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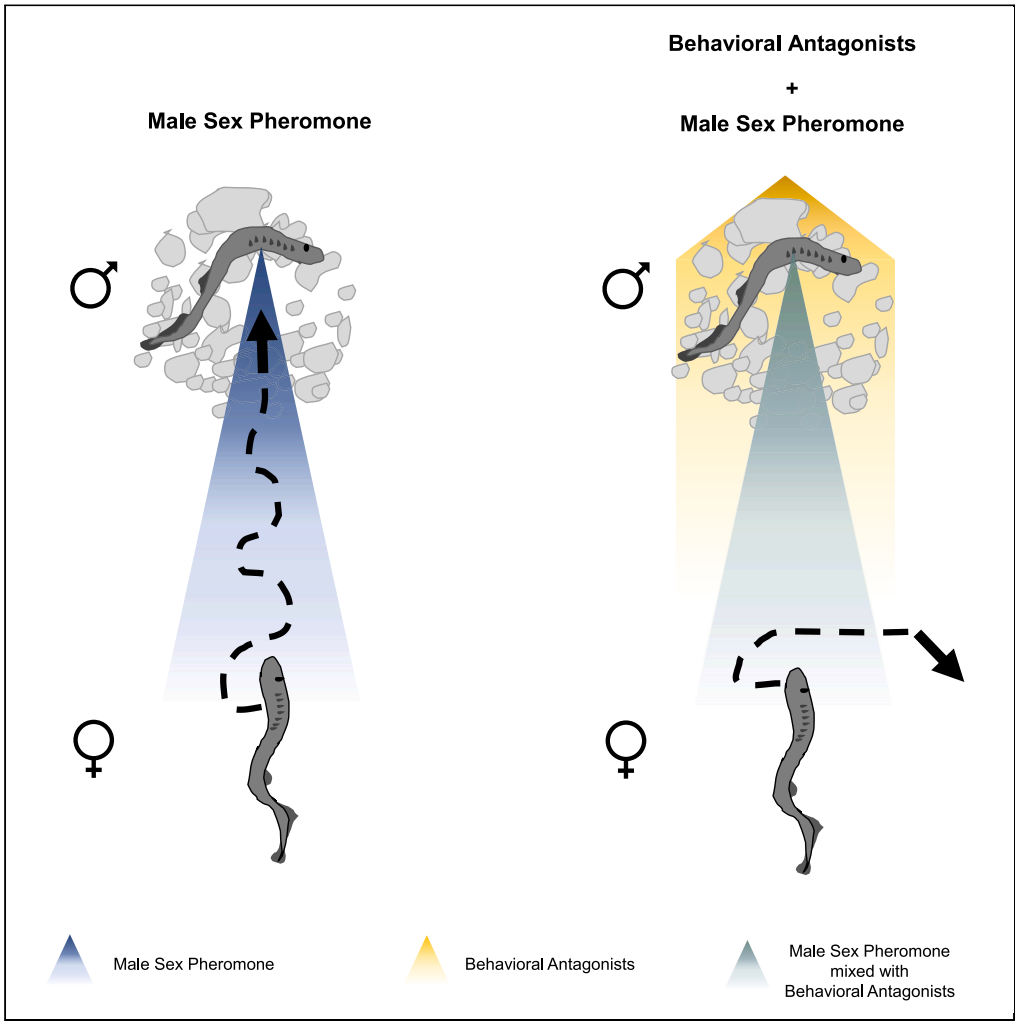

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Highlights

Mature male sea lamprey release a multi-component sex pheromone that attracts females

A pheromone analog disrupted pheromone-mediated olfaction and attraction to males

Mixture of behavioral antagonists reduced spawning and abundance in a natural stream

Behavioral antagonists may be useful to manage invasive sea lamprey populations

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Synergistic behavioral antagonists of a sex pheromone reduce reproduction of invasive sea lamprey

Anne M. Scott,[1] Nicholas S. Johnson,[2] Michael J. Siefkes,[3] and Weiming Li[1,4,*]

SUMMARY

Sex pheromones impart maximal attraction when their components are present at optimal ratios that confer balanced olfactory inputs in potential mates. Altering ratios or adding pheromone analogs to optimal mixtures may disrupt balanced olfactory antagonism and result in reduced attraction, however, tests in natural populations are lacking. We tested this hypothesis in sea lamprey (*Petromyzon marinus*), a fish whose male sex pheromone attracts females when two critical components, 3-keto petromyzonol sulfate (3kPZS) and petromyzonol sulfate (PZS), are present at certain ratios. Here, we report a pheromone analog, petromyzonol tetrasulfate (3sPZS), reduced female attraction to 3kPZS but not to PZS. 3sPZS mixed with additional PZS synergistically disrupted female attraction to the male pheromone and reduced spawning by 97% in a high-density population. Our results provide evidence of balanced olfactory antagonism in a vertebrate and establish a tactic to disrupt spawning of sea lamprey, a destructive invader of the Laurentian Great Lakes.

INTRODUCTION

Pheromones are chemical signals that regulate a variety of behaviors in conspecifics and are indispensable for survival and reproduction in many organisms.[1] Accordingly, animals have evolved olfactory systems that detect and discriminate pheromones with remarkable sensitivity and selectivity.[2] Sex pheromones often comprise one or two major components that guide orientation and several minor components that further optimize the attraction of potential mating partners.[3] Maximal attraction to sex pheromones is hypothesized to result from balanced olfactory antagonism, which occurs when all pheromone components are present at the optimal relative ratios with no addition of other components.[4] A reliable indicator of balanced or unbalanced olfactory antagonism is the behavioral outcome. If olfactory inputs are balanced, stereotyped attraction to the pheromone is observed. As the deviation from the optimal ratio of pheromone components increases, the olfactory inputs become imbalanced and therefore, the magnitude of attraction decreases. Much of the evidence for balanced olfactory antagonism comes from studies on insect pheromones. In moth species, attraction to a sex pheromone is reduced or even eliminated if the components are present at suboptimal ratios[5,6] or an analog of a pheromone component is introduced.[7,8] Compounds that diminish attraction to a sex pheromone are referred to as pheromone behavioral antagonists. These compounds may be novel to the pheromone mixture or existing pheromone components that are present at suboptimal ratios.[4] Although behavioral evidence supports balanced olfactory antagonism,[4] tests in a natural population are lacking.

Pheromone components at balanced ratios enable reliable communication and mediate context-appropriate behavioral responses in sea lamprey (*Petromyzon marinus*).[9] This anadromous jawless fish species uses conspecific odors during their terminal reproductive phase to locate suitable spawning habitat and find mates.[10] After parasitizing fish in lakes or the Atlantic Ocean, pre-spawning adult sea lamprey migrate into streams in the spring to spawn, guided by chemical cues released by stream-resident larvae in nursery habitat,[11–14] and become sexually mature (spermiated and ovulated) over the next several weeks. Spermiated male sea lamprey ascend to spawning grounds characterized by patches of gravel, begin constructing nests from these rocks, and release a multi-component sex pheromone that attracts ovulated females, where each component is present at characteristic ratios.[15,16] Sea lamprey spawn intermittently with multiple mates before perishing in the early summer.[17] The resulting larvae remain in the stream sediment for 2–10 years before out-migrating.[18] The larval cue and male sex pheromone share at least two steroidal components: 3-keto petromyzonol sulfate (3kPZS) and petromyzonol sulfate (PZS).[9] PZS differs from 3kPZS by two hydrogens and is the immediate precursor in biosynthesis of 3kPZS.[19] Pre-ovulated females are attracted to the larval cue that features a 3kPZS to PZS ratio of ~1:10, whereas ovulated females prefer the male sex pheromone with a ratio of ~100:1 and avoid the PZS-rich larval cue.[9]

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We reasoned that the ratio of 3kPZS and PZS featured in the male sex pheromone provides a balanced olfactory input in ovulated females that optimizes the attraction to the nesting males. We predicted that changing this ratio or introducing a pheromone analog that differentially influences the behavioral effects of 3kPZS and PZS would disrupt the balance and therefore reduce the attraction of ovulated females to the male pheromone. We tested these predictions using petromyzonol tetrasulfate (3sPZS), a synthetic analog of PZS and 3kPZS implicated by computer algorithm-based screening and *in vivo* screening to influence sea lamprey olfactory responses to 3kPZS.[20] We found 3sPZS altered the behavioral responses to 3kPZS but not to PZS, and perturbed the responses to the full male sex pheromone. 3sPZS mixed with additional PZS synergistically disrupted the attraction of the females to the full male sex pheromone and reduced spawning of a natural sea lamprey population. Our results provide empirical evidence of balanced olfactory antagonism in the pheromone communication system of a vertebrate and may guide development of a strategy to inhibit spawning of sea lamprey, a destructive invader of the Laurentian Great Lakes.[21]

RESULTS

3sPZS is a potent odorant that interferes with female response to 3kPZS and not to PZS

3kPZS and PZS are known to be potent stimuli that interact with odorant receptors[22] and induce behavioral responses.[9,16] For an analog to influence the pheromone function of 3kPZS and PZS, it must be detected in the olfactory organ. Therefore, we first determined whether 3sPZS elicits olfactory responses. Using electro-olfactogram (EOG) recordings, we found 3sPZS stimulated the adult sea lamprey olfactory epithelium in a concentration-dependent manner (Figure 1A), consistent with PZS (Figure 1B) and 3kPZS (Figure 1C). The detection thresholds of 3sPZS (one tailed paired t-test, $p = 0.038$, $t = -2.137$, degrees of freedom [*df*] = 6), PZS ($p = 0.001$, $t = -4.493$, $df = 7$), and 3kPZS ($p = 0.031$, $t = -2.296$, $df = 6$) were all less than 10^{-12} M. At the olfactory epithelium, 3sPZS evoked similar responses in females and males (Figures 1D–1G). The olfactory response to 3sPZS 10^{-6} M was not different in males and females ($p = 0.805$, $t = -0.255$, $df = 8$; Figure 1D). To evaluate whether 3sPZS reduced the 3kPZS olfactory response to a similar extent in males and females, we measured the 3kPZS EOG responses before and during exposure of the olfactory epithelium to 3sPZS following established approaches.[23] We found the extent that 3sPZS reduced the olfactory response to 3kPZS was not different in males and females (54.8% and 59.8%, respectively; $p = 0.453$, $t = 0.789$, $df = 8$; Figure 1E). 3sPZS did not reduce EOG responses to a non-pheromone control stimulus that elicits olfactory responses, L-arginine, in males or females (Figure 1E).

