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Supplementary Information for

A pheromone antagonist liberates female sea lamprey from a sensory trap to enable reliable communication

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Other supplementary materials for this manuscript include the following:

A file containing all data discussed in the paper.

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SUPPLEMENTAL METHODS

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). Sea lamprey were provided by the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada, except for the spermiated males sampled for pheromone release which were captured by hand off natural spawning nests in the Cheboygan River, Cheboygan County, MI. Sea lamprey for behavioral experiments were held in aerated tanks supplied with ambient Lake Huron water (6 to 19 °C) at the U.S. Geological Survey, Great Lakes Science Center, Hammond Bay Biological Station (HBBS), Millersburg, MI, USA. Sea lamprey used for EOG recordings which were transported to the University Research Containment Facility at Michigan State University, East Lansing, MI, USA and held in flow-through tanks supplied with aerated, chilled well water maintained at 7 to 9 °C. All behavioral experiments used sexually mature males (spermiated) or females (ovulated); pre-spawning sea lamprey were held in the lower Ocqueoc River, Presque Isle County, Michigan to induce maturation, which was assessed daily by visual inspection of secondary sex characteristics and gentle expression of gametes (1, 2).

Electro-olfactogram (EOG) setup and recordings

EOG recordings followed established procedures (3, 4). In brief, sea lamprey (276.1 g ± 24.2, 511.2 mm ± 14.9; mean ± se) were anesthetized with 3-aminobenzoic acid ethyl ester (100 mg L^{-1}, MS222, Sigma-Aldrich, St. Louis, MO, USA) and immobilized with an injection of gallamine triethiodide (30 mg kg^{-1} of body weight, Sigma-Aldrich). Gills were continuously irrigated with aerated water containing 50 mg L^{-1} MS222 throughout the experiment. The olfactory lamellae were exposed and the differential EOG response was recorded using glass capillary borosilicate electrodes filled with 0.4% agar in 0.9% saline and connected to solid state electrodes with Ag/AgCl pellets (model ESP-M15N, Warner Instruments LLC, Hamden, CT, USA) in 3 M KCl. EOG signals were amplified by a NeuroLog system (model NL102, Digitimer Ltd., Hertfordshire, England, UK), filtered with a low-pass 60 Hz filter (model NL125, Digitimer Ltd.), digitized (Digidata 1440A, Molecular Devices LLC, Sunnyvale, CA,

USA), and recorded on a computer running AxoScope 10.4 software (Molecular Devices LLC).

For the concentration-response recordings, 10^{-3} M stock solutions of 3kPZS and PZS (Bridge Organics Co., Vicksburg, MI, USA) in water/methanol (1:1, v:v) were prepared, stored at -20 °C, and then serially diluted with filtered water to yield 10^{-10} M to 10^{-6} M solutions. Stock solutions of 3kPZS, PZS, and the free base form of L-arginine were prepared up to 6 weeks prior to testing. A 10^{-2} M stock solution of L-arginine (Sigma-Aldrich) in deionized water was prepared, stored at 4 °C, and diluted with filtered water to yield a 10^{-5} M solution. Serial dilutions were performed each day of experimentation. The pH of the blank control was 7.01, 10^{-5} M L-arginine was 7.01, 10^{-6} M 3kPZS was 7.02, and 10^{-6} M PZS was 7.01. A 10^{-5} M L-arginine solution was introduced to the olfactory epithelium for 4 s at approximately 70 µL s^{-1}, and the response was recorded to correct 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. Test stimuli starting from 10^{-10} M to 10^{-6} M were then applied in $\log_{10}$ increments of molar concentration, recorded, and flushed. Blank control and 10^{-5} M L-arginine standard were measured repeatedly (approximately after 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 Normalized EOG Amplitude = (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 10^{-5} M L-arginine.

To determine how the olfactory response to 3kPZS or L-arginine changed when the olfactory epithelium was saturated with increasing concentrations of PZS, we first recorded a baseline EOG response to the test stimulus (10^{-6} M 3kPZS or 10^{-5} M L-arginine) before introducing PZS. Next, the olfactory epithelium was saturated with 10^{-10} M PZS for 2 min. Then, the response to a mixture of 10^{-10} M PZS and 10^{-5} M L-arginine was recorded. The olfactory epithelium was flushed was charcoal filtered water, saturated with 10^{-10} M PZS for 2 min, and the response to a mixture of 10^{-10} M PZS and 10^{-6} M 3kPZS was recorded. The olfactory epithelium was flushed with charcoal filtered water for 2 min, and responses to the blank control, 10^{-5} M L-arginine, 10^{-6} M 3kPZS, and 10^{-6}

M PZS were recorded to ensure recovery of the olfactory epithelium before proceeding to exposure to 10^{-9} M PZS. In a similar manner, EOG responses were recorded to increasing concentrations of PZS from 10^{-10} M to 10^{-6} M.

