33% lower than the whole-body extract (Tukey HSD, $p < 0.01$; Figure 2) and 28% lower than the crude skin extract (Tukey HSD, $p < 0.05$; Figure 2), and was not significantly different from the chloroform-soluble fraction (Tukey HSD, $p = 0.98$; Figure 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, $p = 0.99$; Figure 2) or the crude skin extract (Tukey HSD, $p = 0.99$; Figure 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 1), and subjects significantly avoided the whole-body alarm cue ($t_{36} = -6.21$, $p < 0.001$, Table 1). Only one of the 13 individual compounds screened from the water-soluble fraction demonstrated significant reactivity, isoleucine ($t_9 = -2.47$, $p = 0.04$, Table 1). Another compound, hypoxanthine, exhibited a marginally non-significant response ($t_9 = -2.13$, $p = 0.06$, Table 1). Similarly, only one of the six screened subfractions, SL-4, demonstrated behavioral reactivity ($t_4 = -3.15$, $p = 0.03$, Table 1), and contained creatine, arginine, valine, and isoleucine. Other than isoleucine, none of these compounds demonstrated 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 1). However, the mixture of isoleucine, tyrosine, and hypoxanthine ($t_4 = -0.15$, $p = 0.89$, Table 1) demonstrated no evidence of behavioral reactivity. Chondroitin-sulfate also demonstrated no evidence of avoidance behavior ($t_4 = 1.06$, $p = 0.35$, Table 1).

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, $p = 0.59$; Figure 3), and a significantly lower avoidance response compared to the crude water-soluble fraction (Tukey HSD, $p < 0.001$; Figure 3).

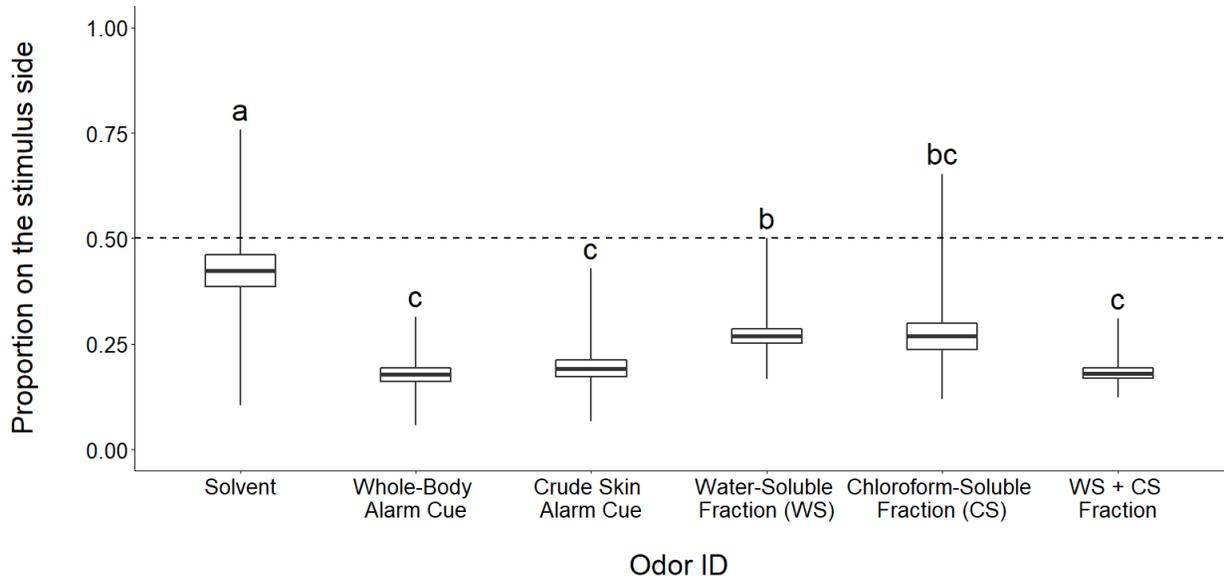


Figure 2. Mean \pm SE proportion of sea lamprey on the stimulus side after the addition of odorants. Middle quartile of boxes represent treatment means, upper and lower quartiles are ± 1 standard error (SE). Upper and lower whiskers represent maximum and minimum values for each treatment. WS = water-soluble 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.

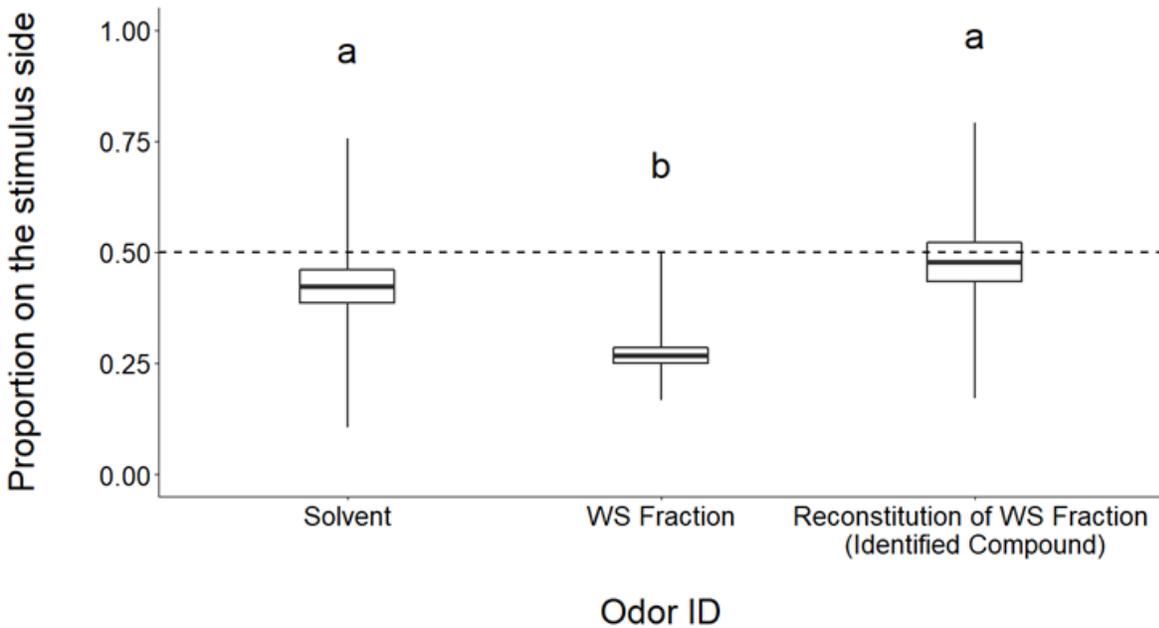


Figure 3. Mean \pm SE proportion of sea lamprey on the stimulus side after the addition of odorants to the arena. Middle quartiles are treatment means, upper and lower quartiles are \pm standard errors (SE). Upper and lower whiskers are maximum and minimum values. Dashed line is null hypothesis of 50% 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

When sea lampreys migrate into streams from oceans or large lakes, an alarm cue released from the tissue of injured conspecifics alerts them to areas of high predation risk. Recent research has revealed several potential uses for this alarm cue as a species-specific repellent that can aid in the management of both invasive and threatened populations. In this study, we investigated 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, each exhibiting a response magnitude three-fourths of the full skin extract. 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 alarm 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 distinct fractions from skin but were not fully contained in the mixture of known compounds.

Consistent with previous reports (Byford et al., 2016; Hume et al., 2015; Hume & Wagner, 2018; Imre et al., 2014; Imre et al., 2016; Luhring et al., 2016; Wagner et al., 2016; Wagner, Stroud, & Meckley, 2011), we observed a predator avoidance response to extracts from sea lamprey skin tissue that is consistent with the hypothesis that a majority of components contained in the alarm cue are waterborne and nonvolatile. Specifically, the water-soluble fraction of Soxhlet-extracted skin invoked 72% of the avoidance response observed from the crude extract. The sea lamprey alarm cue also appears considerably more stable than those derived from many other aquatic organisms. For example, alarm cues derived from two teleost fishes (fathead minnow *Pimephales promelas* and northern redbelly dace *Phoxinus eos*.) and an amphipod (*Gammarus lacustris*) failed to elicit anti-predator behaviors after resting for 6 h at room temperature (Wisenden et al., 2009). The active time of woodfrog (*Rana sylvatica*) tadpole alarm cues in the field has been estimated to be 2-4 h, likely due to environmental degradation (Ferrari, Messier, & Chivers, 2008). Based on these findings it has been hypothesized that fish alarm cues are comprised, in part, of proteins (Kasumyan and Ponomarev 1987; Lebedeva, Malyukina & Kasumyan 1975; Ferrari, Wisenden, & Chivers, 2010; Wisenden et al., 2009). That seems unlikely for sea lamprey. Many proteins experience the onset of denaturation at temperatures above 40° C (Lepock, Frey, & Ritchie, 1993), with denaturing often irreversible above 80° C (Matsuura et al., 2015). Here, Soxhlet extraction required heating to 80° C for up to 6 h. This is consistent with the heat treatment of a tadpole (*Rhinella arenarum*) alarm cue at 100° C, which did not reduce its potency, while a treatment with

Proteinase K did, leading to the conclusion that the alarm cue is likely a small peptide (Raices, Jungblut, & Pozzi, 2020).

These results may raise concern that the extraction process created repulsive byproducts that elicited avoidance behaviors due to esterization, amylation, or a similar transformation of natural molecules in the skin tissue. Two lines of evidence suggest this is unlikely. First, sea lamprey do not respond to Soxhlet extracts from the tissue of two teleost fishes, the bluegill sunfish (*Lepomis macrochirus*) and the white sucker (*Catostomus commersoni*) (Bals & Wagner, 2012; Hume & Wagner, 2018). Further, Soxhlet extracts from other species of lamprey (Petromyzontidae) do invoke an avoidance response in sea lamprey, with the response magnitude diminishing with increasing phylogenetic distance between the donor species and *P. marinus*. These patterns are entirely consistent with the phylogenetic patterning seen in anti-predator responses to alarm cues across fish species where the extracts were created by grinding tissue in water (Brown et al., 2010; Ferrari et al., 2010; Kelly, Adrian, & Brown, 2006; Mirza & Chivers, 2001). Second, there is evidence that sexually mature female sea lampreys stop responding to the conspecific alarm cue, whereas mature males continue to avoid Soxhlet extracted skin in the lab (Bals & Wagner, 2012). The sea lamprey alarm response may be hormonally regulated, as 17β -estradiol increases in males at maturation, and decreases in females (Sower, Plisetskaya, & Gorbman, 1985). Similar phenomena have been reported in female crucian carp (*Carassius carassius*), where a decrease in gonadal steroids (including 17β -estradiol) was associated with reduced alarm responses, likely occurring so that spawning can occur uninterrupted (Lastein et al., 2008). Female threespine stickleback (*Gasterosteus aculeatus*) have also been shown to exhibit riskier behaviors during sexual maturation and spawning, including bolder predator inspection (Frommen, Mehlis, & Bakker, 2009). These tradeoffs presumably evolved to maximize reproductive success. In sea lamprey, this behavioral tradeoff probably occurs so that female sea lamprey can use their finite energy stores to search for nesting males, ensuring a successful reproductive event before death. Females, the seeking sex, are known to spawn with multiple males in multiple locations (Johnson et al., 2015), a reproductive strategy

which can occur if the female can allocate more energy to reproductive activities. Additionally, because sea lamprey spawn at night (Applegate 1950; Vrieze, Bergstedt, & Sorensen, 2011), risk of predation may be globally low compared to daylight. In sum, it seems unlikely that extraction-generated repellent molecules would elicit behavioral responses that exhibit taxon- and sex-specific patterning observed in other alarm cue systems.