Next, we sought to determine whether 3sPZS altered the behavioral responses elicited by either 3kPZS or PZS in ovulated female, pre-ovulated female, and spermiated male sea lamprey. Using an established 2-choice flume preference assay,[9] we measured whether the lamprey spent more time in a channel treated with an odorant versus vehicle (attraction) or less time (aversion), relative to the amount of time spent in each channel prior to odorant application. Given the documented reduction of the 3kPZS olfactory responses when exposed to 3sPZS (Figure 1E),[20] we predicted 3sPZS would abate ovulated female preference for 3kPZS. As predicted, ovulated females were attracted to 3kPZS (10^{-12} M, $p < 0.001$) and avoided a mixture of 3kPZS and 3sPZS (1:1, each at 10^{-12} M, $p < 0.001$, Figure 2). The addition of 3sPZS changed ovulated female preference for 3kPZS from attraction to aversion ($p < 0.001$, $t = 9.738$, $df = 42$). However, adding 3sPZS to PZS did not influence ovulated or pre-ovulated female behavioral responses to PZS (ovulated: $p = 0.733$, $t = -0.346$, $df = 22$; pre-ovulated: $p = 0.893$, $t = 0.136$, $df = 18$). Pre-ovulated females were attracted to PZS (10^{-12} M, $p = 0.002$) and to a mixture of PZS and 3sPZS (1:1, each at 10^{-12} M, $p = 0.037$, Figure 2). 3sPZS alone (10^{-12} M) did not induce attraction or aversion in pre-ovulated females ($p = 0.375$, Figure 2). These data show 3sPZS influenced ovulated female behavioral responses to 3kPZS but not to PZS, suggesting 3sPZS may perturb the balance between the two pheromone components.

3sPZS did not affect spermiated male behaviors in the same manner as ovulated females. The male response to 3sPZS mixed with 3kPZS (1:1, each at 10^{-12} M) was not significantly different from their response to 3kPZS alone (10^{-12} M, Figure 2) ($p = 0.088$, Wilcoxon rank sum $W = 53$). Additionally, 3sPZS alone (10^{-12} M) did not induce attraction or aversion in males ($p = 0.297$, Figure 2). Notably, the olfactory epithelium responses to 3sPZS in males and females were comparable (Figures 1D–1G) despite differing behavioral responses. These results are consistent with the idea that balanced olfactory antagonism is a central nervous system phenomenon whereby the observed behavior is likely the outcome of the processing and integration of multiple stimuli interacting with multiple odorant receptors and is not dependent on peripheral olfactory responses alone.[4] The contrasting behavioral responses to 3sPZS in males and females indicate 3sPZS is not a general repellent, but instead is a behavioral antagonist that specifically influences ovulated female responses.

3sPZS impedes female search for 3kPZS activated nest in a stream

In our next experiment, we examined the effect of 3sPZS on ovulated females in search of 3kPZS using an established bioassay[9] in the Ocqueoc River, a river where sea lamprey spawn.[18] A migration barrier restricts sea lamprey from accessing the upper reaches, a historical spawning ground with good nesting habitat but no pheromone background. In a 50 m section of the upper system, we assessed whether adding 3sPZS to a 3kPZS plume renders 3kPZS less attractive to females traversing a stream. The section contained two adjacent artificial spawning nests (1 m^2) activated with odorants, with each resulting plume activating approximately half of the stream width to provide a side-by-side comparison. In this side-by-side comparison, 3sPZS reduced female attraction to 3kPZS (Figures 3A–3D). When each nest was activated with only 3kPZS, ovulated females entered the left and right nests at approximately equal proportions ($p = 0.757$, Table S1) and remained at each for comparable durations of time (51.9 $\pm$ 15.6 s and 40.5 $\pm$ 11.7 s, respectively) (Wilcoxon signed rank test, $p = 0.496$, $V = 506.5$), indicating there was no side bias. However, when a 3kPZS (5×10^{-13} M) nest was offered alongside a nest with the mixture of 3kPZS and 3sPZS (1:1, each at 5×10^{-13} M), fewer females entered the nest activated with a mixture of 3sPZS and 3kPZS than the nest activated with 3kPZS alone ($p < 0.001$, Figure 3D; Table S1). Of the females that entered a nest, individuals spent less time in the

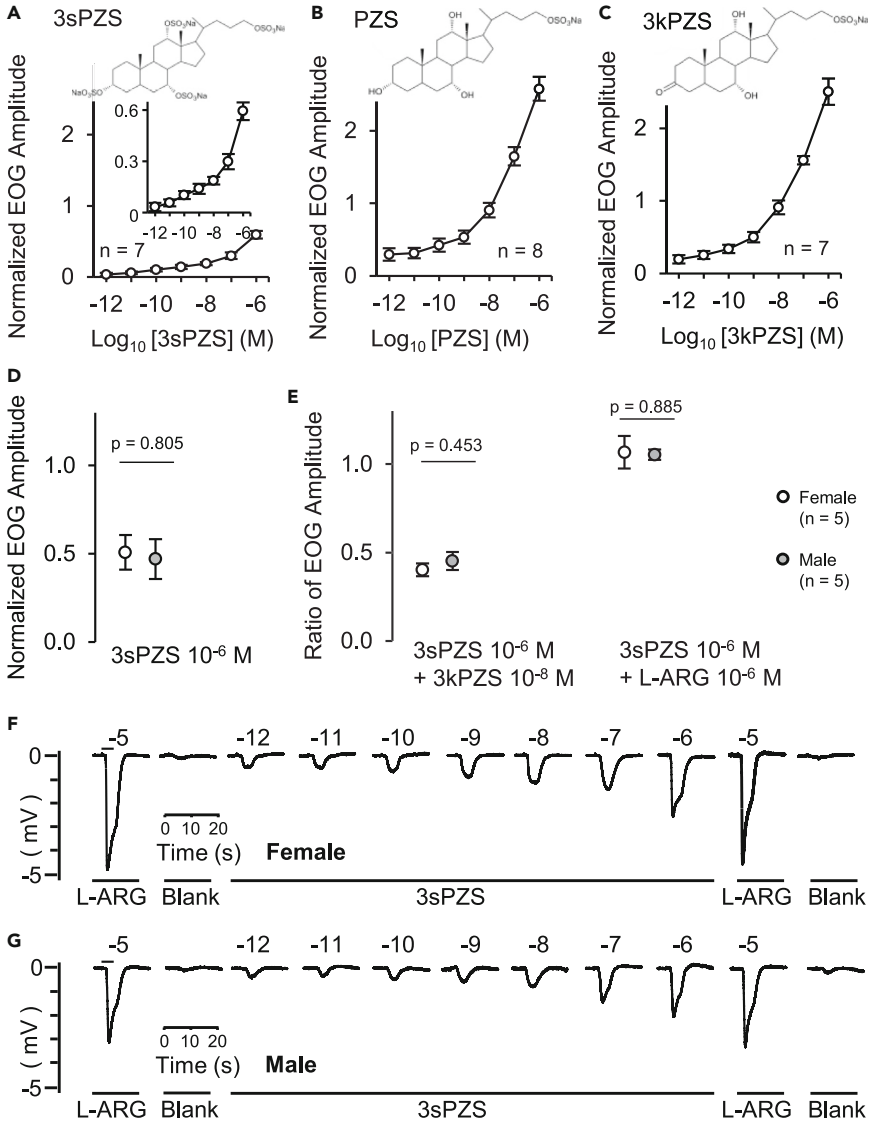

Figure 1. 3sPZS stimulated the olfactory system at picomolar concentrations

(A–G) Responses were measured by electro-olfactogram recordings (EOG) in adult sea lamprey. Data are represented as mean ± SEM. (A) 3sPZS, (B) PZS, and (C) 3kPZS induced concentration-dependent responses. (Inset) Structures of 3sPZS, PZS, and 3kPZS. The response amplitude was blank-corrected and normalized to the response amplitude of 10^{-5} M L-arginine (standard). Inset in (A) shows re-scaled y axis to best show the trend of the data. The EOG response to (D) 3sPZS were not different in males and females. (E) 3sPZS reduced the olfactory response to 3kPZS but not to a non-pheromone control stimulus that elicits olfactory responses, L-arginine, in males and females. A ratio of EOG amplitude value of 1.0 indicates 3sPZS did not reduce the olfactory response to the test stimuli. Significance between sexes was evaluated with two-tailed t test. Representative EOG traces of (F) female and (G) male olfactory epithelia exposed to 3sPZS at concentrations between 10^{-12} M and 10^{-6} M from (A). The number above each trace is the logarithmic value of the molar concentration of each stimulant. The bar above the L-ARG trace (*Left*) represents the duration of odorant treatment. Blank, vehicle solution; L-ARG, L-arginine.

nest activated with 3sPZS and 3kPZS (17.4 ± 6.7 s, mean ± SEM) compared to 3kPZS alone (115.8 ± 36.9 s) (Wilcoxon signed rank test, $p < 0.001$, $V = 410$). When comparing across experimental and control trials, fewer females moved upstream (−15%, $p = 0.027$) and instead more moved downstream (+200%, $p = 0.030$) when one nest was infused with 3sPZS compared to trials when only 3kPZS was applied to both nests (Figures 3B and 3C; Table S1). Swim tracks from visual observations were overlaid onto the odorant plume map and indicated 3sPZS modified the path females swam (Figure S1). Ovulated females were distributed more widely across the stream and were less likely to track the 3kPZS plume when 3sPZS was added, resulting in some females bypassing the 3kPZS activated nest. Therefore, 3sPZS disrupted females in search of a 3kPZS activated nest.