The change in the olfactory response of 3kPZS or L-arginine during exposure to increasing concentrations of PZS was calculated as the percent of the initial non-adapted response, defined as the Percent Initial Response = $(R_{adapt} – blank) / (R_{non-adapt} – blank)] * 100$. For L-arginine, R_{adapt} was the response to the mixture of PZS (10^{-10} M to 10^{-6} M) and 10^{-5} M L-arginine relative to the initial response to10^{-5} M L-arginine ($R_{non-adapt}$). For 3kPZS, R_{adapt} was the response to the mixture of PZS (10^{-10} M to 10^{-6} M) and 10^{-6} M 3kPZS relative to the initial response to10^{-6} M 3kPZS ($R_{non-adapt}$). If PZS and the stimulus (3kPZS or L-arginine) share the same receptors, adapting the olfactory epithelium to PZS should result in an observed decrease in the EOG response during adaptation compared to before adaptation. A value of 100% initial response for the mixture of PZS and 3kPZS indicates the olfactory response of 3kPZS is the same magnitude before and during exposure of PZS, suggesting PZS does not influence the 3kPZS olfactory response. We used a one-way repeated measures ANOVA to determine if the olfactory responses to the test stimulus (3kPZS or L-arginine) was different across PZS adapting solution concentrations (10^{-10} M to 10^{-6} M). This experiment and analysis was repeated with exposure to increasing concentrations of 3kPZS as adapting solutions (10^{-10} M to 10^{-6} M) and 10^{-6} M PZS or 10^{-5} M L-arginine as the test stimulus.

Two-choice flume assay

We used previously described two-choice flume assays to evaluate behavioral responses of ovulated female and spermiated male sea lamprey to natural and synthesized odorants (4). Experiments were conducted in June and July 2013 – 2018 using water from the Little Ocqueoc River in Presque Isle County, MI, USA. A sea lamprey was introduced into an acclimation cage at the downstream end of the flume for 5 min. The sea lamprey was released and the cumulative amount of time the sea lamprey spent in each channel was recorded for 10 min (pre-treatment period before odorant application). The test stimulus was introduced to a randomly chosen channel and vehicle to the other at constant rates of 200 ± 5 mL min^{-1}. The test stimulus and vehicle were pumped into the

flume for 5 min. The cumulative amount of time the sea lamprey spent in each channel was then recorded for 10 min while application of the test stimulus and vehicle continued (odorant application period). The flume was flushed with water for 10 min before the start of the next experiment to remove any remaining test stimulus. A 10-minute flushing period was deemed a sufficient duration in previous experiments and confirmed with a rhodamine dye test. The 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. 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 for each trial as defined by Preference Index = [Ae/(Ae+Be) – Ac/(Ac+Bc)]. The preference index was evaluated using a Wilcoxon signed-rank test to determine if the index was significantly different from zero at $P < 0.05$. A significant positive value of the preference index indicated attraction, a significant negative preference index indicated repulsion, and a non-significant preference index indicated no response.

The concentrations of 3kPZS and PZS in natural odorants collected for two-choice flume assays were 13.38 ng ml^{-1} and 58.69 ng ml^{-1} for larvae and 931.21 ng ml^{-1} and 8.99 ng ml^{-1} for males. For the experiment in which we altered the ratio of 3kPZS and PZS in male odorant, we added synthesized PZS to reach the ratio found in the larval odorant (1: 4.38, 3kPZS: PZS). For the experiment in which we altered the ratio of 3kPZS and PZS in larval odorant, we added synthesized 3kPZS to reach the ratio found in the male odorant (103.58: 1, 3kPZS: PZS).

Field behavioral assay

Instream behavioral assays were used to evaluate responses of ovulated females to odorants in a natural spawning stream. Trials were conducted in June-July 2014 and 2018 in a stretch of the upper Ocqueoc River (Presque Isle County, MI). A barrier on the Ocqueoc River prevents sea lamprey from infesting the upper river, allowing the number of sea lamprey in the system and background pheromone concentrations to be controlled.

The site and method used matched previous studies on sea lamprey pheromone communication (5, 6), with slight modification.