Sea lamprey may benefit from a stable alarm cue in nature. An organism's behavioral response to an odor is modulated by internal and external states (Beauséjour, Zielinski, & Dubuc, 2022). This is especially important in predator-prey interactions, where prey should adjust their behavior to the level of imminent threat to balance energetic costs and predator avoidance (Brown et al., 2006). Although sea lamprey respond to the alarm cue throughout their life, it may be particularly important during the non-homing spawning migration (Bals & Wagner, 2012; Imre et al., 2010; Wagner et al., 2016; Wagner et al., 2011). During the migration, sea lamprey are solitary (McCann et al., 2018), entering streams at night that have been labeled as suitable habitat by the odor emitted from conspecific larvae (Binder & McDonald, 2007; Wagner et al., 2006; Wagner, Twohey, & Fine, 2009). Consequently, they cannot benefit from direct observation of conspecifics that have detected predation risk. In addition, as they transition from the open, deep environment of lakes and oceans into the relatively narrow, shallow environs of rivers, they are exposed to suite of nocturnal mammalian and reptilian shoreline predators that may be difficult to detect through kairomones (Boulêtreau et al., 2020; Cochran 2009; Sjöberg, 1989). A stable cue is likely to remain active over a greater distance downstream from the point of emission. Migrating landlocked sea lamprey have been documented to move between 29-36 km in a single night in lake Huron (Vrieze et al., 2011), anadromous sea lamprey move between 16-33 km in Portugal's river Vouga (Andrade et al., 2007), Pacific lamprey (*Entosphenus tridentatus*) between 4-20 km in the Columbia river (Keefer et al., 2009; Moser & Close, 2003), and European river lamprey (*Lampetra fluviatilis*) in England's River Derwent approximately 18 km per night (Lucas et al., 2009). Luhring et al. (2016) found that sea lamprey were more likely to enter a river when alarm cue was present, increasing their ground speed in sections of the

river where the cue was fully mixed into the full discharge. That is, in areas where the cue did not contain spatial information as to the location of the risky area, other than it was upstream, migrants accelerated toward and through the risky area. This highlights that the sea lamprey response to the alarm cue is actively being used during migration, with context dependent selection of anti-predator tactics. A stable cue has the potential to create a traceable map of risk (plume) to modulate movement and behavior decisions. Movement decisions can be life or death as the sea lamprey navigates shallowing and narrowing systems where risky encounters with shoreline predators can occur (Imre et al., 2014). One potential predator, the North American river otter (*Lontra canadensis*), has consistent home ranges (Jeffress et al., 2011), so labeling these zones as areas of risk would be a strong selective pressure for a long-lived cue and a delayed onset of habituation. Sea lamprey habituate to conspecific alarm cue after 4 h of constant exposure to a fixed concentration, and this onset is longer than is typical in vertebrates (Imre et al., 2017; Wagner et al., 2022). Therefore, a stable sea lamprey alarm cue may mitigate uncertainties in movement decisions that aim to manage risk and avoid areas of active hunting while migrating upstream.

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 1, Table 1). 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 1). 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), while tyrosine (found in the neutral mixture of isoleucine, tyrosine, and hypoxanthine) does not (Kajikawa et al., 2005). 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 is needed to understand if it elicits a robust avoidance response.

When all 32 identified compounds in the water-soluble fraction (Experiment 3, Table 2) were recombined at the observed ratio found in the crude skin extract, a neutral response similar to the solvent treatment was observed (Figure 3). One plausible explanation for why we saw no anti-predator 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, Adrian, & Shih, 2000), 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*) (Kicklighter et al., 2007)). Our findings, along with the observed diminishing reactivity with increasing phylogenetic distance (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). 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 anti-predator behavior.

The compounds identified from the water-soluble 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 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 threshold of

detection for sea lamprey (10^{-3} mol l⁻¹, (Green et al., 2017)). Putrescine (10^{-4} mol l⁻¹), pyruvic acid (10^{-4} mol l⁻¹), serine (10^{-5} mol l⁻¹), adenosine (10^{-5} mol l⁻¹), spermine (10^{-5} mol l⁻¹), and α -ketovaleric acid (10^{-5} 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 & 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 through electro-olfactogram (EOG) responses (Robinson et al., 2009).

We observed an unexpected and substantial avoidance response to the chloroform-soluble fraction (72% of the crude skin extract response). This finding contrasts 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, α = 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 conflicting 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. All compounds within this fraction have been identified, including 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 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 & 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 & Døving, 2007; Døving & 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 was not significantly different from that of 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., 2000; Brown et al., 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 anti-predator 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 & 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 1). 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 affects 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 & Wagner, 2012; Dissanayake et al., 2019; Hume & 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 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.

REFERENCES

REFERENCES

- Algranati, F. D., Perlmutter, A., Biology, A., & Utliversitj, G. Y. (1981). Attraction of zebrafish, *Brachydanio rerio*, to isolated and partially purified chromatographic fractions. *Env. Biol. Fish.*, 6(1), 31–38.
- Almeida, P. R., Quintella, B. R., & Dias, N. M. (2002). Movement of radio-tagged anadromous sea lamprey during the spawning migration in the River Mondego (Portugal). *Hydrobiologia*, 1–8.
- Atherton, J. A., & McCormick, M. I. (2015). Active in the sac: Damsel fish embryos use innate recognition of odours to learn predation risk before hatching. *Anim. Behav.*, 103, 1–6.
- Bals, J. D., & Wagner, C. M. (2012). Behavioral responses of sea lamprey (*Petromyzon marinus*) to a putative alarm cue derived from conspecific and heterospecific sources. *Behaviour*, 149(9), 901–923.
- Beauséjour, P.-A., Zielinski, B., & Dubuc, R. (2022). Olfactory - induced locomotion in lampreys. *Cell Tissue Res.*, 387, 13–27.
- Binder, T. R., & McDonald, D. G. (2007). Is there a role for vision in the behaviour of sea lampreys (*Petromyzon marinus*) during their upstream spawning migration? *Can. J. Fish. Aquat. Sci.*, 64(10), 1403–1412.
- Boulêtreau, S., Carry, L., Meyer, E., Filloux, D., Menchi, O., Mataix, V., & Santoul, F. (2020). High predation of native sea lamprey during spawning migration. *Sci. Rep.* 10, 1–9.
- Bravener, G. A., & McLaughlin, R. L. (2013). A behavioural framework for trapping success and its application to invasive sea lamprey. *Can. J. Fish. Aquat. Sci.*, 70, 1438–1446.
- Brown, G. E., Adrian, J. C., & Shih, M. L. (2001). Behavioural responses of fathead minnows to hypoxanthine-3-N-oxide at varying concentrations. *J. Fish Biol.*, 68, 1465–1470.
- Brown, G. E., Adrian, J. C., Naderi, N. T., Harvey, M. C., & Kelly, J. M. (2003). Nitrogen oxides elicit antipredator responses in juvenile channel catfish, but not in convict cichlids or rainbow trout: Conservation of the ostariophysan alarm pheromone. *J. Chem. Ecol.*, 29(8), 1781–1796.
- Brown, G. E. (2003). Learning about danger: chemical alarm cues and local risk assessment in prey. *Fish Fish.*, 4, 227–234.
- Brown, G. E., Elvidge, C. K., Macnaughton, C. J., Ramnarine, I., & Godin, J. J. (2010). Cross-population responses to conspecific chemical alarm cues in wild Trinidadian guppies, *Poecilia reticulata*: evidence for local conservation of cue production. *Can. J. Zool.*, 88, 139–147.
- 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. *N. Am. J. Fish. Manag.*, 36(5), 1090–1096.
- Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signaling in aquatic predator-prey systems: A review and prospectus. *Ecoscience*, 5(3), 338–352.
- Diaz-Verdugo, C., Sun, G. J., Fawcett, C. H., Zhu, P., & Fishman, M. C. (2019). Mating Suppresses Alarm Response in Zebrafish Report Mating Suppresses Alarm Response in Zebrafish. *Curr. Biol.*, 29(15), 2541-2546.
- Dissanayake, A. A., Wagner, C. M., & Nair, M.G. (2021). Evaluation of health benefits of sea lamprey (*Petromyzon marinus*) isolates using in vitro anti-inflammatory and antioxidant assays. *PLoS One*, 16(11),1–12.
- Dissanayake, A. A., Wagner, C. M., & Nair, M. G. (2016). Chemical characterization of lipophilic constituents in the skin of migratory adult sea lamprey from the Great Lakes Region. *PLoS One*, 11(12), 1–17.
- Dissanayake, A. A., Wagner, C. M., & Nair, M. G. (2019). Nitrogenous compounds characterized in the deterrent skin extract of migratory adult sea lamprey from the Great Lakes region. *PLoS ONE*, 11(12), 1–19.
- Døving, K. B., & Lastein, S. (2009). The alarm reaction in fishes - Odorants, modulations of responses, neural pathways. *Ann. N.Y. Acad. Sci.*, 1170, 413–423.
- Du, Y., Huang, Y., Zhang, H., Li, D., Yang, B., Wei, M., Zhou, Y., & Liu, Y. (2012). Innate Predator Recognition in Giant Pandas Innate Predator Recognition in Giant Pandas. *Zool. Sci.*, 29(2), 67–70.
- Farnsley, S., Kuhajda, B., George, A., & Klug, H. (2016). *Fundulus catenatus* (Northern Studfish) response to the potential alarm cue chondroitin sulfate. *Southeast. Nat.*, 15(3), 523–533.
- Faulkner, A. E., Holstrom, I. E., Molitor, S. A., Hanson, M. E., Shegrud, W. R., Gillen, J. C., Willard, S.J., & Wisenden, B. D. (2017). Field verification of chondroitin sulfate as a putative component of chemical alarm cue in wild populations of fathead minnows (*Pimephales promelas*). *Chemoecology*, 27(6), 233–238.
- Ferguson, S., & Gray, E. (1989). 1988 FIFRA Amendments: A Major Step in Pesticide Regulation. *Envtl. L. Rep. News & Analysis*, 19, 10070.
- Ferrari, M. C. O. (2005). The role of learning in the development of threat-sensitive predator avoidance by fathead minnows. *Anim. Behav.*, 70(4),777–784.
- Ferrari, M. C. O., Gonzalo, A., & Chivers, D. P. (2007). Generalization of learned predator recognition: an experimental test and framework for future studies. *Proc. R. Soc. B: Biol. Sci.*, 274(1620), 1853–1859.
- Ferrari, M. C. O., Messier, F., & Chivers, D. P. (2008). Degradation of chemical alarm cues under natural conditions: risk assessment by larval woodfrogs. *Chemoecology*, 17(4), 263–266.
- Ferrari, M. C. O., Sih, A., & Chivers, D. P. (2009). The paradox of risk allocation: a review and prospectus.

- Anim. Behav., 78(3), 579–585.
- Ferrari, M. C. O., Wisenden, B. D., & Chivers, D. P. (2010). Chemical ecology of predator – prey interactions in aquatic ecosystems: a review and prospectus. *Can. J. Zool.*, 88, 698–724.
- Fraker, M. E. (2008). The dynamics of predation risk assessment: responses of anuran larvae to chemical cues of predators. *J. Anim. Ecol.*, 77(4), 638–645.
- Frommen, J. G., Mehlis, M., & Bakker, T. C. M. (2009). Predator-inspection behaviour in female three-spined sticklebacks *Gasterosteus aculeatus* is associated with status of gravidity. *J. Fish Biol.*, 75(8), 2143–2153.
- Gallagher, A. J., Creel, S., Wilson, R. P., & Cooke, S. J. (2017). Energy Landscapes and the Landscape of Fear. *Trends Ecol. Evol.*, 32(2), 88–96.
- Green, W. W., Boyes, K., Mcfadden, C., Daghfous, G., Auclair, F., Zhang, H., Li, W., Dubuc, R., & Zielinski, B. S. (2017). Odorant organization in the olfactory bulb of the sea lamprey. *J. Exp. Biol.*, 220, 1350–1359.
- Griffin, A. S. (2004). Social learning about predators: A review and prospectus. *Learn. Behav.*, 32(1), 131–140.
- Hamdani, E. H., & Døving, K. B. (2007). The functional organization of the fish olfactory system. *Prog. Neurobiol.*, 82(2), 80–86.
- Hawkins, L. A., Magurran, A. E., & Armstrong, J. D. (2004). Innate predator recognition in newly-hatched Atlantic salmon. *Behaviour*, 141(10), 1249–1262.
- Hawkins, L. A., Magurran, A. E., & Armstrong, J. D. (2008). Ontogenetic learning of predator recognition in hatchery-reared Atlantic salmon, *Salmo salar*. *Anim. Behav.*, 75, 1663–1671.
- Helfman, G. S. (1989). Threat-sensitive predator avoidance in damselfish-trumpetfish interactions. *Behav Ecol. Sociobiol.*, 24, 47–58.
- Hogg, R., Coghlan Jr, S. M., & Zydlewski, J. (2013). Anadromous Sea Lampreys Recolonize a Maine Coastal River Tributary after Dam Removal. *Trans. Am. Fish. Soc.* 142(5), 1381–1394.
- Holmes, T. H., & McCormick, M. I. (2010). Smell, learn and live: The role of chemical alarm cues in predator learning during early life history in a marine fish. *Behav. Process.*, 83(3), 299–305.
- Hume, J. B., Lucas, M. C., Reinhardt, U., Hrodey, P. J., & Wagner, C. M. (2020). Sea lamprey (*Petromyzon marinus*) transit of a ramp equipped with studded substrate: Implications for fish passage and invasive species control. *Ecol. Eng.*, 155, 1–11.
- 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(12), 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(7), 3751–3762.
- Imre, I., Di Rocco, R. T., McClure, H., Johnson, N. S., & Brown, G. E. (2017). Migratory-stage sea lamprey *Petromyzon marinus* stop responding to conspecific damage-released alarm cues after 4 h of continuous exposure in laboratory conditions. *J. Fish Biol.*, 90(4), 1297–1304.
- Imre, I., Rocco, R. T. Di, Belanger, C. F., Brown, G. E., & Johnson, N. S. (2014). The behavioural response of adult *Petromyzon marinus* to damage-released alarm and predator cues. *J. Fish Biol.*, 84, 1490–1502.
- Imre, I., Rocco, R. T. Di, 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(8), 613–620.
- Imre, I., Brown, G. E., Bergstedt, R. A., & McDonald, R. (2010). Use of chemosensory cues as repellents for sea lamprey: Potential directions for population management. *J. Great Lakes Res.*, 36(4), 790–793.
- Jeffress, M. R., Paukert, C. P., Whittier, J. B., Sandercock, B. K., & Gipson, P. S. (2011). Scale-dependent factors affecting North American river otter distribution in the Midwest. *Am. Midl. Nat.*, 166(1), 177–193.
- Johnson, N. S., Siefkes, M. J., Wagner, C. M., Bravener, G., Steeves, T., Twohey, M., & Li, W. (2015). Factors influencing capture of invasive sea lamprey in traps baited with a synthesized sex pheromone component. *J. Chem. Ecol.*, 41(10), 913–923.
- Johnson, N. S., Siefkes, M. J., Wagner, C. M., Dawson, H., Wang, H., Steeves, T., Twohey, M., & Li, W. (2013). A synthesized mating pheromone component increases adult sea lamprey (*Petromyzon marinus*) trap capture in management scenarios. *Can. J. Fish Aquat. Sci.*, 70, 1101–1108.
- Johnson, N. S., Tix, J. A., Hlina, B. L., Wagner, C. M., Siefkes, M. J., Wang, H., & Li, W. (2015). A sea lamprey (*Petromyzon marinus*) sex pheromone mixture increases trap catch relative to a single synthesized component in specific environments. *J. Chem. Ecol.*, 41(3), 311–321.
- Kajikawa, H., Mitsumori, M., Tajima, K., & Kurihara, M. (2005). Amino acids antagonistic to the amino acids inhibitory for growth rate of mixed ruminal bacteria. *J. Dairy Sci.*, 88(7), 2601–2603.
- Keefer, M. L., Boogs, C. T., & Peery, C. A. (2009). Adult pacific lamprey migration in the lower Columbia River: 2007 radiotelemetry and half-duplex pit tag studies. Technical Report: National Oceanic and Atmospheric Administration, Seattle WA, Northwest Fisheries Science Center.
- Keen, S. C., Cole, E. F., Sheehan, M. J., & Sheldon, B. C. (2020). Social learning of acoustic anti-predator cues occurs between wild bird species. *Proc. R. Soc. B: Biol. Sci.*, 287, 1–9.
- Kelley, J. L., & Magurran, A. E. (2003). Learned predator recognition and antipredator responses in fishes. *Fish Fish.*, 4, 216–226.
- Kelly, J. M., Adrian, J. C., & Brown, G. E. (2006). Can the ratio of aromatic skeletons explain cross-species responses within evolutionarily conserved Ostariophysan alarm cues?: testing the purine-ratio hypothesis. *Chemoecology*, 16, 93–96.