We reasoned adding 3sPZS to a mixture of PZS and 3kPZS (10:1) that has previously been demonstrated to neutralize female attraction to 3kPZS[9] would further deter females. During trials when 3kPZS was applied to each nest, no side bias was observed as females entered the left

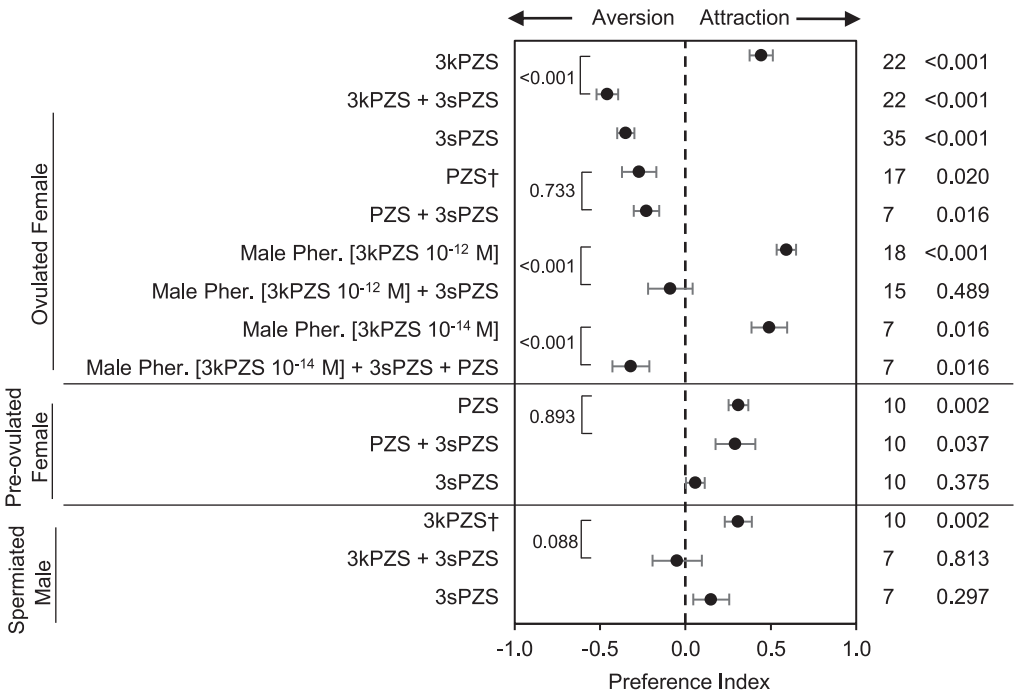

Figure 2. Behaviors of sea lamprey in a two-choice flume

The preference index was calculated for each stimulant, applied to reach a concentration in the flume of 10^{-12} M unless otherwise noted. Data are represented as mean ± SEM. A Wilcoxon signed-rank test was used to determine whether the index was different from zero. A positive index value indicates attraction, and a negative index value indicates aversion. 3kPZS attracted both ovulated females and spermiated males. For ovulated females, 3sPZS eliminated the preference for 3kPZS, and 3sPZS mixed with PZS neutralized the preference for the full male sea lamprey sex pheromone (Male Pher.). Pre-ovulated female behavioral responses to PZS mixed with 3sPZS were not different than those to PZS. Spermiated male behavioral responses to 3sPZS mixed with 3kPZS were not different than those to 3kPZS. n, sample size, followed by the p value from the Wilcoxon signed-rank test, is displayed (*Right*). Significance between treatment groups was evaluated with a two-tailed t test and p values are displayed (*Left*). Male Pher., full male sea lamprey sex pheromone applied to reach a concentration of 3kPZS at 10^{-12} M or 10^{-14} M † Data from Buchinger et al.[9].

and right nest at approximately equal proportions (p = 0.939) and remained at each for comparable durations of time (52.3 ± 24.6 s and 48.5 ± 11.3 s, respectively, mean ± SEM) (Wilcoxon signed rank test, p = 0.460, *V* = 631.5, Figure 3). As predicted, 3sPZS added to PZS and 3kPZS (3sPZS: PZS: 3kPZS, 10:10:1, 3kPZS at 5 × 10^{-13} M) biased female behavioral preferences in the in-stream side-by-side bioassay (Figures 3E–3H). 3sPZS and PZS prevented all females from entering a 3kPZS activated nest (p < 0.001, Figure 3H; Table S2). When comparing across experimental and control trials, 3sPZS and PZS reduced upstream movement (−51%, p < 0.001), increased remaining in the release cage (+42%, p = 0.021), and increased downstream movement (+119%, p < 0.001) (Figures 3E–3G; Table S2). The movement patterns recorded from visual observations provided a striking contrast between the females exposed to 3kPZS compared to those exposed to 3kPZS mixed with 3sPZS and PZS (Figure S2). 3sPZS and PZS reduced overall upstream movement and diverted all females from entering the 3kPZS activated nest.

3sPZS and PZS synergistically disrupt female attraction to full male sex pheromone

Having shown that 3sPZS disrupted the female behavioral responses to 3kPZS, we sought to determine if 3sPZS disrupts the function of the full male sex pheromone. Most characterized sex pheromones are mixtures that elicit stronger responses when all components are present at appropriate ratios compared to responses induced by individual components.[1] Likewise, the full male sex pheromone of sea lamprey (collected as washings of spermiated males) is a mixture of 3kPZS and other attractive sulfonated bile acids[10,24] that attracts and retains more females and induces more spawning behaviors than 3kPZS alone.[15,25,26] The increased effectiveness of the full male pheromone implies it may be more resilient than 3kPZS to disruption. As expected, we found females were attracted to the full male sex pheromone when it was applied to reach a concentration of 3kPZS at 10^{-12} M (p < 0.001), but showed no preference for the male pheromone when 3sPZS was added (10^{-12} M, p = 0.489, Figure 2) in the 2-choice flume, indicating 3sPZS perturbs female preference to the male pheromone (p < 0.001, Wilcoxon rank sum *W* = 49). We found the combination of 3sPZS and PZS averted females from the male pheromone (p < 0.001, t = 5.399, *df* = 12, Figure 2).

In our next field experiment, we activated a single nest with the male pheromone (applied to reach 3kPZS at 5 × 10^{-13} M) and applied 3sPZS or PZS (1 × 10^{-10} M, equivalent to 200× the concentration of 3kPZS in the male pheromone) directly upstream of the nest, such

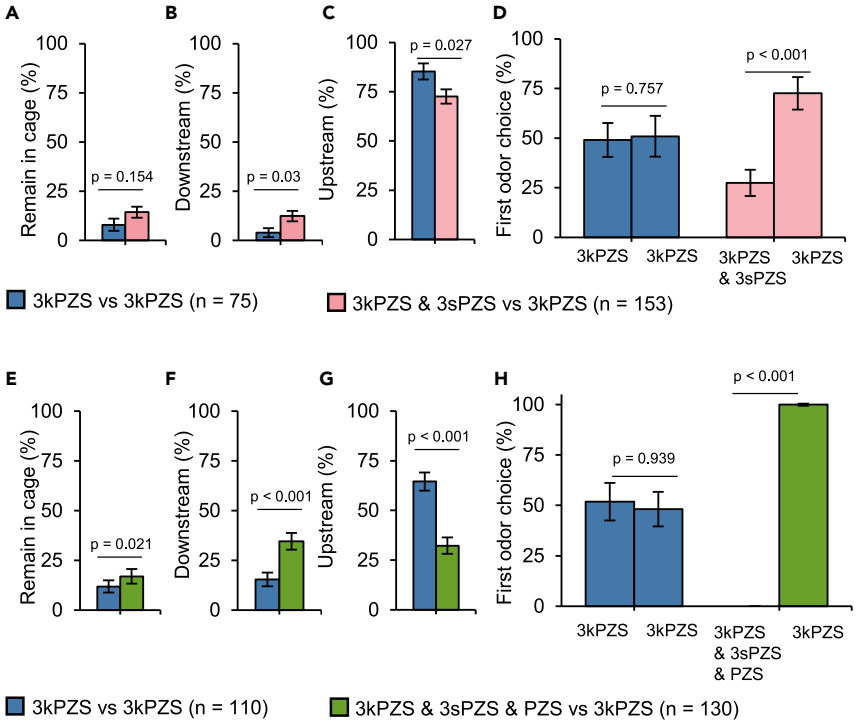

Figure 3. Behavioral antagonist impeded female search for a 3kPZS activated nest in a stream environment