Briefly, female preference between two odors applied to side-by-side spawning nests was monitored using passive integrated transponder (PIT) antennas. A 23mm half-duplex PIT tag (Oregon RFID, Portland, OR, USA) was inserted into a latex sleeve and sutured to the mid-dorsal region of each female. Two unique colored streamer tags were attached to the dorsal fins to aid in visual identification of each individual. Each trial included approximately 10-15 ovulated females depending upon availability. Females were acclimated for at least 8 h in cages (0.25 m^3) positioned approximately 45 m downstream from the two constructed nests. Nests were each surrounded by a 1 m^2 PIT antenna, which were wired to a multiplexor and data logger (Oregon RFID, Portland, OR, USA). Test odorants were diluted in bins with river water and pumped (Masterflex 7553-70, Cole-Parmer, Vernon Hills, IL, USA) into the center of each nest at a rate of 167 ± 5 mL min^{-1}. The treatments applied to nests were alternated each trial. The amount of odorant needed to activate the whole stream to the target concentration was calculated using stream discharge estimates measured every three days or after precipitation events using a Marsh-McBirney portable flow meter (Flo-Mate 2000, Fredrick, MD, USA). Trials in which we contrasted larval versus male ratios of synthesized 3kPZS and PZS began at 0800 with a 15 min pre-release odor application and provided females 45 min to choose a nest. Trials in which we tested natural odors were slightly modified to conserve the limited available odorant; the pre-release odor application was shortened to 10 min and the observation period was shortened to 35 min. The trials were conducted at night when sea lamprey are more active but visual observations are not possible. Likewise, trials with natural odorants were conducted at a site approximately 100 m downstream of that used in previous studies and our experiments with synthesized odorants due to low water conditions. The concentrations of 3kPZS and PZS in natural odorants collected for in-stream assays were 6.31 ng ml^{-1} and 9.32 ng ml^{-1} for larval odorants and 613.3 ng ml^{-1} and 4.5 ng ml^{-1} for male odorants. In all instream trials, the effect of odor on which nest females entered first was evaluated in R (R version 3.6.1, Vienna, Austria) using a logistic regression with a binomial distribution.

Experiments in which we tested ovulated female responses to 3kPZS versus a 1:1

mixture of 3kPZS and PZS used slightly expanded methods to provide more detailed observations on the behavioral mechanism by which females use PZS as an antagonist. Trials began around 0800 with a 30 min pre-release odor application and provided females 90 min to choose a nest. In addition to the PIT antennas around each nest, antennas were 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. Fish were also visually observed and the swim track of each individual based on the unique color combination of the streamer tags was recorded onto scaled maps. When a female entered a nest, the observer recorded the amount of time spent in the 1 m^2 area. Swim tracks were later compiled onto a digital map. A sinuosity index of each swim track was calculated for each female that swam at least half the distance of the field site (22.5 m) following established procedures (5). The track length measured with a PlanWheel SA2 (Scalex Corporation, Carlsbad, CA, USA) was divided by the length of a straight line connecting the start and end of each track. The sinuosity indices were square-root transformed and a two-sample *t* test was conducted to compare the sinuosity data across the two treatments. Logistic regression evaluated the effect of odorant treatment on 1) the proportion of released females that remained in the release cage at the end of the trial, 2) the proportion of released females that swam 3 m or more downstream from the release cage and stayed downstream of the release cages for the duration of the trial, 3) the proportion of released females that 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 source or 5) control odorant source.

To characterize the odorant dispersion from the nest, rhodamine (Cole-Parmer Rhodamine Red, Vernon Hills, IL, USA) dye tests were conducted using previously validated methods (7). The stream was sectioned with fixed transects. The first 10 transects downstream of the nests were spaced 1 m apart and the remaining spaced every 5 m apart. Rhodamine dye was applied to the center of one of the nests at a constant rate of 167 ± 5 mL min^{-1} using a peristaltic pump for 30 min prior to sampling. The rhodamine concentration was sampled at the 10 evenly spaced points along each of the transects during active administration of dye for 90 min using Cyclops 7 (Model number

2100-000, Serial number 2101754, Turner Designs, Sunnyvale, CA, USA) affixed with Data Bank (Model number 2900-005, Serial number 2900211, Turner Designs). The river was allowed to flush for 1 h, the absence of dye was confirmed with rhodamine sampling, and then the dye test was repeated from the nest. The values were compiled to determine the relative concentration of the odorant plume from the nests to the release cages.

Quantification of 3kPZS and PZS released by males and larvae

Larvae were held in cages (15 x 20 x 30 cm) partially buried into a tributary of Lake Huron (Nagel Creek, Presque Isle County, MI). To collect odors, each cage was held in 7 L of aerated, deionized water acclimated to the stream temperature. Odors accumulated for 12 h through the night, after which 1 L water samples from each group were frozen. Immediately after capture from natural spawning nests, individual males (n = 7) were placed into 5 L of aerated, deionized water for 10 min, after which 50 ml of water was sampled and frozen. A 10 ml (larvae) or 1 ml (male) subsample was freeze dried, reconstituted in 50% methanol: water (v:v; 100 µl for larvae, 1 ml for male), and subjected to liquid chromatography tandem mass spectrometry (8). Data from each larval group repeatedly sampled across three weeks were averaged.

Dataset S1 (separate file). All data discussed in the paper.

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