- Kicklighter, C. E., Germann, M., Kamie, M., & Derby, C. D. (2007). Molecular identification of alarm cues in the defensive secretions of the sea hare *Aplysia californica*. *Anim. Behav.*, 74, 1481–1492.
- Kopack, C. J., Broder, E. D., Fetherman, E. R., Lepak, J. M., & Angeloni, L. M. (2016). The effect of a single prerelease exposure to conspecific alarm cue on poststocking survival in three strains of rainbow trout (*Oncorhynchus mykiss*). *Can. J. Zool.*, 94, 661–664.
- Kynard, B., & Horgan, M. (2019). Long-term studies on restoration of Connecticut River anadromous sea lamprey, *Petromyzon marinus* Linnaeus 1758: Trend in annual adult runs, abundance cycle, and nesting. *J. Appl. Ichthyol.*, 35, 1154–1163.
- Laframboise, A. J., Ren, X., Chang, S., Dubuc, R., & Zielinski, B. S. (2007). Olfactory sensory neurons in the sea lamprey display polymorphisms. *Neurosci. Lett.*, 414(3), 277–281.
- Lasne, E., Sabatié, M., Jeannot, N., & Cucherousset, J. (2015). The effects of dam removal on river colonization by sea lamprey *Petromyzon marinus*. *River. Res. Applic.*, 31, 904–911.
- Lastein, S., Höglund, E., Mayer, I., Øverli, Ø., & Døving, K. B. (2008). Female crucian carp, *Carassius carassius*, lose predator avoidance behavior when getting ready to mate. *J. Chem. Ecol.*, 34(11), 1487–1491.
- Lawrence, B. J., & Smith, R. J. F. (1989). Behavioral response of solitary fathead minnows, *Pimephales promelas*, to alarm substance. *J. Chem. Ecol.*, 15(1), 209–219.
- Lepock, J. R., Frey, H. E., & Ritchie, K. P. (1993). Protein denaturation in intact hepatocytes and isolated cellular organelles during heat shock isolation of hepatocytes. *J. Cell Biol.*, 122(6), 1267–1276.
- Li, K., Brant, C. O., Huertas, M., Hessler, E. J., Mezei, G., Scott, A. M., Hoye, T.R., & Li, W. (2018). Fatty-acid derivative acts as a sea lamprey migratory pheromone. *PNAS*, 115(34), 8603–8608.
- Li, W., Scott, A. P., Siefkes, M. J., Yan, H., Liu, Q., Yun, S. S., & Gage, D. A. (2002). Bile acid secreted by male sea lamprey that acts as a sex pheromone. *Science*, 296(5565), 138–141.
- Lima, S. L., & Bednekoff, P. A. (1999). Temporal variation in danger drives antipredator behavior: The predation risk allocation hypothesis. *Am. Nat.*, 153(6), 649–659.
- Lönstedt, O. M., & McCormick, M. I. (2011). Chemical alarm cues inform prey of predation threat: the importance of ontogeny and concentration in a coral reef fish. *Anim. Behav.*, 82(2), 213–218.
- Lucas, M. C., Bubb, D. H., Jang, M. H., Ha, K., & Masters, J. E. G. (2009). Availability of and access to critical habitats in regulated rivers: Effects of low-head barriers on threatened lampreys. *Freshw. Biol.*, 54(3), 621–634.
- Lucon-Xiccato, T., Mauro, G. Di, Bisazza, A., & Bertolucci, C. (2020). Alarm cue-mediated response and learning in zebra fish larvae. *Behav. Brain Res.*, 380, 112446.
- 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.

- Mathuru, A. S., Kibat, C., Cheong, W. F., Shui, G., Wenk, M. R., Friedrich, R. W., & Jesuthasan, S. (2012). Chondroitin fragments are odorants that trigger fear behavior in fish. *Curr. Biol.*, 22(6), 538–544.
- Matsuura, Y., Takehira, M., Joti, Y., Ogasahara, K., Tanaka, T., Ono, N., Kunishima, N., & Yutani, K. (2015). Thermodynamics of protein denaturation at temperatures over 100 °C: CutA1 mutant proteins substituted with hydrophobic and charged residues. *Sci. Rep.*, 5(1), 1–9.
- McCann, E. L., Johnson, N. S., Hrodey, P. J., & Pangle, K. L. (2018). Characterization of Sea Lamprey Stream Entry Using Dual-Frequency Identification Sonar. *Trans. Am. Fish. Soc.*, 147(3), 514–524.
- Miehls, S., Sullivan, P., Twohey, M., Barber, J., Marie, S. S., Pa, O. N., & McDonald, R. (2020). The future of barriers and trapping methods in the sea lamprey (*Petromyzon marinus*) control program in the Laurentian Great Lakes. *Rev. Fish. Biol. Fish.*, 30, 1–24.
- Mirza, R. S., & Chivers, D. P. (2001). Are chemical alarm cues conserved within salmonid fishes? *J. Chem. Ecol.*, 27(8), 1641–1655.
- Mirza, R. S., Laraby, C. A., & Marcellus, A. M. (2013). Knowing your behaviour: The importance of behavioural assays in the characterisation of chemical alarm cues in fishes and amphibians. In *Chemical Signals in Vertebrates 12*(295–308). Springer, New York, NY.
- Mitchell, M. D., Cowman, P. F., & McCormick, M. I. (2012). Chemical Alarm Cues Are Conserved within the Coral Reef Fish Family Pomacentridae. *PLoS One*, 7(10), 1–7.
- Moore, F. R. (2018). Biology of landbird migrants: A stopover perspective. *Wilson j. ornithol.*, 130(1), 1–12.
- Moser, M. L., & Close, D. A. (2003). Assessing Pacific Lamprey Status in the Columbia River Basin. *Northwest Sci.*, 77(2), 116-125.
- Parra, K. V, Adrian, J. C., & Gerlai, R. (2009). The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (*Danio rerio*). *Behav. Brain Res.*, 205, 336–341.
- Pereira, E., Quintella, B. R., Mateus, C. S., Alexandre, C. M., Belo, A. F., Telhado, A., Quadrado, M.F., & Almeida, P. R. (2017). Performance of a vertical-slot fish pass for the sea lamprey *Petromyzon marinus* L and habitat recolonization. *River Res. Applic.*, 33, 16–26.
- Pfeiffer, W., Riegelbauer, G., Meier, G., & Scheibler, B. (1984). Effect of Hypoxanthine-3(N)-Oxide and Hypoxanthine-1(N)-Oxide on central nervous excitation of the black tetra *Gymnocorymbus ternetzi* (Characidae, Ostariophysi, Pisces) indicated by dorsal light response. *J. Chem. Ecol.*, 11(4), 507–523.
- Poisson, A., Valotaire, C., Borel, F., Bertin, A., Darmaillacq, A. S., Dickel, L., & Colson, V. (2017). Embryonic exposure to a conspecific alarm cue triggers behavioural plasticity in juvenile rainbow trout. *Anim. Behav.*, 133, 35–45.
- Raices, M., Jungblut, L. D., & Pozzi, A. G. (2020). Evidence of the peptide identity of the epidermal alarm cue in tadpoles of the toad *Rhinella arenarum*. *Herpetol. J.*, 30, 230–233.

- Robinson, T. C., Sorensen, P. W., Bayer, J. M., & Seelye, J. G. (2009). Olfactory sensitivity of pacific lampreys to lamprey bile acids. *Trans. Am. Fish. Soc.*, 138(1), 144–152.
- Roux, O., & Diabate, A. (2014). Divergence in threat sensitivity among aquatic larvae of cryptic mosquito species. *J. Anim. Ecol.*, 83(3), 702–711.
- Sabal, M. C., Boyce, M. S., Charpentier, C. L., Furey, N. B., Luhning, T. M., Martin, H. W., Melnychuk, M.C., Srygley, R.B., Wagner, C.M., Wirsing, A.J., Ydenberg, R.C., & Palkovacs, E. P. (2021). Predation landscapes influence migratory prey ecology and evolution. *Trends Ecol. Evol.*, 36(8), 737–749.
- Schoeppner, N. M., & Relyea, R. A. (2009). When should prey respond to consumed heterospecifics? testing hypotheses of perceived risk. *Copeia*, (1), 190–194.
- Scott, A. M., Li, K., & Li, W. (2018). The identification of sea lamprey pheromones using bioassay-guided fractionation. *J. Vis. Exp.*, (137), 1–11.
- Sjöberg, K. (1989). Time-related predator/prey interactions between birds and fish in a northern Swedish river. *Oecologia*, 80(1), 1–10.
- Sloychuk, J. R., Chivers, D. P., Ferrari, M. C. O., Sloychuk, J. R., Chivers, D. P., & Juvenile, M. C. O. F. (2016). Juvenile lake sturgeon go to school: life-skills training for hatchery fish. *Trans. Am. Fish. Soc.*, 145(2), 287-294.
- Smith, R. J. F. (1992). Alarm signals in fishes. *Rev. Fish Biol. Fish.*, 2(1), 33–63.
- Sorensen, P W, Vrieze, L. A., & Fine, J. M. (2004). A multi-component migratory pheromone in the sea lamprey. *Fish Physiol. Biochem.*, 28(1), 253–257.
- Sorensen, Peter W, & Vrieze, L. A. (2003). The chemical ecology and potential application of the sea lamprey migratory pheromone. *J. Great Lakes Res.*, 29, 66–84.
- Sower, S. A., Plisetskaya, E., & Gorbman, A. (1985). Changes in plasma steroid and thyroid hormones and insulin during final maturation and spawning of the sea lamprey, *Petromyzon marinus*. *Gen. Comp. Endocrinol.*, 58(2), 259–269.
- Veen, T., Richardson, D. S., Blaakmeer, K., & Komdeur, J. (2000). Experimental evidence for innate predator recognition in the Seychelles warbler. *Proc. R. Soc. B: Biol. Sci.*, 267(1459), 2253-2258.
- Vrieze, L. A., Bergstedt, R. A., & Sorensen, P. W. (2011). Olfactory-mediated stream-finding behavior of migratory adult sea lamprey (*Petromyzon marinus*). *Can. J. Fish. Aquat. Sci.*, 68(3), 523–533.
- Wagner, C.M., Bals, J.D., Hanson, M.E., Scott, A.M. (2022). Attenuation and recovery of an avoidance response to a chemical anti-predator cue in an invasive fish: implications for use as a repellent in conservation. *Cons. Phys.* In Press.
- Wagner, C. M., Kierczynski, K. E., Hume, J. B., & Luhning, T. M. (2016). Exposure to a putative alarm cue reduces downstream drift in larval sea lamprey *Petromyzon marinus* in the laboratory. *J. Fish Biol.*, 89(3), 1897–1904.