(A–H) In a set of side-by-side odorant comparisons (A–D), females were exposed to control treatments (3kPZS at 5×10^{-13} M vs. 3kPZS at 5×10^{-13} M, final in-stream concentration) or to experimental treatments (3kPZS at 5×10^{-13} M vs. a mixture of 3kPZS and 3sPZS each at 5×10^{-13} M). In the second set of side-by-side odorant comparisons (E–H), females were exposed to control treatments (3kPZS at 5×10^{-13} M vs. 3kPZS at 5×10^{-13} M) or to experimental treatments (3kPZS at 5×10^{-13} M vs. a mixture of 3sPZS, PZS, and 3kPZS at a ratio of 10:10:1, with 3kPZS at 5×10^{-13} M). The movement of females from the release cages was monitored with four passive integrated transponder (PIT) antennas and visual observations (See Figures S1 and S2). Movements were evaluated with a generalized linear model with a binomial distribution (See Tables S1 and S2). Results plotted show the percent of released females that (A and E) remained in the release cage at the end of the trial, (B and F) moved at least 3 m downstream of the release cages, and (C and G) moved at least 3 m upstream of the release cages. (D and H) The distribution of females that entered each 1 m^2 odorant nest within control or experimental trials. Error bars represent standard error.

that the 3sPZS or PZS plume enshrouded the male pheromone plume (Figure 4A). In this modified assay, we eliminated the refuge from the behavioral antagonist with the removal of the adjacent pheromone activated nest from the side-by-side comparison, imposing a suboptimal mixture of pheromone components or adding a behavioral antagonist continuously on the test subjects. Additionally, we increased the length of the field site from 50 m to 200 m to simulate the longer upstream movement female sea lamprey often undergo as they approach a male-occupied nest. Overall, we found PZS was more effective than 3sPZS in disrupting female responses to the male pheromone. The male pheromone enshrouded with PZS increased downstream movement (+215%, $p < 0.001$), decreased upstream movement (−46%, $p < 0.001$), decreased entry into the channel (−60%, $p < 0.001$), decreased approach (−57%, $p < 0.001$) and entry into the male pheromone activated nest (−56%, $p < 0.001$), relative to the male pheromone enshrouded with vehicle (Figure 4; Table S3). In contrast, the male pheromone enshrouded with 3sPZS only decreased female entry into the channel (−28%, $p = 0.037$) compared to the male pheromone enshrouded with vehicle (Figure 4), consistent with the flume observation that 3sPZS rendered the male pheromone less attractive (Figure 2), but did not influence the other parameters recorded.

Since 3sPZS and PZS had differing impacts on female responses to the male pheromone, we suspected their effects are non-redundant, which poses the question of whether the effects of 3sPZS and PZS are additive. To address this question, we applied a mixture of 3sPZS and PZS, each at 5×10^{-11} M (each equivalent to 100× the concentration of 3kPZS in the male pheromone), upstream of the male pheromone activated nest. In this test scheme if the effects of 3sPZS and PZS were additive, the female behavioral responses to the male pheromone would fall between the responses observed to the male pheromone enshrouded with the 200x 3sPZS and 200× PZS treatments. Under this assumption, the expected values of behavioral parameters were calculated and compared to the observed values (Table S4). This comparison indicated the combination of 3sPZS and PZS was synergistic because the mixture was more effective than would be expected from the summed effects of the components for each parameter measured ($p < 0.001$, Table S4). The 3sPZS and PZS mixture increased downstream movement (+257%, $p < 0.001$), decreased upstream movement (−67%, $p < 0.001$), decreased entry into the channel (−80%, $p < 0.001$), decreased approach (−80%, $p < 0.001$), and decreased entry into the male pheromone activated nest (−81%, $p < 0.001$), relative to the male pheromone enshrouded with vehicle (Figure 4). The mixture reduced female attraction to the male pheromone more than either

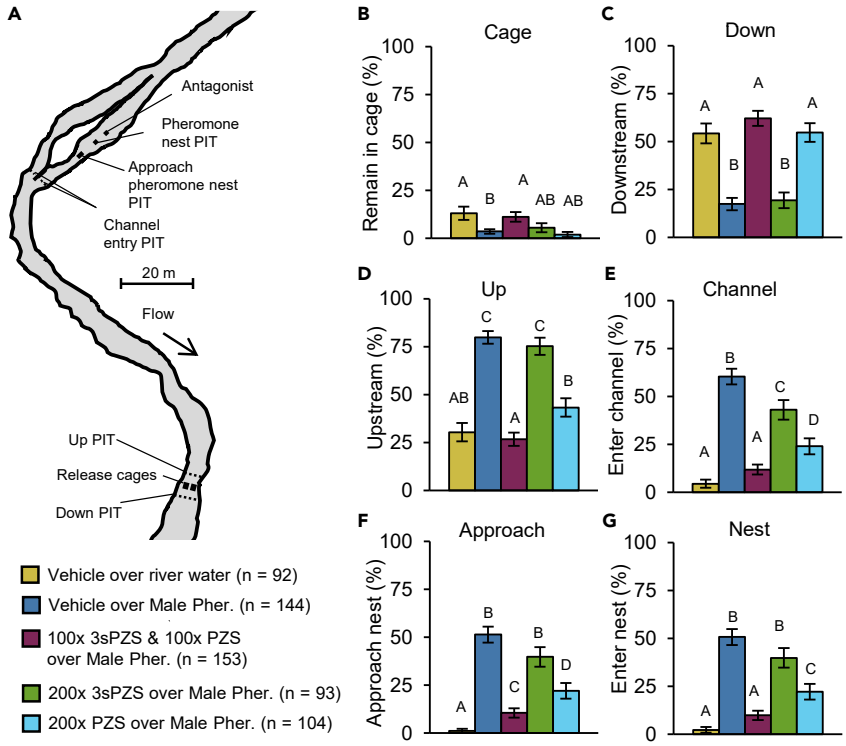

Figure 4. A mixture of 3sPZS and PZS is more effective than either component in disrupting female attraction to an artificial nest activated with the full male sex pheromone

(A–G) The full male sex pheromone (Male Pher.) was applied to the nest to reach an in-stream concentration of 3kPZS at 5×10^{-13} M. At 7 m upstream of the nest, we applied vehicle (50% methanol) or behavioral antagonist treatments (3sPZS and PZS each at 5×10^{-11} M, 3sPZS at 1×10^{-10} M, or PZS at 1×10^{-10} M). (A) The movement of females from the release cages was monitored with five passive integrated transponder (PIT) antennas and visual observations over 200 m. Results plotted show the percent of released females that (B) remained in the release cage at the end of the trial, (C) moved at least 3 m downstream of the release cages, and (D) moved at least 3 m upstream of the release cages, (E) entered the activated treatment subchannel compared to the adjacent subchannel with river water, (F) approached the pheromone activated nest by passing through the PIT antenna located 5 m downstream of the pheromone, and (G) entered the pheromone activated nest. Responses within a panel (B–G) that share a letter are not significantly different in a generalized linear model with a binomial distribution (Holm-Bonferroni adjusted p value <0.05; See Table S3). Error bars represent standard error.

compound alone (Figure 4). Therefore, we decided to use the synergistic combination of 3sPZS and PZS to develop a possible strategy to inhibit mate searching and spawning in a natural sea lamprey population.

Mixture of 3sPZS and PZS reduces sea lamprey spawning and abundance in a high-density spawning ground

Mature female upstream movement and search of mature males is largely mediated by the male pheromone, whereas spawning is an interaction that involves additional sensory modalities.[17] We predicted disrupting the balanced olfactory inputs of the male pheromone would reduce spawning of a natural adult population. We first assessed the importance of olfaction for sea lamprey mating in Carp Lake Outlet, a stream that attracts over one thousand migrating sea lamprey from Lake Michigan annually.[27] Sexually mature sea lamprey congregate, generate a complex pheromone landscape, and spawn in a small riffle (200 m^2) located 550 m upstream from the confluence with Lake Michigan that can be effectively visually surveyed. Here, we released ovulated females with plugged or unplugged (intact) nares 200 m downstream of free-ranging males in a high-density spawning ground and tracked the LED-tagged females with dim red headlamps for 2.5 h after release at night when spawning activity was elevated[17] to determine the importance of pheromone detection on mating success. Females with plugged nares were less likely to swim upstream ($p < 0.001$), locate male-occupied nests ($p = 0.003$), and spawn ($p = 0.007$, Figure S3) relative to those with unplugged nares. Notably, no females with a plugged naris spawned because no females were observed releasing eggs into the water while males simultaneously released milt.[17] These results indicate olfaction is critical for females to reproduce.

In the same stream, we examined whether the 3sPZS and PZS mixture disrupted females' ability to locate and spawn with free-ranging males to the same extent as occluding the females' nares during two spawning seasons. We evaluated the behavior of released females and the abundance of free-ranging sea lamprey when exposed to vehicle or 3sPZS and PZS as a function of environmental conditions including water temperature, stream discharge, background lamprey pheromone concentration in river water, and treatment application (Figure 5; Table S5). Dye tests confirmed the treatment odorants were thoroughly mixed with the discharge prior to flowing through the

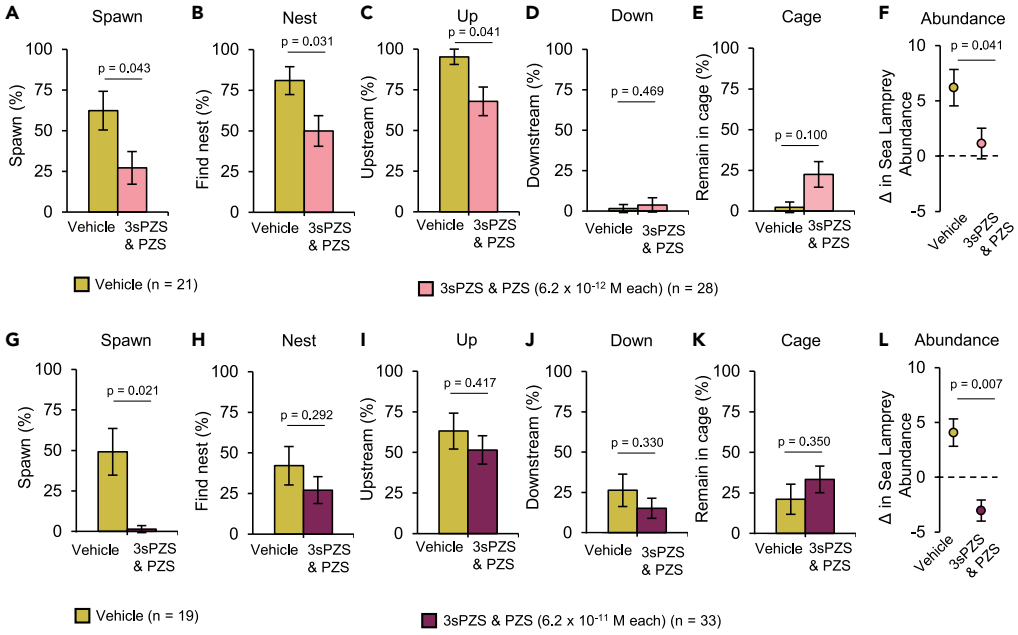

Figure 5. A mixture of 3sPZS and PZS reduced the proportion of released females that spawned and reduced the number of free-ranging sea lamprey occupying the spawning grounds

(A–F) Vehicle (50% methanol) or 3sPZS and PZS (each at 6.2×10^{-12} M) were applied over the sea lamprey spawning grounds in 2019 or (G–L) vehicle or 3sPZS and PZS (each at 6.2×10^{-11} M) in 2020. The results display the percent of the tagged female sea lamprey that (A and G) spawned, (B and H) found a male-occupied nest, (C and I) moved at least 3 m upstream of the release cages, (D and J) moved at least 3 m downstream of the release cages, and (E and K) remained in the release cage at the end of the trial when exposed to vehicle or behavioral antagonist over 3 h at Carp Lake Outlet based on the top ranked model selected by AICc (Table S5). Movements were evaluated with a generalized linear mixed-effect models with a binomial distribution. (F and L) Behavioral antagonist application decreased free-ranging sea lamprey abundance over 3-h trials. The plot shows the mean difference between the total number of free-ranging (untagged) sea lamprey observed before and after trials based on the top ranked linear regression model selected by AICc (Table S6). A positive value indicates the number of sea lamprey increases during a trial. Error bars represent standard error.

spawning area. We observed a 53% reduction in the proportion of females that spawned over the 3-h trial when 3sPZS and PZS (each at 6.2×10^{-12} M) were applied compared to vehicle ($p = 0.022$, Figure 5A). Behavioral antagonist treatment also reduced the proportion of females that swam upstream (−29%, $p = 0.041$) and found a male-occupied nest (−38%, $p = 0.031$, Figures 5B and 5C). In the absence of the behavioral antagonist application, we predicted the relative number of free-ranging sea lamprey would increase over the duration of a trial given their nocturnal tendencies.[28] However, relative to the number of sea lamprey present at the beginning of a trial, we observed fewer free-ranging male and female sea lamprey in the spawning grounds after behavioral antagonist application than after vehicle application ($p = 0.041$, Figure 5F). This observation suggests sea lamprey vacate the spawning grounds when exposed to 3sPZS and PZS.

In the following spawning season approximately one year later, we replicated the experiment in Carp Lake Outlet and increased the concentration of 3sPZS and PZS 10-fold (each at 6.2×10^{-11} M). Notably, increasing the behavioral antagonist concentration reduced spawning by 97% relative to vehicle ($p = 0.021$, Figure 5G). Free-ranging sexually mature males and females were also observed leaving the spawning grounds during behavioral antagonist application at a higher rate ($p = 0.007$, Figure 5L). The combination of the behavioral antagonists disrupted the pheromone communication in a high-density sea lamprey population, ultimately resulting in a dramatic reduction in spawning activities.

DISCUSSION

Our study demonstrates that mating is reduced when the pheromone is mixed with an analog and the ratio of the pheromone components is altered, providing supporting evidence for balanced olfactory antagonism in a vertebrate pheromone communication system. We confirmed 3kPZS and PZS at the ratio naturally released by males optimize the attraction of ovulated females while their structural analog, 3sPZS, alters the ovulated female behavioral responses to 3kPZS but not to PZS. This allowed us to speculate that 3sPZS may impact the female attraction to a male pheromone largely based on the optimal 3kPZS and PZS ratio. Indeed, we found 3sPZS disrupted the attraction to the male pheromone, and when it was added to a pheromone mixture with artificially elevated PZS levels, reduced females' ability to locate mates and subsequently spawn. The mixture of 3sPZS and PZS synergistically perturbed olfactory balance and antagonize female attraction to the male pheromone, and drastically reduced sea lamprey spawning activities and abundance in a high-density natural spawning habitat.

Here, we establish the basis for a tactic that disrupts olfactory-mediated mate finding and subsequently reduces reproduction of invasive sea lamprey.

Behavioral antagonists that diminish the attraction to pheromones are usually analogs of pheromone components.[29] Pheromone components of related sympatric species often act as behavioral antagonists in heterospecifics, enabling the discrimination of similar pheromone mixtures with remarkable olfactory resolution to avoid costly cross-attraction and interspecific mating.[30–32] Within a species, behavioral antagonists can be used to discern maturation status and regulate optimal mating time. Immature female cotton bollworm moths (*Helicoverpa armigera*) emit a relatively high concentration of a pheromone behavioral antagonist that deters male attraction or approach.[33] Similarly, ovulated female sea lamprey use PZS as a behavioral antagonist to avoid orienting toward larvae in favor of spermiated males despite both emitting 3kPZS. Despite the chemical diversity of sex pheromones across taxa including bile acid derivatives for sea lamprey[24] or long-chain hydrocarbons for insects,[34] behavioral antagonists often share high degree of structural analogy with their respective major pheromone component.[35] Minor changes to the functional groups of a pheromone component can generate behavioral antagonists that result in lack of attraction to the sex pheromone. Synthetic analogs of sex pheromone components have been identified as a possible strategy to disrupt sexual communication in pest insects.[35] 3sPZS is an example of a synthetic analog of pheromone components that effectively disrupts the balanced olfactory antagonism, which has the effect of reducing female attraction to mates.

Our study suggests sea lamprey behavioral responses to pheromones in the presence of behavioral antagonists are analogous to those previously described in insects, whose pheromone systems have been extensively characterized.[1] PZS was recently identified as the first described vertebrate pheromone behavioral antagonist.[9] The results from our side-by-side odorant comparisons collectively demonstrate that when the behavioral antagonists are applied, sinuosity in swim tracks and downstream movement increases. Similar behaviors were observed in other studies when females lost the pheromone signal.[9,15] Moreover, behavioral antagonist application increased the proportion of females that remained in the release cage, failing to even orient toward the pheromone and initiate upstream movement. These changes are consistent with those recorded in moths that fail to take flight,[36,37] display frequent lateral casting, or regress downwind[38,39] as a result of a reduced ability to track an attractive sex pheromone plume when exposed to a pheromone behavioral antagonist. The olfactory mechanisms whereby PZS and 3sPZS alter the female responses to 3kPZS remain elusive. In moths and other insect groups, numerous neural substrates or circuits in the olfactory pathways, ranging from chemoreceptors and olfactory sensory neurons to higher centers, may impart or disturb balanced olfactory antagonism.[4] In the sea lamprey, two highly related odorant receptors (ORs), OR320a and OR320b, show similar responses to PZS and 3kPZS.[40] It is possible the sea lamprey olfactory epithelium expresses other receptors that either respond only to PZS or 3kPZS and/or respond differently to PZS and 3kPZS. 3sPZS may be a full or partial receptor agonist or receptor antagonist for these known and hypothesized receptors. If found, activation of these receptors by PZS and 3sPZS could result in the aversive behaviors observed.

We found 3sPZS and PZS together reduced the attraction to the sex pheromone more than would be predicted from an additive effect of each behavioral antagonist. A benefit of the synergistic action of 3sPZS and PZS is that each compound can be applied at low concentrations ($\sim 10^{-11}$ M) and still have striking behavioral influences on natural spawning populations. If 3sPZS and PZS are used in sea lamprey control, these behavioral antagonists are likely to have minimal effects on species that inhabit lamprey spawning grounds.[41] 3kPZS, PZS, and 3sPZS are 5α-bile acids or analogs. The EOG concentration response recordings of 3kPZS, PZS, and 3sPZS show steep increases in the olfactory responses with increasing concentrations and low detection thresholds ($<10^{-12}$ M), which are dynamics often observed when an odorant is interacting with one or more specific receptors.[42] While lamprey olfactory receptors appear to be specially tuned to detect 5α-bile acids,[40] jawed fishes primarily release and detect 5β-bile acids,[43] suggesting the olfaction of jawed fishes is unlikely to be disrupted by the 5α-behavioral antagonists.