- 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(7), 1157–1160.
- 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(3), 475–479.
- Wagner, C.M., Twohey, M. B., & Fine, J. M. (2009). Conspecific cueing in the sea lamprey: do reproductive migrations consistently follow the most intense larval odour? *Anim. Behav.*, 78(3), 593–599.
- William, F., & Beamish, H. (1979). Migration and spawning energetics of the anadromous sea lamprey, *Petromyzon marinus*. *Environ. Biol. Fishes*, 4(1), 3–7.
- Wisenden, B. D. (2000). Olfactory assessment of predation risk in the aquatic environment. *Philos. Trans. R. Soc. B., Biol. Sci.*, 355(1401), 1205–1208.
- Wisenden, Brian D., Rugg, M. L., Korpi, N. L., & Fuselier, L. C. (2009). Lab and field estimates of active time of chemical alarm cues of a cyprinid fish and an amphipod crustacean. *Behaviour*, 146(10), 1423–1442.
- Wisenden, Brian D., Vollbrecht, K. A., & Brown, J. L. (2004). Is there a fish alarm cue? Affirming evidence from a wild study. *Anim. Behav.*, 67(1), 59-67.
- Yambe, H., Kitamura, S., Kamio, M., Yamada, M., Matsunaga, S., & Fusetani, N. (2006). L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. *PNAS*, 103(42), 15370–15374.
- Zielinski, B., Arbuckle, W., Belanger, A., Corkum, L. D., Li, W., & Scott, A. P. (2004). Evidence for the release of sex pheromones by male round gobies (*Neogobius melanstomus*). *Fish Physiol. Biochem.*, 28, 237–239

CHAPTER 2: The effect of putrescine on space use and activity in sea lamprey (*Petromyzon marinus*)

Abstract

Fish use odor to navigate away from risky situations including predation and disease. Harnessing these odors as repellents are proving useful for management initiatives, dually for conservation of native species and control of invasive populations. Here, we evaluated the behavioral response of sea lamprey to putrescine, a decay molecule that many prey organisms avoid. Putrescine is found in tissue extracts that contain the sea lamprey alarm cue, and in human saliva, two mixtures known to elicit flight and avoidance responses in migratory sea lamprey. We used two behavioral assays to evaluate metrics of repellency: behavioral preference (space use) and change in activity rates and found context-dependent results. In a smaller assay with individual fish, we found that putrescine had no effect on sea lamprey activity but did increase repellency. In a larger assay with multiple animals, we found no repellent behavior to any putrescine treatment. However, our results also demonstrated consistent changes in activity and avoidance behavior in sea lamprey exposed to alarm cue in the smaller assay, concluding that this design could prove useful as a high-throughput screening tool. We also investigated a novel compound identified in sea lamprey skin, petromyzonacil, and found no behavioral effects to this odor on its own or in synergy with putrescine. Our results show limited evidence that putrescine acts as robust repellent for sea lamprey and highlight the importance of environmental context in anti-predator behavioral studies.

Introduction

The sea lamprey (*Petromyzon marinus* L.) is a large ectoparasitic jawless fish whose sectorial mouth and rasping tongue inflicts significant damage to its host fish, often resulting in death. Valued in its native range in the northern Atlantic basin, an invasive population in the Laurentian Great Lakes threatens an estimated \$7 billion fishery (Hume et al., 2021; Siefkes 2017; Southwick Associates 2012). Currently, the management of the invasive population relies on applications of two lamprey specific pesticides, or lampricides, to kill larvae in Great Lakes tributaries, combined with low-head dams that block access to suitable spawning habitats (Hunn & Youngs, 1980; Siefkes, 2017). However, application costs are increasing, climate change estimates project an increase in sea lamprey growth and access to spawning habitats, concerns are rising that over-reliance on lampricides could lead to biological resistance, and there is societal pressure to remove dams and restore stream connectivity (Christie, Sepúlveda, & Dunlop, 2019; Cline et al., 2014; Lennox et al., 2020; Siefkes, Johnson, & Muir, 2021). Additional control methods are needed to synergize with the lampricide program and maintain control efficacy and acceptability (Siefkes et al., 2021).

Harnessing chemosensory cues may provide the key to unlocking innovative supplemental control methods (Fisette et al., 2021). For example, there is substantial interest in capturing sea lamprey as they migrate into streams to spawn (Miehls et al., 2020). Upon entering rivers, the sea lamprey encounters a gauntlet of shoreline predators, and relies on chemical risk cues to survive passage to the spawning grounds (Imre et al., 2014). In aquatic predator-prey dynamics, these predation-related odors can include predator kairomones (odors emitted directly from a predator, such as saliva, urine or feces (Kats & Dill, 1998; Wisenden, 2000)), disturbance cues (cues emitted after the perception of risk (Wisenden, Chivers, Brown, & Smith, 1995)) and alarm cues. Chemosensory alarm cues are public information emitted from injured organisms, alerting nearby conspecifics and closely related species to the presence of predation risk (Chivers & Smith, 1998; Ferrari, Wisenden, & Chivers, 2010). Typical behavioral responses to alarm cues include area avoidance, increased use of shelter, and increased vigilance (Lawrence & Smith, 1989;

Wisenden, 2015). The sea lamprey shows significant avoidance behaviors when exposed to a conspecific alarm cue in laboratory settings (Bals & Wagner, 2012; Hume & Wagner, 2018; Imre et al., 2014; Wagner, Stroud, & Meckley, 2011; Byford et al., 2016; Wagner et al., 2016) and in natural streams (Di Rocco et al., 2016; Hume, Luhring, & Wagner, 2020; Hume et al., 2015).

Field studies that deployed the sea lamprey alarm cue as a repellent to drive migrants towards trapping devices have proven promising. When alarm cue was introduced to half of a stream channel, migrating sea lamprey were more likely to encounter a trap entrance located on the other half of the river whether traps were placed along dam faces or in the open river channel (Hume et al., 2015, 2020). However, one barrier to using chemosensory cues in a repellent formulation is habituation, which occurs when an organism's behavioral response to a stimulus diminishes after frequent or continuous exposure (Blumstein, 2016; Greggor et al., 2020). Sea lamprey are known to habituate to its alarm cue when continuously immersed for 4 h (Imre et al. 2017; Wagner et al., 2022). Having a rotating "menu" of repellent formulations could be one mechanism to prevent habituation to alarm cue in a management setting. Alternatively, a risk cue that is not part of the alarm cue could be used to induce dishabituation to the alarm cue, causing spontaneous recovery of the avoidance behavior (Wagner et al., 2022).

One class of potential repellents that could augment alarm cue is decay odors. Among these odorants is putrescine, is a small aliphatic diamine produced by animal tissue decay. It generates an odor that triggers strong, yet varying, behavioral responses in animals (Hussain et al., 2013). This distinctive "death scent" is repulsive and elicits starkly adverse responses in some species, especially prey species, likely as an adaptive response to avoid predation risk or disease contagion (Yao et al., 2009). Humans exposed to putrescine increase vigilance, area avoidance, and hostility as threat management behaviors (Wisman & Shrira, 2015). Zebrafish (*Danio rerio*) exhibit avoidance and defensive behaviors when exposed to putrescine (Hussain et al., 2013; Oliveira et al., 2014), and cortisol levels significantly increase, indicating stress (Oliveira et al., 2014). In rats (*Rattus rattus*), olfactory control of putrescine modulates the burial of

conspecifics (Pinel, Gorzalka, & Ladak, 1981) likely to prevent risks associated with corpses such as scavenger attraction or pathogenic risk (Prounis & Shields, 2013; Sun, Haynes, & Zhou, 2017). Conversely, putrescine can be attractive for certain predators or scavengers, where an odor of death may indicate an available meal. Food scented with putrescine and a similar decay odor, cadaverine, are preferred by rats (Heale, Petersen, & Vanderwolf, 1996) and these compounds increased feeding behavior three-fold in goldfish (*Carassius auratus*) (Rolen, Sorensen, Mattson, & Caprio, 2003). Consequently, prey may seek to avoid waters scented with putrescine to reduce the likelihood of encountering scavenging predators.

Putrescine has been identified in extractions from sea lamprey tissues that contain the animal's alarm cue (Dissanayake, Wagner, & Nair, 2019). It is also the most abundant amine in human saliva (Cooke, Leeves, & White, 2003). With sudden exposure, human saliva can elicit a powerful flight response in sea lamprey (Tilden 1809). In laboratory raceway studies, human saliva induces avoidance in sea lamprey (Di Rocco et al., 2014). In two studies (Imre et al., 2014; Imre et al., 2016), alarm cue and human saliva induced a similar magnitude avoidance response compared to a deionized water control, and combining the two odors increased the potency of the response. One explanation for this finding is that the two cues act in an additive manner, providing the organism with an increased risk assessment capacity through sensory complementation (Ferrari et al., 2008; Lima & Steury, 2005). Here, the alarm cue may provide information that a conspecific injury has occurred, while saliva provides information of the proximity of a mammalian predator (Imre et al., 2014). Conversely, if putrescine is a component of both human saliva and the alarm cue, the observed increase in avoidance may be due to threat-sensitivity, where an increased concentration of the cue provides information of a larger level of risk and thus leads to a more intense response (Brown et al., 2006). In either case, the active ingredients of any repellent used to control a pest species must be specified per the U.S. Environmental Protection Agency's Federal Insecticide, Fungicide and Rodenticide Act (Ferguson & Gray, 1989).

Sea lamprey is a semelparous species that have finite energy stores to use during their upstream spawning migration. This energy must be used strategically when making behavioral choices, especially in the context of anti-predator responses that can be energetically costly, but also are used to evade an attack or death. These choices should therefore be dependent on the imminence of threat and adjusted to the likelihood of an attack. This 'predatory imminence continuum' is organized around spatial and temporal variations in predation risk (Fanselow, Hoffman, & Zhuravka, 2019). We hypothesized that putrescine is behaviorally relevant to sea lamprey in one or more of three contexts: part of a conspecific alarm cue, part of a predator kairomone (i.e., saliva), or a decay odor. We predicted alarm cue would elicit the highest anti-predator behavioral response because it gives reliable information about both the presence of an active predator and an attack on a conspecific (or closely related) animal. If putrescine was a component of a salivary kairomone, it may elicit an intermediate response as it provides evidence of a predator, but no evidence of its activity or of a recent attack. Finally, if putrescine is perceived as a decay odor, we expected a weaker response as it provides evidence of death, but no information about the nature of the death or immediate risk through predator presence. However, as decay odors can attract mammalian predators, especially scavengers, (Heale et al., 1996; Rolen et al., 2003) sea lamprey may avoid it to reduce the likelihood of encounters that may lead to attack.

In the present study, we examined whether putrescine may prove useful as a supplementary repellent for use in sea lamprey management due to its inclusion in known repellent mixtures, alarm cue and human saliva. We first examined the responses of individual sea lamprey to putrescine in a small behavioral arena, observing evidence of avoidance. We next examined if groups of sea lamprey responded similarly to putrescine in a larger laboratory raceway across three treatments: 1) putrescine at observed ratios in sea lamprey skin, 2) putrescine in combination with a novel molecule identified in sea lamprey alarm cue extracts that may label the putrescine as coming from conspecifics, and 3) putrescine at an increased concentration. The aim of this study was to investigate the efficacy of this molecule for use as a potential component of a sea lamprey repellent.

Materials and Methods

Study design

To understand the role of putrescine on repellent behavior, we tested the activity of sea lamprey exposed to putrescine treatments in a behavioral assay through two experiments. Experiment one examined lamprey behavioral responses to alarm cue derived from whole body or skin tissues, putrescine, and a novel compound observed in sea lamprey skin, dubbed petromyzonacil (Dissanayake, Wagner & Nair, 2019) in a high throughput individual assay in June 2020. In experiment two, we examined the role of three putrescine treatments on sea lamprey behavior in a larger multi-animal assay of the type described in Bals & Wagner (2012) between June and July 2021. First, we tested putrescine at the observed concentration within the skin extract. Next, we tested the same concentration of putrescine in combination with petromyzonacil. Finally, we tested a high molarity treatment (10^{-1} M) to assess how the effect on repellency in the laboratory system. The responses to these formulations were compared to the full alarm cue extract.

Odor collection and preparation

Per methods laid out in Wagner et al. (2011), alarm cue was collected from whole carcasses of male and female sea lamprey that naturally senesced during captivity. All specimens were kept at -20°C prior to odor extractions. Odor was collected through Soxhlet extractors (2.08m, Ace Glass Inc., Vineland, New Jersey, USA) with attached six-bulb water-cooled Allihn condensers. Solvent reservoirs with 12L capacity were loaded with a 50:50 solution of 200 proof ethanol and deionized water solution and heated $75^{\circ} - 80^{\circ}\text{C}$ by a hemispherical mantle for approximately six hours, creating 10.2L of alarm cue extract. Extractions cooled overnight and were then decanted, filtered through muslin, and stored at -20°C until experimental use.

Individual compounds (petromyzonacil and putrescine) were isolated from sea lamprey skin extracts per the methods of Dissanayake, Wagner, & Nair, 2016, 2019, 2021). Alarm cue derived from sea lamprey skins was extracted through a Soxhlet extraction as described above, with solvent (80:20

EtOH:RO water) and excess ethanol was removed through rotary evaporation. Lyophilized extracts were stored at -80° C until use. Individual compounds were purified from the skin extract via preparative HPLC and identified via NMR experiments. For experiment 2, putrescine and petromyzonacil were separately identified at a presence of 0.04% of the total skin extract, or 0.08 mg/L per extract of one skin. Putrescine odor solution was made by dissolving 0.08 mg dry material in 10 mL solution and bringing up to a 1L solution with 990mL solution (50:50 DI H₂O:EtOH). Putrescine + petromyzonacil treatment was created by combining dissolved putrescine (0.08 mg in 10 mL solution) and dissolved petromyzonacil (0.08 mg in 10 mL solution) and bringing up to 1L with 980mL additional solution. 10⁻¹ M Putrescine treatment was made by dissolving 1.7g dry putrescine in 10mL solution and bringing up to 1L with 990mL additional solution.

Experimental subjects

All sea lamprey used in the experiments were migratory sub-adults obtained from the annual trapping operations of the U.S. Fish and Wildlife Service from tributaries of Lake Huron (Cheboygan and Ocqueoc Rivers) or the St. Mary's River, a channel connecting Lake Superior and Lake Huron. Trapped fish were transported to 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 and held under a natural day-night light cycle. We used only males in this study because female lamprey decrease their reactivity to alarm cue during their sexual migration and males do not, and previous experiments show no difference in response between sexes in sexually immature migrants (Bals & 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

All experiments took place in two laboratory raceways at HBBS with dimensions 1.44m x 12.2m, and within either individual (experiment 1; Figure 1) or group (experiment 2; Figure 4) experimental

arenas, described below. Trials were conducted in full darkness between 18:00 and 02:00 hours during the spring spawning season, to approximate times and conditions of a typical nocturnal sea lamprey migration in streams. Water flowed into flumes from a head tank supplied directly from Lake Huron. Turbulence in each arena was reduced by placing baffles (rolled plastic mesh) at the upstream end. Two hours before experimental trials, subjects were visually inspected to ensure immature status and transferred to holding baskets constructed to allow water to constantly flow through and were held in round holding tanks until the beginning of the trial. All trials consisted of an acclimation period, a stimulus observation period where the odors were introduced. Odors were introduced into one-half of the experimental arena (left or right side), with the stimulus side alternating after each replicate. Peristaltic pumps (MasterFlex model 7533-20) released odor solutions, continuously stirred with a 2cm magnetic stir bar to ensure a homogenous mixture, through PVC tubing at a fixed rate of 20mL min^{-1} . Each test odor was pumped into the raceway from a beaker containing a solution at target 2 000 000:1 DI water:odor extract, and dilutions were calculated based on raceway discharge measured by width, depth, and velocity of the water. Separate sets of tubing were used for each odor treatment to ensure no cross contamination. Dye tests conducted prior to trials confirmed odor plumes were confined to the target half of the experimental arena. At the conclusion of each trial, each subject was removed from the arenas and total length (TL, cm) and wet weight (g) were recorded.

Experiment 1: Behavioral responses by individuals in a small-arena assay

Four individual test arenas were created by installing prefabricated 1.22 m x 2.44 m experimental two-choice arenas made from HDPE paneling into the raceways (two in each raceway as in Figure 1). The test area of each arena measured 1.22 m x 1.22 m, blocked off with mesh block nets. A 0.61 m panel of HDPE extended into the experimental arena to aid with stimulus partitioning. Video cameras (Lorex 8-Channel 4K UHD NVR with 2TB HDD) and infrared lights (4 5 MP Night Vision Bullet) were installed above each arena. Water temperature ranged from 11-15 °C over the course of trials, in accordance with seasonal changes in lake temperature and discharge was maintained at or near $0.01\text{ m}^3\text{ sec}^{-1}$ in each

raceway. Trials began by carefully releasing the fish into the center of the test area by opening the holding basket. Trials lasted 22 minutes, including a five-minute acclimation period, a five-minute pre-stimulus observation period, a two-minute period to introduce the odor to the system and ensure it traveled through the test area, and then a 10-minute stimulus observation period.

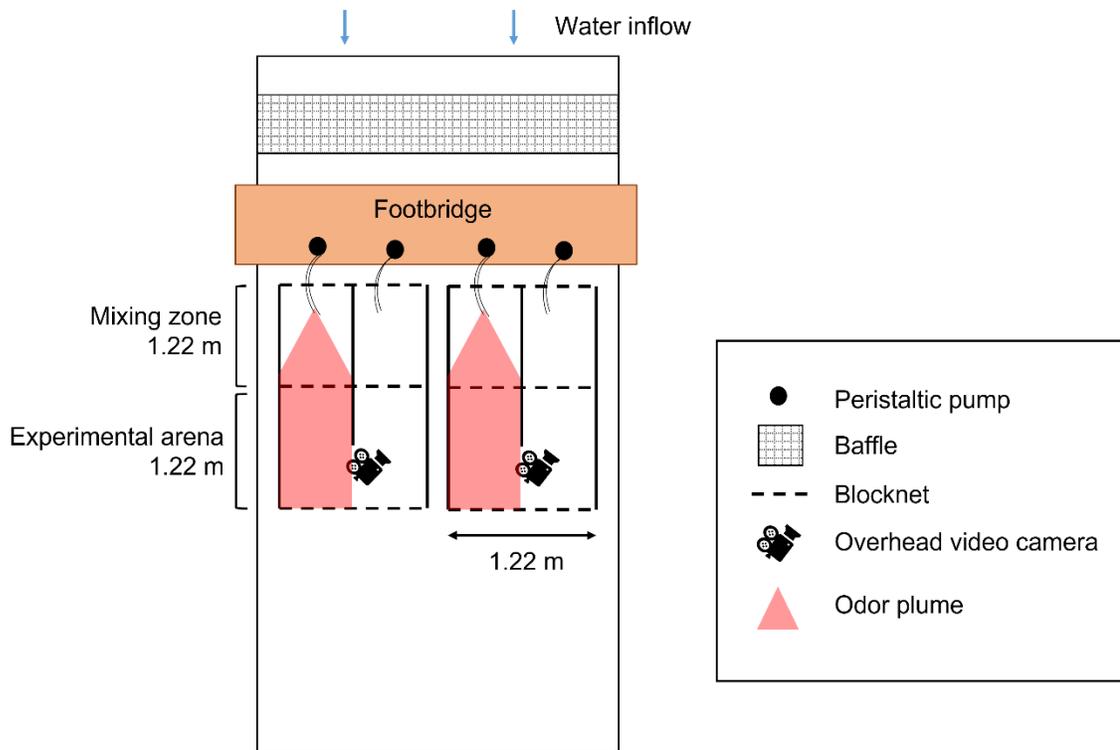


Figure 4. Schematic of individual assay with experimental arenas in one raceway. Fish were introduced into the center of the arena and able to move freely throughout the arena over the course of the trial. Odor was introduced through one peristaltic pump before the stimulus observation period, and pump sides were switched after the completion of each trial.

Experiment 2: Behavioral responses by groups in a large arena assay

Experimental arenas were isolated with block nets at upstream and downstream ends, forming a 3.1 m long reach (Figure 4). 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. Arenas were illuminated with arrays of six infrared lights (Wildlife Engineering; Model IRLamp6) and experiments were recorded with overhead infrared sensitive video cameras (Axis

Communications, Q1604 Network Camera). Water temperature ranged from 6-18 °C and discharge was maintained at 0.02-0.03 m³ sec⁻¹ in each experimental raceway. Trial groups consisted of 10 sea lamprey, held in the same holding baskets until the beginning of a trial. Each trial began by carefully releasing the ten animals from their holding basket into the middle of the experimental arena and lasted 30 minutes including a 10-minute acclimation period and a 20-minute observation period.

Analyses

Experiment 1: Behavioral responses by individuals in a small-arena assay

Video analysis was completed in Behavioral Observation Research Interactive Software (BORIS), version 7.9.8 (Friard & Gamba, 2016). In BORIS, all videos were watched in entirety and all behaviors were manually recorded. The post exposure to stimulus period was only recorded after confirmation that the fish interacted with the cue after its addition into the assay, and thus a trial was discarded if a fish spent the entire trial without moving into the stimulus odor. Two metrics of response were recorded: preference and change in activity. To analyze preference, the proportion of time a lamprey spent on the stimulus side was calculated by dividing the time spent in the stimulus by the total time after the first encounter with the stimulus. A proportion of time equal to 50% indicated neutral preference, and a distribution less than 50% indicated avoidance. All statistical analyses were done in R (Version 1.4.1103). A Shapiro-Wilk's test demonstrated that the data followed a non-normal distribution, and a Levene's test confirmed equal variance, thus a Kruskal-Wallis test was performed to test for any effect of odor treatment on avoidance response. A post-hoc Dunn's test ($\alpha = 0.05$) was completed as a means comparison of preference responses. To analyze activity level, each time a fish exhibited a behavior described in the Ethogram (Table 1) was recorded. Each activity was scored between 1 and 3, depicting a range of activity levels based on continuous and active movement from low (1) to high (3) (Table 1). An activity index was calculated using the activity score for each individual by multiplying the amount of time spent on each behavior by its activity level and combining as follows: (time spent on high activity behaviors * 3) + (time spent on

medium activity behaviors * 2) – (time spent on low activity behaviors *1). Activity indexes were separately calculated for pre-exposure (during the pre-stimulus trial observation period) and post-exposure (during the stimulus trial observation period). To account for individual differences in baseline activity rate, the change in activity was calculated for each fish by subtracting the pre-exposure activity index from the post-exposure activity index. A larger Δ index score was evidence of increased activity after odor exposure. In R, a one-way ANOVA was performed with Δ activity index as the response variable and odor type as a fixed effect. Normality was confirmed with a Shapiro-Wilk’s test ($\alpha = 0.05$) and Tukey’s Honestly Significant Difference (HSD) ($\alpha = 0.05$) was completed as a post-hoc means comparison for each treatment to understand the effect of odor on activity.

Table 3. Ethogram used to analyze behavioral responses to odor treatments in BORIS software. Activity levels were used to calculate activity indexes.

Activity Level	Description of behavior
1	Lamprey is unmoving and attached to the experimental arena with oral disk
2	Lamprey is active and exploring the arena at a nominal speed
3	Lamprey increases speed, frequent darting, sharp turns, and breaching of the surface are observed within the arena.

Experiment 2: Behavioral responses by groups in a large arena assay

Videos of each trial were analyzed for preference responses by pausing every 30s and counting the number of fish on each side of the raceway (stimulus or non-stimulus, Figure 4) as an indication of preference. Fish positions were designated based on the location of a fish’s head at each 30s-time stamp. All statistical analyses were done in R (Version 1.4.1103). A one-way ANOVA was performed with proportion of animals on the stimulus side as the response variable and odor type as a fixed effect. Normality was confirmed with a Shapiro-Wilk’s test ($\alpha = 0.05$) and Tukey’s Honestly Significant Difference (HSD) ($\alpha = 0.05$) was completed as a post-hoc means comparison for each treatment. Putrescine, putrescine + petromyzonacil, and 10^{-1} M putrescine treatment means were compared to the whole-body

alarm cue and solvent controls means to determine if putrescine elicited a partial, full, or not-significant avoidance response in sea lamprey.

Results

Experiment 1: Behavioral responses by individuals in a small-arena assay

Preference- Odor exhibited a significant effect on sea lamprey preference behavior (Kruskal-Wallis $\chi^2(4) = 17.78, p = 0.001$). Each alarm cue treatment demonstrated a significant avoidance response compared to the solvent control (Dunn's test, whole-body and skin $p < 0.05$; Figure 5). Putrescine demonstrated significantly higher avoidance response than the solvent (Dunn's test, $p = 0.04$; Figure 5), and was not significantly different than either alarm cue treatment (Dunn's test, whole-body and skin $p = 0.99$; Figure 5). Responses to petromyzonacil were not significantly different from the solvent (Dunn's test, $p = 0.74$; Figure 5), and lamprey spent more time in the odor vs. alarm cue treatments (Dunn's test, whole-body $p < 0.001$; skin $p = 0.02$; Figure 5).