The sea lamprey spawning season is short and relatively predictable,[17] enabling behavioral antagonist application to coincide with the spawning activity. They often spawn in shallow rivers, in which the flow restricts the pheromone plume in a two-dimensional space unlike airborne insect pheromone plumes in a three-dimensional space. Therefore, the spatial dynamics of lamprey pheromones are more stable and trackable than insect pheromone systems. Within this context, data from our experiments in a high-density spawning environment showed 3sPZS mixed with PZS impact female behaviors effectively in the spawning ground, including reduced searching for a mate, nesting, and spawning, and rendering the pheromone system ineffective through not only physical occlusion of the naris but also disruption of the olfactory balance. Previous work has found naris-plugged ovulated females were unable to locate spermiated males in a stream.[44] In this study, 3sPZS and PZS also reduced free-ranging sea lamprey abundance in the spawning grounds, suggesting a possible compounding effect in which emigration from a system increases, resulting in a decrease in background pheromone levels and an increase in behavioral antagonists to pheromone ratios. Consequently, this would likely further reduce recruitment rates for females in the treated spawning habitat. The possibility of this feedback loop should be further examined with frequent measurement of pheromone concentrations and monitoring animal emigration in the study stream.

Advances in the understanding of the sea lamprey pheromone communication system have established a framework to develop a control strategy that exploits sea lamprey reliance on pheromones to reduce reproduction.[24,45] There is precedent for manipulation of sea lamprey pheromone communication through the application of 3kPZS during management scenarios across the Great Lakes Basin.[46] Here, we assessed the feasibility of using 3sPZS and PZS to alter female behavioral responses to a 3kPZS or male pheromone baited nest in natural habitats. We anticipate 3sPZS and PZS could be applied in streams with characteristics that maximize the efficacy of behavioral antagonists as part of the sea lamprey supplemental control initiative.[41]

In summary, 3sPZS and PZS remarkably disrupt the sea lamprey male pheromone communication system by perturbing the olfactory balance imparted by the male pheromone, evident by a precipitous reduction in female attraction to male pheromone-activated nests in a

stream environment and diminished spawning activities of a high-density natural population during periods of peak activity.[28] Our results represent a step forward in understanding the potential behavioral implications of disrupting the balanced olfactory antagonism in a vertebrate pest. Adaptation of this approach may offer additional tactics to control invasive sea lamprey in the Laurentian Great Lakes.[41]

Limitations of the study

While the present study reveals 3sPZS and PZS perturb the olfactory balance and reduce spawning of sea lamprey, there are important limitations that should be considered. Previous work found the mixture of 3kPZS and PZS at the ratio of the natural blend of male pheromone (100:1, 3kPZS: PZS) replicates the male pheromone in inducing maximal attraction of females.[9] We assumed that this ratio represents an optimal ratio and tested the prediction that disrupting it reduces the attraction. Our assumption may be further validated by additional behavioral experiments on a wider variety of 3kPZS: PZS ratios, which may provide additional insights into the mechanisms underlying the observed behaviors. The behavioral antagonist application to the spawning grounds occurred over 3-h long trials during their peak spawning period in one system, although experiments were replicated over two consecutive spawning seasons. From our results, we cannot conclude whether the behaviors we observed would continue over the course of a sustained pheromone behavioral antagonist application. During these trials, we evaluated the behaviors of introduced tagged females the night of their release. We did not collect detailed behavioral observations on free-ranging female sea lamprey and therefore cannot make conclusions on all behavioral implications of pheromone antagonists on wild populations. However, survey data show there were fewer free-ranging individuals in the spawning area after behavioral antagonist application, implying the antagonist has similar behavioral impacts on free-ranging and released individuals. Future studies assessing the effects of behavioral antagonists in other riverine contexts over longer durations with free-ranging (non-released) sea lamprey populations are warranted.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Animals
- METHOD DETAILS
  - Electro-olfactogram (EOG) setup and recording
  - Two-choice flume assay
  - Side-by-side odorant choice assay in-stream
  - Collection of full male sex pheromone
  - Antagonist enshroudment of male sex pheromone
  - Antagonist application to spawning grounds
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107744.

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AUTHOR CONTRIBUTIONS

A.M.S., N.S.J., M.J.S., and W.L. designed research; A.M.S. and N.S.J. performed research; A.M.S. analyzed data; A.M.S. and W.L. wrote the original draft; A.M.S., N.S.J., M.J.S., and W.L. reviewed and edited; and W.L. coordinated the overall study.

DECLARATION OF INTERESTS

The authors declare no competing interest.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ethyl 3-aminobenzoate	Sigma-Aldrich	Cat#E10505; CAS: 582-33-2
Gallamine triethiodide	Sigma-Aldrich	Cat#G8134; CAS: 65-29-2
3-keto petromyzonol sulfate (3kPZS)	Bridge Organics	CAS: 435327-06-3
Petromyzonol sulfate (PZS)	Bridge Organics	CAS: 1271318-61-6
Petromyzonol tetrasulfate (3sPZS)	Bridge Organics	N/A
L-Arginine	Sigma-Aldrich	Cat#A5006; CAS: 74-79-3
Fluorescent Fwt Red Dye Concentrate	Cole-Parmer	Cat#UX-00298-06
5-deuterated 3kPZS [$^{2}H_{5}$] internal standard	Bridge Organics	N/A
Deposited data		
All data	This paper	Mendeley Data repository: https://doi.org/10.17632/k6h7vz6dvy.1
All related code	This paper	Zenodo Data repository: https://doi.org/10.5281/zenodo.7412008
Experimental models: Organisms/strains		
Sea lamprey	U.S. Fish & Wildlife Service	N/A
Software and algorithms		
AxoScope (version 10.4)	Molecular Devices LLC	http://mdc.custhelp.com/app/home
R (version 4.1.0)	R.Core Team	https://www.r-project.org/
R package *multcomp* (version 1.4.22)	Hothorn et al.[47]	https://cran.r-project.org/web/packages/multcomp/index.html
R package *effects* (version 4.2.2)	Fox and Weisberg[48]	https://cran.r-project.org/web/packages/effects/index.html
R package *brglm* (version 0.7.2)	Kosmidis and Firth[49]	https://cran.r-project.org/web/packages/brglm/index.html
R package *lme4* (version 1.1.26)	Bates et al.[50]	https://cran.r-project.org/web/packages/lme4/index.html
R package *bbmle* (version 1.0.20)	Bolker[51]	https://cran.r-project.org/web/packages/bbmle/index.html
Other		
EOG recording electrode holder with Ag/AgCl pellet	Warner Instruments	Cat#ESP-M15N
EOG reference electrode holder with Ag/AgCl pellet	Warner Instruments	Cat#E45P-F15NH
EOG NeuroLog amplifier	Digitimer Ltd.	Cat#NL102
EOG Low-pass 60 Hz filter	Digitimer Ltd.	Cat#NL125
EOG Digitizer	Molecular Devices LLC	Cat#Digidata 1440A
Masterflex L/S peristaltic pump	Cole-Parmer	Cat#EW-07554-90
23 mm half-duplex PIT tag	Oregon RFID	N/A
Multiple antenna PIT tag reader multiplexor	Oregon RFID	N/A
Manual tuner PIT antenna box	Oregon RFID	N/A
Colored streamer tags	Hallprint	PST2S

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Portable flow meter	Marsh-McBirney	Cat#Flo-Mate 2000
Cyclops 7 Submersible fluorometer sensor	Turner Designs	Cat#2100-000
DataBank Handheld Datalogger	Turner Designs	Cat#2900-005
SternVantage Quick Light Body dental impression gel	Sterngold Dental	Cat#220101
Nite Brite LED	Thill	N/A
Xevo TQ-S triple quadrupole mass spectrometer	Waters	N/A
Oasis MCX cartridge for solid phase extraction, 6cc, 500 mg sorbent, 60 μm	Waters Corp.	Cat#186000776

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Weiming Li (liweim@msu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data have been deposited at Mendeley Data and are publicly available as of the date of publication. DOIs are listed in the key resources table.
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Michigan State University's Institutional Animal Use and Care Committee approved all procedures involving sea lamprey (Animal use forms: 03/12-063-00, 12/14-223-00, 03/11-053-00, 05/09-088-00, and 02/17-031-00). All experiments conform to the Committee's regulatory standards. Pre-spawning adult sea lamprey were captured by the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada with scientific collection permits. Sea lamprey were transported to the U.S. Geological Survey, Hammond Bay Biological Station (HBBS), Millersburg, MI and held in 200–1000 L flow-through tanks supplied with aerated, ambient Lake Huron water. Pre-spawning adult sea lamprey used for EOG recordings (253.6 g ± 16.7, 507.5 mm ± 14.6; mean ± SEM) were transported to Michigan State University, East Lansing, MI, USA and held in 250 L flow-through tanks supplied with aerated water maintained at 7°C to 9°C in May – June 2013, 2015–2018. All behavioral experiments used sexually mature male (spermiated) or female (ovulated) sea lamprey in June – August 2013–2020. Pre-spawning adult sea lamprey were held in the lower Ocqueoc River, Presque Isle County, MI and assessed daily for expression of gametes.[17,52] Once sexually mature, sea lamprey were returned to HBBS and held in 200 L aerated tanks until experimentation. The influence of sex was explicitly assessed in EOG recordings and three treatments in the two-choice flume experiments. All remaining experiments used only female sea lamprey because our study focused on characterizing the effects of the behavioral antagonists on female responses to the male sex pheromone.

METHOD DETAILS

Electro-olfactogram (EOG) setup and recording

EOG recordings followed previously established procedures[23,53] to measure olfactory responses after exposure to odorants in May – June 2013, 2015–2018. Sea lamprey were anesthetized with ethyl 3-aminobenzoate (100 mg L^{-1}, MS222) and immobilized with an injection of gallamine triethiodide (30 mg kg^{-1} of body weight). Gills were irrigated with aerated water containing 50 mg L^{-1} MS222. The olfactory lamellae were exposed and the differential EOG response was recorded using glass capillaries filled with 0.4% agar in 0.9% saline that were connected to solid state electrodes in 3 M KCl. EOG signals were amplified by a NeuroLog system, filtered with a low-pass 60 Hz filter, digitized, and recorded on a computer running AxoScope (version 10.4).

To record odorant concentration-response dynamics, 10^{-3} M stock solutions of 3sPZS, 3kPZS, and PZS in water/methanol (1:1, v/v) were prepared, stored at −20°C until experimentation, and then serially diluted with filtered water resulting in 10^{-12} M to 10^{-6} M solutions. A 10^{-2} M stock solution of L-arginine in deionized water was prepared, stored at 4°C, diluted to10^{-5} M, and applied to the olfactory epithelium for 4 s. The response was recorded to normalize for variations in olfactory sensitivity among individual sea lamprey. The olfactory epithelium was flushed with filtered water for 2 min, the blank control introduced, and the response recorded. The test stimulus (either 3sPZS, 3kPZS, or PZS) starting at 10^{-12} M working toward 10^{-6} M was then applied in $\log_{10}$ increments of molar concentration, recorded, and flushed. A response to the blank control and 10^{-5} M L-arginine standard were measured repeatedly after approximately every three concentrations of stimuli throughout each recording session. The EOG response magnitudes were measured in millivolts (mV). The normalized EOG response was calculated as,

$$\text{Normalized EOG Amplitude} = \frac{Rt - Rb}{Ra - Rb}$$

where Rt is the response magnitude to the test stimulus, Rb is the response magnitude to the blank, and Ra is the response magnitude to L-arginine.

To evaluate whether 3sPZS reduced the 3kPZS olfactory response to a similar extent in males and females, we measured the 3kPZS EOG responses before and during exposure of the olfactory epithelium to 3sPZS following established approaches.[23] The EOG responses to 3kPZS 10^{-8} M, L-arginine 10^{-5} M, and blank were recorded. The naris was continuously exposed to 3sPZS 10^{-6} M for 2 min. Next, the EOG responses to a mixture of 3sPZS 10^{-6} M and 3kPZS 10^{-8} M was recorded. The naris was rinsed with for 2 min, and the responses to 3kPZS, L-arginine, and blank were measured. The change in 3kPZS olfactory response during exposure to 3sPZS was calculated as the ratio of the 3kPZS response during versus before 3sPZS exposure, defined as the,

$$\text{Ratio of EOG Amplitude} = \left(\frac{R_{during} - Rb}{Ra - Rb}\right) \Big/ \left(\frac{R_{before} - Rb}{Ra - Rb}\right)$$

where R_{during} is the response to the mixture of 3sPZS 10^{-6} M and 3kPZS 10^{-8} M, R_{before} is the initial response to 3kPZS 10^{-8} M, Rb is the response magnitude to the blank, and Ra is the response magnitude to L-arginine10^{-5} M. A ratio of EOG amplitude value of 1.0 indicates the 3sPZS did not reduce the olfactory response of 3kPZS, whereas a value of 0 indicates the 3sPZS reduced 100% of the olfactory response of 3kPZS. The experiment and analysis were repeated to determine if exposure to 3sPZS 10^{-6} M reduced olfactory responses to L-arginine 10^{-6} M, a non-pheromone control stimulus that elicits olfactory responses.

Two-choice flume assay

We used two-choice flumes to assay the behavioral responses of ovulated female, pre-ovulated female, and spermiated male sea lamprey to test stimuli[9] in June – August 2013–2015 and 2018 using water from the Little Ocqueoc River (Presque Isle County, MI, USA) and in June 2023 using water from the upper Trout River (Presque Isle County, MI, USA). A sea lamprey was placed in an acclimation cage at the downstream end of the flume for 5 min, released, and the cumulative time the sea lamprey spent in each channel was recorded for 10 min (pre-treatment period before odorant application). Then, the test stimulus was introduced to a randomly chosen channel and vehicle to the other at 200 ± 5 mL min^{-1} using a peristaltic pump for 5 min. Then, the cumulative time the sea lamprey spent in each channel was recorded for 10 min while continuing to apply the treatment (odorant application period). The time spent in the control (Bc) and experimental (Be) channel before odorant application and in the control (Ac) and experimental (Ae) channel after odorant application were used to calculate a preference index[16] for each trial as defined by,

$$\text{Preference Index} = \frac{Ae}{(Ae+Be)} - \frac{Ac}{(Ac+Bc)}$$

which assessed the change in behavior following odorant application.

Side-by-side odorant choice assay in-stream

We applied odorants to side-by-side artificial nests to evaluate if 3sPZS or a mixture of 3sPZS and PZS disrupted ovulated female search for a 3kPZS activated nest in a stream. Trials were conducted in June – July 2016 and 2017 from 07:00 h to 12:00 h in a 50 m stretch of the upper Ocqueoc River (Presque Isle County, MI, USA), a historical spawning ground with good nesting habitat but no pheromone background because a migration barrier restricts sea lamprey from accessing the upper reaches. The field site and methods were modified from previous studies that characterized behavioral responses to sea lamprey pheromones.[9]

Ovulated female preference for odorants applied to the artificial nests was monitored using passive integrated transponder (PIT) array. Before a trial, 10–15 ovulated females were each fitted with a 23 mm half-duplex PIT tag and two unique colored streamer tags. Females were acclimated in the river for at least 8 h in cages positioned 50 m downstream of the two nests. Each nest was surrounded by a 1 m^2 PIT antenna. A PIT antenna was also placed across the stream channel 3 m below the release cage to track downstream movement and across the stream channel 3 m above the release cage to track upstream movement. All PIT antennas were wired to a multiple antenna multiplexor and data logger. Test odorants (2016: 5×10^{-13} M 3kPZS or 5×10^{-13} M 3kPZS mixed with 5×10^{-13} M 3sPZS; 2017: 5×10^{-13} M 3kPZS or 5×10^{-13} M 3kPZS mixed with 5×10^{-12} M PZS and 5×10^{-12} M 3sPZS) were diluted in bins with river water and pumped into each nest

(167 ± 5 mL min^{-1}). The treatments that were applied to each nest were alternated each trial. The amount of odorant needed to activate the stream to the target concentration was calculated using stream discharge estimates measured with a portable flow meter. Trials began with a 30-min pre-release treatment application, females were released, and observed for 90 min via the PIT array and visual observations while treatment application continued. Swim tracks of each individual observed based on the attached colored stream tags were recorded onto scaled maps and later compiled onto a digital map. The parameters recorded were the proportion of released females that 1) remained in the release cage at the end of the trial, 2) swam 3 m or more downstream of the release cage and stayed downstream of the release cages for the duration of the trial, 3) swam 3 m or more upstream from release cages and stayed upstream of the release cages for the duration of the trial, and the proportion of the females that moved upstream that entered the 4) experimental odorant nest or 5) control odorant nest.

The odorant dispersion from the nest was simulated with rhodamine (Rhodamine Red, Cole-Parmer)[15] applied at a nest for 30 min, and then sampled at 10 evenly spaced points along each fixed transect during active administration of dye using Cyclops 7 submersible fluorometer sensor affixed with DataBank. The river flushed for 1 h, the absence of dye was confirmed, and then repeated from the nest. The values were compiled to determine the relative concentration of the odorant plume within the field site.

Collection of full male sex pheromone

Water conditioned with spermiated males, referred to herein as full pheromone, was collected and used as a test odorant. Twenty spermiated males were placed in 80 L of aerated, deionized water for 8 h, after which 50 mL of water was sampled, and immediately spiked with a 5-deuterated 3kPZS [$^2H_5$] internal standard to reach a concentration of 1 μg L^{-1}. Triplicate 1 mL subsamples were collected, freeze dried, reconstituted in 100 μL water/methanol (1:1, v/v), and subjected to liquid chromatography-tandem mass spectrometry for quantification of 3kPZS.[54] The remaining volume of the male pheromone was thoroughly mixed, aliquoted, and stored at −20°C until experimentation. The male pheromone was later applied to the flume or stream by thawing the necessary volume and diluting with river water to achieve the desired 3kPZS concentration in the flume (10^{-12} M or 10^{-14} M) or stream (5×10^{-13} M).