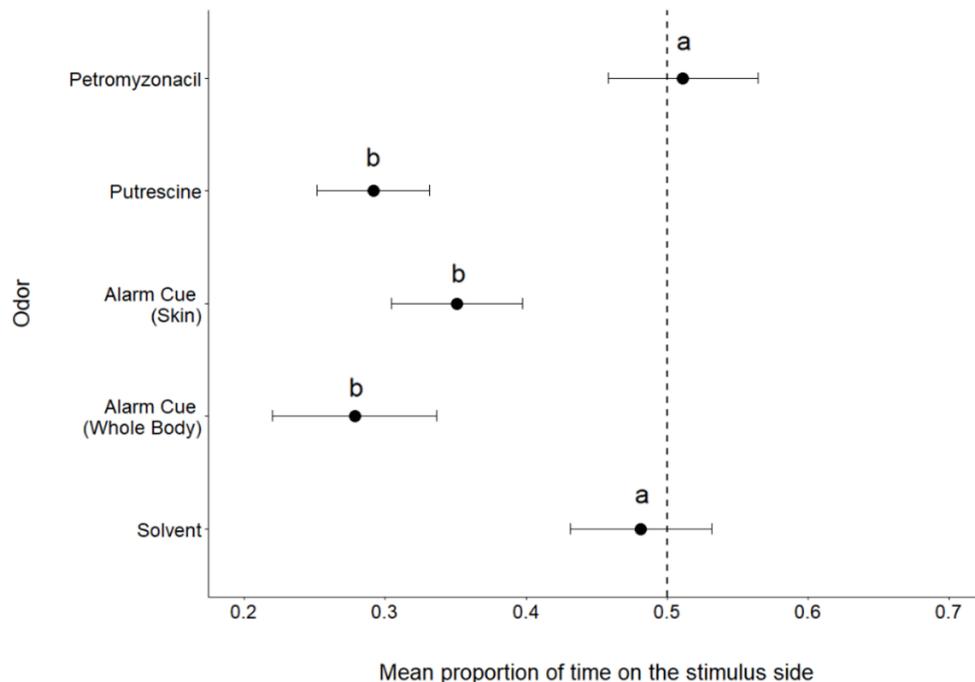


Figure 5. Mean ($\pm 1SE$) proportion of sea lamprey on the stimulus side after the addition of odorants in

Figure 5 (cont'd) small assay experiment. 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 Dunn's test ($\alpha = 0.05$). N = 18 for solvent, N = 13 for whole body alarm cue, N = 19 for skin alarm cue, N = 10 for putrescine and N = 19 for petromyzonacil.

Activity- Odor exhibited a significant effect on the change in sea lamprey activity (ANOVA, $F_{4,74} = 4.493$, $p < 0.01$). Both alarm cue treatments demonstrated a significant increase in activity compared to the solvent control (Tukey HSD, whole-body $p = 0.01$; skin $p < 0.01$; Figure 6). Petromyzonacil demonstrated no significant difference compared to solvent the solvent (Tukey HSD, $p = 0.40$; Figure 6) or alarm cue treatments (Tukey HSD, whole-body $p = 0.43$; skin $p = 0.44$; Figure 6). Putrescine also demonstrated no significant difference compared to the solvent (Tukey HSD, $p = 0.98$; Figure 6) or alarm cue treatments (Tukey HSD, each $p = 0.14$; Figure 6).

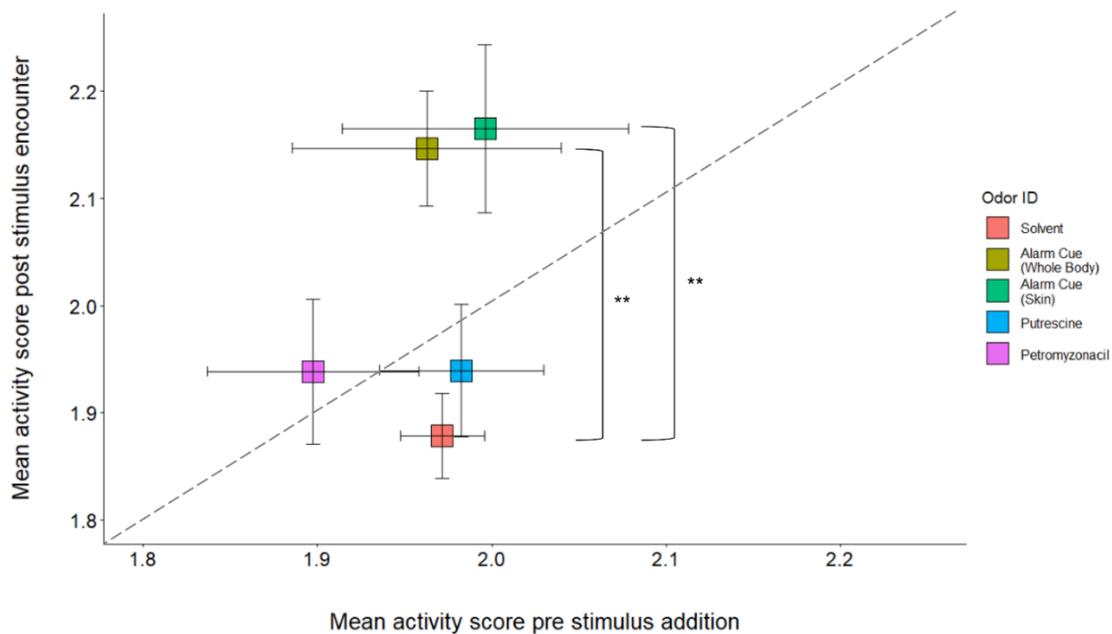


Figure 6. Mean ($\pm 1SE$) activity score before addition of odorants by mean ($\pm 1SE$) activity score after encounter with odorant. Responses to odors with an asterisk represent a significantly different change in

Figure 6 (cont'd) activity (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) based on Tukey HSD. The dashed line represents the null hypothesis of no change in activity before and after stimulus exposure. Responses above the line indicate an increase in activity post exposure to the odor, and responses below the line indicate a decrease in activity post exposure to the odor. The minimum score possible is 1 (indicating the entire trial period was spent on low activity behaviors per the ethogram in table 1) and maximum is 3 (indicating the entire trial period was spent on high activity behavior per the ethogram in table 1)

Experiment 2: Behavioral responses by groups in a large arena assay

The model results (ANOVA, $F_{4,40} = 10.68$, $p < 0.001$) clearly indicated that the type of odor introduced into the raceway channel influenced sea lamprey space use. The proportion of time spent on the stimulus side was significantly lower in the whole-body alarm cue treatment than the solvent treatment (Tukey HSD, $p < 0.001$; Figure 7). Putrescine treatments failed to exhibit avoidance responses and were not significantly different from the solvent control (putrescine, Tukey HSD, $p = 0.43$; putrescine + petromyzonacil, Tukey HSD, $p = 0.99$; 10^{-1} M putrescine, Tukey HSD, $p = 0.99$; Figure 7).

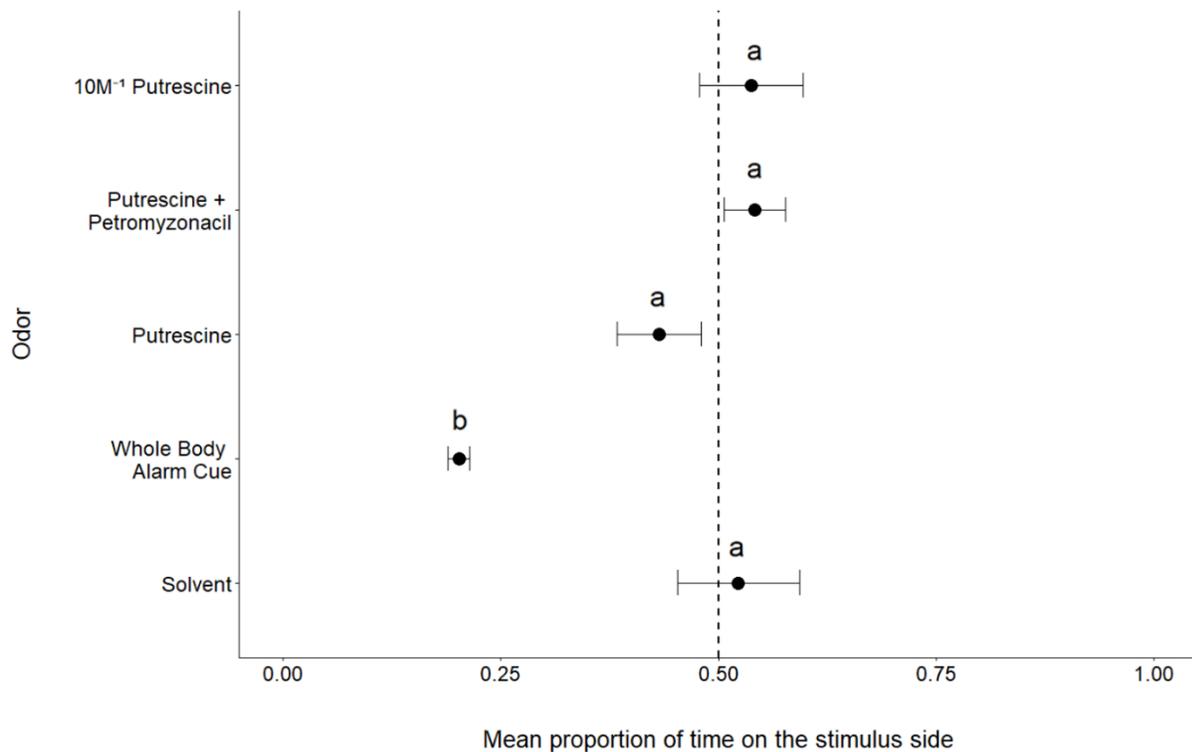


Figure 7. Mean ($\pm 1SE$) proportion of sea lamprey on the stimulus side after the addition of odorants. Dashed line at 0.50 represents the null hypothesis of a true neutral response to introduced stimulus. Values to the left of the line represent an avoidance response and values to the right of the line represent

Figure 7 (cont'd) an attractant response. Treatments with different letters are significantly different from one another based on Tukey HSD ($\alpha = 0.05$). N = 10 for solvent, whole body alarm cue, putrescine, and putrescine + petromyzonacil treatments. N = 5 for 10^{-1} M putrescine treatment.

Discussion

We investigated the effects of alarm cue and putrescine on preference behavior and activity in migratory sea lamprey. Our results suggest migrating sea lamprey respond to putrescine, but the response is substantially less than that to alarm cue, only arises in certain contexts, and does not synergize with petromyzonacil, a unique compound found in sea lamprey skin. In small arenas (Experiment 1), individual sea lamprey strongly avoided conspecific alarm cues derived from whole body or skin, and exhibited increased activity, often swimming near the surface and probing with its head out of the water in an apparent attempt to find egress from the arena (i.e., an overt escape response; Domenici 2010). Similar responses have been reported in larval sea lamprey exposed to alarm cue in small tanks (Perrault et al. 2014). Individuals exposed to putrescine also exhibited avoidance, but exposure did not result in a significant increase in activity, suggesting an intermediate response more consistent the perception of lower risk of exposure to predation, or an adaptive 'disgust' response affiliated with avoiding exposure to disease (Curtis et al. 2011). Given the weaker responses vs alarm cue, these results were more consistent with the hypothesis that putrescine operated as an indicator of decaying tissue that allows migrants to better avoid scavenging predators and/or exposure to contagion. However, in a larger arena and in the presence of conspecifics (Experiment 2), exposure to putrescine failed to elicit an avoidance response. Taken together, these data support the contention that risk-sensitive decisions arise across odor classes that relate to differing types of threat, and the degree of confinement and/or social interaction may mediate responses to those threats.

An animal's perception of risk is partly a result of the imminence of the threatening agent, a perception that can be modulated by the environment in which the information is received (e.g. predation threat per Fanselow, 2018; Fanselow, Hoffman, & Zhuravka, 2019), resulting in the expression of graded or threat-sensitive responses (Helfman 1989). Here, we found that small arenas elicited an avoidance

response to putrescine that was not evident in larger arenas. This strongly suggests that putrescine is less-threatening than alarm cue and that the odorant elicits a chemosensory response as a source of decay-likely to avoid disease or scavenge predators. Certain fishes avoid infected conspecifics in a threat-sensitive manner (Stephenson et al. 2018), and recent evidence suggest they can detect the odor of infectious microorganisms (Sepahi et al. 2019). Trinidadian guppies (*Poecilia reticulata*) reduce shoal cohesion upon detection of infection (Croft et al., 2011) and respond to visual cues of infected conspecifics at close range (Stephenson et al. 2018), behaviors likely used to increase space between infected and non-infected individuals to reduce rates of disease transmission. This aligns with the observed avoidance pattern in sea lamprey in the small assays, where threat of disease would be more spatially imminent than in the larger assay.