Antagonist enshroudment of male sex pheromone

Ovulated female behavioral responses to a pheromone activated nest enshrouded with behavioral antagonist followed the methods from the side-by-side odorant choice assay in-stream with modification. Trials were conducted in June – August 2019 from 07:00 h to 12:00 h in the upper Ocqueoc River. We applied full male pheromone normalized to contain 5×10^{-13} M 3kPZS, or river water at the pheromone nest with a peristaltic pump. At the behavioral antagonist source, we applied either 1×10^{-10} M 3sPZS (equivalent of 200× the concentration of 3kPZS in the male pheromone), 1×10^{-10} M PZS (equivalent of 200× the concentration of 3kPZS in the male pheromone), 5×10^{-11} M 3sPZS and 5×10^{-11} M PZS (each equivalent of 100× the concentration of 3kPZS in the male pheromone), or vehicle (water/methanol,1:1, v/v). After a 15-min treatment application, we released tagged females from cages positioned 200 m downstream of the pheromone nest and monitored their movement with a PIT array and visual observations for 75 min while continuing to apply the treatment. PIT antennas were located across the stream channel 3 m below the release cage to track downstream movement, across the stream channel 3 m above the release cage to track upstream movement, 5 m downstream of the pheromone nest (1 m × 2 m) to track female movement as they approached the pheromone nest, and around the pheromone the pheromone nest (1 m^2) to track entry and retention. The behavioral antagonist source was positioned 7 m upstream of the pheromone nest such that the plume from the antagonist source enshrouded the 1 m^2 pheromone nest and the pheromone plume downstream. The pheromone plume passed through the center of the pheromone approach antenna. We extensively confirmed the behavioral antagonist and pheromone plume dynamics with rhodamine dye tests to characterize the odorant dispersion.

Antagonist application to spawning grounds

We evaluated if behavioral antagonists reduced spawning activities of ovulated females released in natural spawning grounds with nesting males in Carp Lake Outlet (Emmett County, MI, USA). This 550 m stretch of Carp Lake Outlet contained a dense sea lamprey spawning ground with optimal gravel beds. Sea lamprey concentrate below a barrier in the system. Trials were conducted in June – July 2019 and 2020 from 22:00 h to 01:00 h.

The treatments were diluted in bins with river water and applied with peristaltic pump (1000 ± 25 mL min^{-1}) affixed to a diffuser bar located 15 m upstream of the barrier and centered bank to bank. The test articles applied in 2019 were either vehicle (water/methanol,1:1, v/v) or a mixture of 6.21×10^{-12} M PZS and 6.21×10^{-12} M 3sPZS and in 2020 were either vehicle (water/methanol,1:1, v/v) or a mixture of 6.21×10^{-11} M PZS and 6.21×10^{-11} M 3sPZS. The amount of test article needed to activate the stream to the target concentration was calculated using stream discharge estimates measured with a portable flow meter. Several rhodamine dye tests were performed to confirm the behavioral antagonists mixed thoroughly with the discharge prior to flowing through the sea lamprey spawning area.

Each trial included 5–8 ovulated females (depending upon availability), each fitted with a LED light in a latex sleeve that was sutured to the dorsal region and streamer tags to enable individual visual identification. Previous research found naris-plugged ovulated females did not mate when released near actively spawning sea lamprey.[44] To examine whether olfaction is essential to coordinate spawning, a subset of females were naris-plugged in 2019 by filling the nasal cavity with dental impression gel to occlude the nasopharyngeal opening or the tip a 10 μl pipette was inserted into the opening of a sham group of females to control for handling stress or olfactory irritation from the naris plugging.[44] Females were acclimated in the river at least 2 h prior to the start of the trial. While females were acclimating, we conducted a survey to locate and count the number of spawning nests and number of free-ranging (non-released) male and female sea lamprey. We

conducted an additional survey at the end of each trial. At the beginning of each trial, we collected duplicate 100 mL of river water samples in the middle of the water column 5 m upstream of female release cage and spiked each with 5 ng of a 5-deuterated 3kPZS [$^2H_5$] internal standard to estimate the concentration of 3kPZS following validated methods.[55] Samples were frozen at −20°C until they were later filtered, subjected to solid phase extraction, eluted, freeze-dried, dissolved in 1 mL of methanol, and centrifuged (13,800 × *g* for 15 min at 4°C). Supernatants were collected, freeze-dried, reconstituted in 100 μL methanol/water (1:1, v/v), and lamprey bile acids (3kPZS, DkPES, and PZS) were quantified with liquid chromatography-tandem mass spectrometry.[55] After females acclimated for at 2 h, the test articles were applied for a 30-min pre-release period and then the females were released and observed for 2.5 h. The trials started at dusk because spawning activity increases substantially at nightfall.[17] If a female exited the release cage, the movement and behavior of each female were recorded on a scaled map. The cage was closed when each researcher was observing a released female. The cage was re-opened when a female went downstream and exited the river or when a female became inactive (no movement for >15 min). The parameters recorded were the proportion of females that (1) remained in the release cage, (2) swam downstream of the release cage, (3) swam upstream of the release cage, (4) found a male-occupied nest, and (5) spawned.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed in R version 4.1.0. Data in plots represent the mean and standard error of the mean (SEM) denoted by the error bars. For each experiment, n refers to the number of animals and is stated in each Figure, Figure legend, or corresponding Supplementary Table. All tests were two-sided unless otherwise noted. Nonparametric tests were used when variance was dissimilar between groups. Treatments were considered statistically significant when $p < 0.05$ unless stated otherwise. Statistically significant differences are depicted using different letters for multi-comparison tests. For pairwise comparisons, p-values are displayed within the Results, Figures, or corresponding Supplementary Tables.

The EOG detection threshold of each odorant, defined as the lowest concentration in which the test stimuli elicited a larger response than the blank, was evaluated with a paired t-test (one tailed with a Bonferroni adjustment, $\alpha = 0.025$).

For two-choice flume assays, a trial was discarded if the sea lamprey failed to enter the control and experimental channel for at least 10 s during the 10-min period before the odorant was applied as this was an indication of strong side bias or inactivity. A preference index for each test stimulus was evaluated using a Wilcoxon signed-rank test to determine if the index was significantly different from zero. A significant positive value of the preference index indicated attraction and a significant negative preference index indicated aversion.

Generalized linear models with binomial distributions were used to evaluate treatment effects in the Ocqueoc River. For side-by-side odorant choice experiments, we examined the proportion of released females that remained in the release cage, swam downstream, swam upstream, and entered the experimental odorant nest. For behavioral antagonist enshroudment of pheromone experiments, we examined the proportion of females that remained in the release cage, swam upstream, swam downstream, remained between upstream antenna and channel choice, entered the treatment channel, approached the pheromone nest, and entered the pheromone nest. When more than 2 treatment groups were compared, we used multiple comparison contrasts with a Holm-Bonferroni p value adjustment (*multcomp* package, version 1.4.22).[47] A paired analysis of nest retention was analyzed with a Wilcoxon signed rank test. To determine whether the effects of 3sPZS and PZS on the behavioral responses to the male pheromone were additive, the expected behavioral responses to the pheromone enshrouded with 5×10^{-11} M 3sPZS and 5×10^{-11} M PZS (equivalent of 100× the concentration of 3kPZS in the male pheromone) were calculated for each response parameter. The expected values were calculated by adding the intercept to the mean of 1×10^{-10} M 3sPZS effect estimate and the 1×10^{-10} M PZS effect estimate identified in the generalized linear model with binomial distribution describing each response parameter. Differences between the expected responses if behavioral antagonist effects were additive and observed responses were evaluated with a Chi-squared test.

To determine if behavioral antagonist application influenced the behavior of released ovulated females and free-ranging (non-released) sea lamprey abundance in natural spawning grounds, three analyses were conducted. Trials were discarded if no free-ranging males were observed during the pre-trial survey to ensure there was a possibility for the released females to find a male-occupied nest and mate (2019: 0 trials; 2020: 2 trials). Values used in all plots were obtained with the *effects* package (version 4.2.2).[48] Analysis 1: To assess whether olfaction is essential to coordinate spawning, the proportion of females that remained in the release cage, swam downstream of the release cage, swam upstream of the release cage, found a male-occupied nest, and spawned during the 3-h vehicle trials in 2019 as a function of naris status (unplugged or plugged) were evaluated as generalized linear models with binomial responses with a bias-reduction method[56] to adequately handle zero-inflated data, using the *brglm* package (version 0.7.2).[49] Analysis 2: Behavioral responses of females within each parameter were assessed within each year because the majority of the female behavioral response parameters (3 out of 5) analyzed were significantly different during vehicle control trials in 2019 compared to those in 2020 (Fisher's exact test, Spawn $p = 0.205$; Nest $p = 0.021$; Upstream $p = 0.017$; Downstream $p = 0.085$; Remain in Cage $p = 0.042$). Accordingly, trials in 2019 and 2020 were analyzed separately for analyses 2 and 3. The behavioral responses of each female (remained in the release cage, swam downstream of the release cage, swam upstream of the release cage, found a male-occupied nest, and spawned during the 3-h trial) were evaluated as generalized linear mixed-effect models with a binomial distribution in the *lme4* package (version 1.1.26).[50] Models were fit with covariates related to environmental conditions including water temperature, stream discharge, background lamprey pheromone concentration in river water (3kPZS, DkPES,

and PZS), treatment application (vehicle, 3sPZS and PZS each at 6.2×10^{-12} M, or 3sPZS and PZS each at 6.2×10^{-11} M), and Julian date as a random effect. The proportion of females that remained in the release cage in 2019 was evaluated with a bias-reduction binomial generalized linear model (*brglm* package; version 0.7.2) to account for zero-inflated data, though Julian date was not included as these models do not include random effects.[49] Models were ranked using AICc in the *bbmle* package (version 1.0.20).[51] Analysis 3: The change in free-ranging sea lamprey abundance was evaluated as a linear regression model for each year. Models were fit with covariates including water temperature, stream discharge, background lamprey pheromone concentration in river water (3kPZS, DkPES, and PZS), and treatment application (vehicle, 3sPZS and PZS each at 6.2×10^{-12} M, or 3sPZS and PZS each at 6.2×10^{-11} M) and ranked using AICc in the *bbmle* package (version 1.0.20).[51]