Sea lamprey did not avoid any putrescine treatment in the large, multi-animal assay, which contrasts with well documented evidence of avoidance to alarm cue in large raceway streams, including evidence in the current study (Figure 7) (Byford et al., 2016; Hume et al., 2015; Hume & Wagner, 2018; Imre et al., 2014; Imre et al., 2016; Luhning et al., 2016; Wagner et al., 2016; Wagner, Stroud, & Meckley, 2011). Thus, although putrescine was identified as a compound within the skin extract containing the alarm cue (Dissanayake et al., 2019; Chapter 1), there is no evidence here to support putrescine is part of the behaviorally active suite of compounds within this cue. Because the alarm cue mixture is extracted from dead organisms, the nature of putrescine within the mixture is unknown and may be present as a part of natural decomposition. There was also limited support for the hypothesis that putrescine was related to a salivary kairomone. In the larger, multi-animal assay, we did not find evidence that putrescine avoidance responses were similar to the human saliva trials seen in studies which used similar assay designs (10 sea lamprey in 145 cm x 185 cm assays; Di Rocco et al., 2014; Imre et al., 2014, 2016). Human saliva is a mixture, and it is possible that putrescine is part of this mixture. However, our data shows little evidence that putrescine elicits the expected spit response, such as avoidance or increased activity, on its own. Tilden (1809) described lamprey jumping out of water in “great agitation” when human saliva was

introduced, a pattern which was not seen in activity change within the small individual assay (Figure 6), and previous studies found a consistent and significant avoidance of human saliva in larger multi-animal assays (Di Rocco et al., 2014; Imre et al., 2014, 2016). We saw a similar significant avoidance pattern in the small assay, of different design than these studies (Figure 5), but not in larger assays of similar design (Figure 7), evidence that the avoidance response to putrescine is context specific in lamprey. Future studies should investigate putrescine in combination with other known components of human saliva already identified (Cook et al., 2003; Halgand et al., 2012). The response to other mammalian saliva samples, especially of those known to predate on sea lamprey such as raccoons and river otters (Imre et al., 2014; Scott & Crossman 1998), should also be investigated to understand the role of mammalian saliva as a putative predator kairomone, and any compound overlaps should be identified. Tilden's 1809 account reported that lamprey did not elicit any behavioral response to dog saliva, and the possibility remains that human saliva contains similar reactive compounds to the alarm cue and elicits an anti-predator response by happenstance.

The smaller arena may also have decreased the animal's perception of safety compared to the larger multi-animal assay. It has been shown that aquatic organisms respond both to perceptions of fear (threat level) and safety (vulnerability to a threat) when making movement decisions (e.g., crayfish, *Faxonius rusticus*, Mackay, Wood, & Moore, 2021). It is plausible that the larger multi-animal assay provided more cues of safety than the small individual assay. Both assays were relatively shallow, and lamprey may perceive a shallow environment as riskier because of increased vulnerability to shoreline predators (Imre et al., 2014; Scott & Crossman 1998). Sea lamprey are hypothesized to use depth cues to orient towards shallower waters during the start of their spawning migration (Meckley et al., 2017), and in shallow rivers may tend to move in the deeper thalweg (Hume et al, 2020). Despite the shallow conditions, the larger arena had an area three times that of the smaller arena (Figure 1; Figure 4), which likely provided animals with an increased perception of safety, because at any one point in the arena there is a larger area distance available to move away from a threat compared to the smaller assay. Perception of

safety in the individual assay may also have been affected by the lack of conspecifics. Many aquatic organisms are known to produce and respond to disturbance cues, which are distinct from alarm cues in that they are emitted upon the perception of risk rather than upon tactile attack and act as social cues (Bairos-Novak, Ferrari, & Chivers, 2019; Ferrari, Wisenden, & Chivers, 2010; Wisenden, 2019). Traditional ecological knowledge from Karuk and Yurok tribe fishermen suggests the Pacific lamprey emits a disturbance cue when handled by fishers, initiating a downstream flight response in migrants (Petersen 2009). The inclusion of more animals in Experiment 2 may have provided a sense of safety (i.e., the absence of disturbance cues), as individuals could gain information of risk from other individuals in the arena.

Confinement can also lead to stress which may have affected the observed behavioral patterns (Barton, 2002). Metabolic changes associated with stress can be significant in mediating anti-predator behaviors (Lawrence, Godin, & Cooke, 2018; Conrad et al., 2011), but there have been limited studies testing this hypothesis in fishes to date (Lawrence, Godin, & Cooke, 2018). Antipredator responses have been linked to increased cortisol levels in Nile tilapia (*Oreochromis niloticus*) (Sanches et al., 2015), coho salmon (*Oncorhynchus kisutch*) (Rehnberg & Schreck, 1987), and freshwater pearl dace (*Margariscus margarita*) (Rehnberg, Smith, & Sloley, 1987). A similar corticosteroid, 11-deoxycortisol, has recently been shown to play a role in sea lamprey gluconeogenesis, evidence that corticosteroid function is conserved in basal vertebrates (Shaughnessy & McCormick, 2021). However, both checkered puffer fish (*Sphoeroides testudineus*) (Cull et al., 2015; Pleizier et al., 2015) and schoolmaster snapper (*Lutjanus apodus*) (Lawrence et al., 2017; Lawrence, Eliason, et al., 2018) did not show evidence of increased anti-predator behaviors with increased cortisol levels. The influence of stress is likely context specific, and more research is needed on a variety of contexts to understand complex interactions between internal stress states and anti-predator behaviors (Sanches et al., 2015; Lawrence, Eliason, et al., 2018). It has been recommended that the size of a two-choice arena in studies with fishes should be decided based on the size of the species using the assay. Assays for larger, more motile fish should be designed to allow sufficient areas for

movement and exploration and to minimize confinement stress (Atema, Kingsford, & Gerlach, 2002; Gardiner & Atema, 2007; Jutfelt et al., 2017). The animals used in the study are notably motile during this life stage, as they actively migrate over large distances in search of suitable spawning habitat (Moser et al., 2015); a larger arena may prove less stressful for sea lamprey. One review suggested that the width and length of an experimental arena should be approximately 4-15 times the length of the organism, based on over a dozen studies of aquatic animals in two-choice assay experiments (Jutfelt et al., 2017). The average length of sea lamprey used in this study was 0.47 m, and the area of the experimental arena used in the assay was 1.22 m x 1.22 m (i.e., 2.6 times the average length of experimental subjects, below the recommended threshold).

We investigated the reaction to petromyzonacil, a novel molecule identified from sea lamprey skin (Dissanayake et al., 2019) on its own and in combination with putrescine. This compound was investigated as a potential species-specific labeling compound, as it has been hypothesized that the alarm cue is a mixture containing compounds that label “risk” and others that label the cue as species specific, consistent with evidence that responses to heterospecific alarm cues diminish with increased phylogenetic distance (Hume & Wagner, 2018; Chapter 1). Here, we saw no effect on sea lamprey preference or activity in treatments with petromyzonacil on its own (Figure 5) and found no synergistic effects with putrescine in the large assay (Figure 7). Therefore, there is no evidence based on behavioral screening that this molecule is olfactorily active at the concentrations tested.

In the small assay, we observed significant avoidance and increases in activity in response to both alarm cue treatments as previously reported in laboratory (Bals & Wagner 2012; Byford et al., 2016; Hume & Wagner, 2018; Imre et al., 2014; Imre et al., 2016) and field studies (Hume et al., 2015; Imre et al., 2010; Wagner et al., 2011; Luhring et al., 2016) Thus, this individual assay may be a useful tool to investigate activity and behavioral patterns to odor stimuli. One particular benefit to this assay is its high-throughput design, achieved in two ways: 1) the assay build is smaller, so more apparatuses can be built

side-by-side into raceways or natural streams and run simultaneously; and 2) trial time is cut down, so more trials can be completed in one night. A high-throughput, individual design requires fewer animals to achieve statistical significance, as each animal represents a replicate. It also requires less odor material, as there are more replicates per unit of odor. The individual assay can also allow researchers to investigate complex questions in inter-individual variation of behavioral patterns related to animal personality, an area of research which has been identified as particularly important to bridge basic and applied research in conservation studies (Merrick & Koprowski, 2017). To improve assay design and understand its efficacy in behavioral research, we suggest future research investigates how sea lamprey activity is affected by varying arena dimensions to understand how size effects the animal's perception of vulnerability. We also suggest studies investigate behavioral risk avoidance patterns in sea lamprey in assays with differing depths to understand how water depth affects the organism's perception of vulnerability. While a smaller assay does not represent how a lamprey would respond to stimuli in nature, it could be used as a fruitful screening tool to then be scaled up to larger raceway or field studies.

In sum, we found limited evidence of repellent activity to putrescine in migratory sea lamprey after two experiments investigating avoidance and activity change to the odorant. Our work demonstrated the potential efficacy of a small scale, individual animal behavioral assay as a screening tool for behavioral and chemical ecology research. Future research should continue to investigate how assay metrics constrain behavior and continue to research potential repellent molecules, including other decay molecules such as cadaverine, for use in management of invasive aquatic species such as the sea lamprey. More research needs to be done to understand the response of sea lamprey to saliva, with the most notable gap in the avoidance response to saliva of mammalian predators. The overlap of reactive compounds should be identified to further understand if saliva acts as a predator kairomone for migratory sea lamprey.

REFERENCES

REFERENCES

- Atema, J., Kingsford, M. J., & Gerlach, G. (2002). Larval reef fish could use odour for detection, retention and orientation to reefs. *Mar. Ecol. Prog. Ser.*, 241, 151–160.
- Bairos-Novak, K. R., Ferrari, M. C. O., & Chivers, D. P. (2019). A novel alarm signal in aquatic prey: Familiar minnows coordinate group defences against predators through chemical disturbance cues. *J. Anim. Ecol.*, 88(9), 1281–1290.
- Barton, B. A. (2002). Stress in Fishes: A diversity of responses with particular reference to changes in. *Integ. Comp. Biol.*, 525, 517–525.
- Bals, J. D., & Wagner, C. M. (2012). Behavioral responses of sea lamprey (*Petromyzon marinus*) to a putative alarm cue derived from conspecific and heterospecific sources. *Behaviour*, 149(9), 901–923.
- Blumstein, D. T. (2016). Habituation and sensitization: new thoughts about old ideas. *Anim. Behav.*, 120, 255–262.
- 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. *N. Am. J. Fish. Manag.*, 36(5), 1090–1096.
- Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Ecoscience*, 5(3), 338–352.
- Christie, M. R., Sepúlveda, M. S., & Dunlop, E. S. (2019). Rapid resistance to pesticide control is predicted to evolve in an invasive fish. *Sci. Rep.*, 1–13.
- Cline, T. J., Kitchell, J. F., Bennington, V., McKinley, G. A., Moody, E. K., & Weidel, B. C. (2014). Climate impacts on landlocked sea lamprey: Implications for host-parasite interactions and invasive species management. *Ecosphere*, 5(6), 1–13.
- Cooke, M., Leevs, N., & White, C. (2003). Time profile of putrescine, cadaverine, indole and skatole in human saliva. *Arch. Oral Biol.*, 48(4), 323–327.
- Curtis, V., de Barra, M., & Aunger, R. (2011). Disgust as an adaptive system for disease avoidance behaviour. *Philos. Trans. R. Soc. Lond., B: Biol. Sci.*, 366, 389–401.
- 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.
- Di Rocco, Richard T, Belanger, C. F., Imre, I., Brown, G. E., & Johnson, N. S. (2014). Daytime avoidance of chemosensory alarm cues by adult sea lamprey (*Petromyzon marinus*). *Can. J. Fish. Aquat. Sci.*,

71(6), 824–830.

- Dissanayake, A. A., Wagner, C. M., & Nair, M.G.. (2021). Evaluation of health benefits of sea lamprey (*Petromyzon marinus*) isolates using in vitro antiinflammatory and antioxidant assays. PLoS ONE, 16(11), 1–12.
- Dissanayake, A. A., Wagner, C. M., & Nair, M. G. (2016). Chemical characterization of lipophilic constituents in the skin of migratory adult sea lamprey from the Great Lakes Region. PLoS ONE, 11(12), 1–17.
- Dissanayake, A. A., Wagner, C. M., & Nair, M. G. (2019). Nitrogenous compounds characterized in the deterrent skin extract of migratory adult sea lamprey from the Great Lakes region. PLoS ONE, 11(12), 1–19.
- Domenici, P. (2010). Context-dependent variability in the components of fish escape response: integrating locomotor performance and behavior. J. Exp. Biol., 313(2), 59–79.
- Fanselow, M. S. (2018). The role of learning in threat imminence and defensive behaviors. Curr. Opin. Behav. Sci., 24, 44–49.
- Fanselow, M. S., Hoffman, A. N., & Zhuravka, I. (2019). Timing and the transition between modes in the defensive behavior system. Behav. Process., 166, 103890.
- Ferguson, S., & Gray, E. (1989). 1988 FIFRA Amendments: A Major Step in Pesticide Regulation. Env'tl. L. Rep. News & Analysis, 19, 10070.
- Ferrari, M. C. O., Vavrek, M. A., Elvidge, C. K., Fridman, B., Chivers, D. P., & Brown, G. E. (2008). Sensory complementation and the acquisition of predator recognition by salmonid fishes. Behav. Ecol. Sociobiol., 63(1), 113–121.
- Ferrari, M. C. O., Wisenden, B. D., & Chivers, D. P. (2010). Chemical ecology of predator – prey interactions in aquatic ecosystems: a review and prospectus. Can. J. Zool., 88, 698–724.
- Fisette, 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., 47, S660–S672.
- 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(11), 1325–1330.
- Gardiner, J. M., & Atema, J. (2007). Sharks need the lateral line to locate odor sources: rheotaxis and eddy chemotaxis. J. Exp. Biol., 210, 1925–1934.
- 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. Ann. Rev. Ecol. Evol. Syst., 51, 483–503.
- Halgand, F., Zabrouskov, V., Bassilian, S., Souda, P., Loo, J. A., Faull, K. F., Wong, D.T. & Whitelegge, J. P. (2012). Defining intact protein primary structures from saliva: a step toward the human proteome

- project. *Anal. Chem.*, 84(10), 4383-4395.
- Hanson, M.E. (2021). Making a Repellent: Overcoming Physiological Impediments to Guiding Migratory Sea Lamprey (*Petromyzon Marinus*) with an Alarm Cue. MS Thesis. Michigan State University.
- Helfman, G. S. (1989). Behavioral Ecology and Sociobiology Threat-sensitive predator avoidance in damselfish-trumpetfish interactions. *Behav. Ecol. Sociobiol.*, 24, 47–58.
- Heale, V. R., Petersen, K., & Vanderwolf, C. H. (1996). Effect of colchicine-induced cell loss in the dentate gyms and Ammon's horn on the olfactory control of feeding in rats. *Brain Res. J.*, 712, 213–220.
- Hume, J. B., Almeida, P. R., Buckley, C. M., Criger, L. A., Madenjian, C. P., Robinson, K. F., Wang, C. J., & Muir, A. M. (2021). Managing native and non-native sea lamprey (*Petromyzon marinus*) through anthropogenic change: A prospective assessment of key threats and uncertainties. *J. Great Lakes Res.*, 47, S704–S722.
- 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(7), 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(12), 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(7), 3751–3762.
- Hunn, J. B., & Youngs, W. D. (1980). Role of Physical Barriers in the Control of Sea Lamprey (*Petromyzon marinus*). *Can. J. Fish. Aquat. Sci.*, 37, 2118–2122.
- Hussain, A., Saraiva, L. R., Ferrero, D. M., Ahuja, G., Krishna, V. S., Liberles, S. D., & Korsching, S. I. (2013). High-affinity olfactory receptor for the death-associated odor cadaverine. *Proc. Natl. Acad. Sci.*, 110(48), 19579–19584.
- Imre, I., Brown, G. E., Bergstedt, R. A., & McDonald, R. (2010). Use of chemosensory cues as repellents for sea lamprey: Potential directions for population management. *J. Great Lakes Res.*, 36(4), 790–793.
- Imre, I., Di Rocco, R. T., Belanger, C. F., Brown, G. E., & Johnson, N. S. (2014). The behavioural response of adult *Petromyzon marinus* to damage-released alarm and predator cues. *J. Fish Biol.*, 84(5), 1490–1502.
- Imre, I., Di Rocco, R. T., McClure, H., Johnson, N. S., & Brown, G. E. (2017). Migratory-stage sea lamprey *Petromyzon marinus* stop responding to conspecific damage-released alarm cues after 4 h of continuous exposure in laboratory conditions. *J. Fish Biol.*, 90(4), 1297-1304.
- Imre, I., Rocco, R. T. Di, 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(8), 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(3), 379–390.
- Kats, L. B., & Dill, L. M. (1998). The scent of death: chemosensory assessment of predation risk by prey animals. *Ecoscience*, 5(3), 361–394.
- Lawrence, M. J., Eliason, E. J., Brownscombe, J. W., Gilmour, K. M., Mandelman, J. W., & Cooke, S. J. (2017). An experimental evaluation of the role of the stress axis in mediating predator-prey interactions in wild marine fish. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.*, 207, 21–29.
- Lawrence, M. J., Eliason, E. J., Brownscombe, J. W., Kathleen, M., Mandelman, J. W., Gutowsky, L. F. G., & Cooke, S. J. (2018). Influence of supraphysiological cortisol manipulation on predator avoidance behaviors and physiological responses to a predation threat in a wild marine teleost fish. *Integr. Zool.*, 13, 206–218.
- Lawrence, M. J., Godin, J. J., & Cooke, S. J. (2018). Does experimental cortisol elevation mediate risk-taking and antipredator behaviour in a wild teleost fish? *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.*, 226, 75–82.
- Lawrence, B. J., & Smith, R. J. F. (1989). Behavioral response of solitary fathead minnows, *Pimephales promelas*, to alarm substance. *J. Chem. Ecol.*, 15(1), 209–219.
- 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.
- Lennox, R. J., Bravener, G. A., Charles, H. L., Muir, A. M., Remucal, C. K., Robinson, K. F., Rous, A. M., Siefkes, M. J., Wilkie, M. P., Zielinski, D.P., & Cooke, S. J. (2020). Potential changes to the biology and challenges to the management of invasive sea lamprey *Petromyzon marinus* in the Laurentian Great Lakes due to climate change. *Glob. Change Biol.*, 26(3), 1118–1137.
- Mackay, R. N., Wood, T. C., & Moore, P. A. (2021). Running away or running to? Do prey make decisions solely based on the landscape of fear or do they also include stimuli from a landscape of safety? *J. Exp. Biol.*, 224(19), jeb242687.
- Meckley, T. D., Gurarie, E., Miller, J. R., & Wagner, C. (2017). How fishes find the shore: Evidence for orientation to bathymetry from the non-homing sea lamprey. *Can. J. Fish. Aquat. Sci.*, 74(12), 2045–2058.
- Merrick, M. J., & Koprowski, J. L. (2017). Should we consider individual behavior differences in applied wildlife conservation studies? *Biol. Conserv.*, 209, 34–44.
- Miehls, S., Sullivan, P., Twohey, M., Barber, J., Marie, S. S., Pa, O. N., & McDonald, R. (2020). The future of barriers and trapping methods in the sea lamprey (*Petromyzon marinus*) control program in the Laurentian Great Lakes. *Rev. Fish Biol. Fish.*, 30, 1–24.
- Moser, M. L., Almeida, P. R., Kemp, P. S., & Sorensen, P. W. (2015). Lamprey spawning migration. In *Lampreys: biology, conservation and control* (pp. 215–263). Springer, Dordrecht.
- Oliveira, T. A., Koakoski, G., da Motta, A. C., Piato, A. L., Barreto, R. E., Volpato, G. L., & Barcellos, L. J. G.

- (2014). Death-associated odors induce stress in zebrafish. *Horm. Behav.*, 65(4), 340–344.
- Perrault, K., Imre, I., & Brown, G. E. (2014). Behavioural response of larval sea lamprey (*Petromyzon marinus*) in a laboratory environment to potential damage-released chemical alarm cues. *Can. J. Zool.* 92(5), 443–447.
- Petersen Lewis, R. S. (2009). Yurok and Karuk traditional ecological knowledge: insights into Pacific lamprey populations of the lower Klamath Basin. In *Biology, management, and conservation of lampreys in North America*. American Fisheries Society, Symposium (Vol. 72, pp. 1-39).
- Pinel, J. P. J., Gorzalka, B. B., & Ladak, F. (1981). Cadaverine and Putrescine Initiate the Burial of Dead Conspecifics by Rats. *Physiol. Behav.*, 27, 819–824.
- Pratt, T., O'Connor, L., Hallett, A., McLaughlin, R., Katopodis, C., Hayes, D., & Bergstedt, R. (2009). Balancing aquatic habitat fragmentation and control of invasive species: enhancing selective fish passage at sea lamprey control barriers. *Trans. Am. Fish. Soc.*, 138(3), 652–665.
- Prounis, G. S., & Shields, W. M. (2013). Necrophobic behavior in small mammals. *Behav. Process.*, 94, 41–44.
- Rehnberg, B. G., & Schreck, C. B. (1987). Chemosensory detection of predators by coho salmon (*Oncorhynchus kisutch*): behavioral reaction and the physiological stress response. *Can. J. Zool.*, 65, 481–485.
- Rehnberg, B. G., Smith, R. J. F., & Sloley, B. D. (1987). The reaction of pearl dace (Pisces, Cyprinidae) to alarm substance: time-course of behavior, brain amines, and stress physiology. *Can. J. Zool.*, 65, 2916–2921.
- Rolen, S. H., Sorensen, P. W., Mattson, D., & Caprio, J. (2003). Polyamines as olfactory stimuli in the goldfish *Carassius auratus*. *J. Exp. Biol.*, 206(10), 1683–1696.
- Sanches, F. H. C., Miyai, C. A., Pinho-Neto, C. F., & Barreto, R. E. (2015). Stress responses to chemical alarm cues in Nile tilapia. *Physiol. Behav.*, 149, 8–13.
- Sepahi, A., Kraus, A., Casadei, E., Johnston, C. A., Galindo-Villegas, J., & Kelly, C. (2019). Olfactory sensory neurons mediate ultrarapid antiviral immune responses in a TrkA-dependent manner. *PNAS*, 116(25), 12428–12436.
- Shaughnessy, C. A., & McCormick, S. D. (2021). 11-Deoxycortisol is a stress responsive and gluconeogenic hormone in a jawless vertebrate, the sea lamprey (*Petromyzon marinus*). 224(11), jeb241943.
- Siefkes, M. J. (2017). Use of physiological knowledge to control the invasive sea lamprey (*Petromyzon marinus*) in the Laurentian Great Lakes. *Conserv. Physiol.*, 5(1), 1–18.
- Siefkes, M. J., Johnson, N. S., & Muir, A. M. (2021). A renewed philosophy about supplemental sea lamprey controls. *J. Great Lakes Res.*, 47, S742–S752.
- Stephenson, J. F., Perkins, S. E., & Cable, J. (2018). Transmission risk predicts avoidance of infected conspecifics in Trinidadian guppies. *J. Anim. Ecol.*, 87(6), 1525–1533.

- Sun, Q., Haynes, K. F., & Zhou, X. (2017). Dynamic changes in death cues modulate risks and rewards of corpse management in a social insect. *Funct. Ecol.*, 31, 697–706.
- Wagner, C. M., Kierczynski, K. E., Hume, J. B., & Luhning, T. M. (2016). Exposure to a putative alarm cue reduces downstream drift in larval sea lamprey *Petromyzon marinus* in the laboratory. *J. Fish Biol.*, 89(3), 1897–1904.
- 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(7), 1157–1160.
- Wagner, C.M., Bals, J.M., Hanson, M.E., & Scott, A.M (2022) Attenuation and recovery of an avoidance response to a chemical antipredator cue in an invasive fish: implications for use as a repellent in conservation. *Cons. Phys.* (In Press).
- Wisenden, B. D. (2000). Olfactory assessment of predation risk in the aquatic environment. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.*, 355(1401), 1205–1208.
- Wisenden, B. D. (2019). Evidence for incipient alarm signaling in fish. *J. Anim. Ecol.*, 88(9), 1278–1280.
- Wisenden, B. D., Chivers, D. P., Brown, G. E., & Smith, R. J. (1995). The role of experience in risk assessment: Avoidance of areas chemically labelled with fathead minnow alarm pheromone by conspecifics and heterospecifics. *Ecoscience*, 2(2), 116–122.
- Wisman, A., & Shrira, I. (2015). The smell of death: evidence that putrescine elicits threat management. *Front. Psychol.*, 6, 1274.
- Yao, M., Rosenfeld, J., Attridge, S., Sidhu, S., Aksenov, V., & Rollo, C. D. (2009). The Ancient Chemistry of Avoiding Risks of Predation and Disease. *Evol. Biol.*, 36, 267–281.
- Zielinski, D P, & Freiburger, C. (2021). Advances in fish passage in the Great Lakes basin. *J. Great Lakes Res.*, 47, S439–S447.
- Zielinski, Daniel P, Mclaughlin, R., Castro-santos, T., Hrodey, P., & Muir, A. (2019). Alternative sea lamprey barrier technologies: history as a control tool. *Rev. Fish. Sci. Aquac.*, 27(4), 438–457.