Evidence that male sea lamprey increase pheromone release after perceiving a competitor

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Summary Statement

Male sea lamprey, *Petromyzon marinus*, increase pheromone release after perceiving intrasexual competition, and this change in chemical signaling strategy may influence female mate choice.

List of Abbreviations

3kPZS - 3keto-petromyzonol sulfate

PZS – petromyzonol sulfate

5d3kPZS - 5-deuterated 3keto-petromyzonol sulfate

11d3kPZS - 11-deuterated 3keto-petromyzonol sulfate

LC–MS/MS - liquid chromatography tandem mass spectrometry

PIT - passive integrated transponder

Abstract

Sexual signals evolve via selective pressures arising from male-male competition and female choice, including those originating from unintended receivers that detect the signal. For example, males can acquire information from other males signaling to females and alter their own signal. Relative to visual and acoustic signals, less is known about how such communication networks influence chemical signaling among animals. In sea lamprey (*Petromyzon marinus*), chemical communication system is essential for reproduction, offering a useful system to study a pheromone communication network that includes signalers and both intended and unintended receivers. Male sea lamprey aggregate on spawning grounds where individuals build nests and signal to females using sex pheromones. We examined how exposure to a major component of the male pheromone, 3keto-petromyzonol sulfate (3kPZS), influenced male pheromone signaling, and whether females had a preference for males that altered their signal. Exposure to 3kPZS, at a concentration of $5x10^{-10}$ M, simulated the presence of other male(s) and led to increased 3kPZS release rates within 10 min, followed by a return to baseline levels within 30 min. Exposure also led to increases in hepatic synthesis and circulatory transport of pheromone components. In behavioral assays, females preferred the odor of males that had been exposed to 3kPZS; therefore, males likely benefit from upregulating 3kPZS release after detecting competition for mates. Here, we define how a specific pheromone component influences chemical signaling during intrasexual competition, and show a rare example of how changes in chemical signaling strategies resulting from male competition may influence mate choice.

Introduction

The evolution of exaggerated male signaling traits is driven, in part, by inter- and intra-sexual selection (Andersson, 1994). Often, inter- and intra-sexual selection on signaling traits are investigated independently, with many studies focusing on binary interactions between signaling males and choosing females or signaling males and their competitors (McGregor and Peake, 2000). However, communication between individuals occurs within a broader social environment that shapes mating and competitive interactions. For example, individuals can copy mating decisions of others (Schlupp et al., 1994; Schlupp and Ryan, 1997) or alter mate preferences when in the presence of competitors (Plath et al., 2008). Therefore, understanding

the function and evolution of communication requires information on the broader social context in which it occurs.

Information on the effects of social context is particularly lacking for communication via pheromones. Many animals communicate using pheromones during reproduction (Johansson and Jones, 2007) but most research on sexual selection focuses on auditory and visual signals (Coleman, 2009; Yohe and Brand, 2018) . Consistent with other signals, pheromones often mediate interactions between individuals within a social environment that includes various signalers and receivers. For example, males in lekking species likely encounter pheromones from competitors signaling to females and can adjust their signaling behaviors after perceiving competition (Maruska and Fernald, 2012; Widemo and Johansson, 2006). However, sparse information on the identity of active molecules, especially in vertebrates (Symonds and Elgar, 2008), often constrains the design or interpretation of studies on the social context surrounding pheromone communication. Indeed, previous studies expose males to competitors' cues with unknown quantities of the specific pheromone molecule(s) or measure pheromone signaling using behavioral proxies such as urination rate (Maruska and Fernald, 2012) or hair-pencil displays (Davie et al., 2010) rather than actual pheromone quantification. Furthermore, the fitness consequences for males that adjust their signaling per the social context remain largely unknown. Unlike many vertebrates, the sea lamprey (*Petromyzon marinus*) has a well-charactarized pheromone communication system (Buchinger et al., 2015) and therefore is a useful model to study how social context influences pheromone signaling (Buchinger et al., 2015).

Male sea lamprey signal with a multi-component sex pheromone that guides mate search and spawning behaviors in females (Buchinger et al., 2015). A major component of the sex pheromone, 3-keto petromyzonol sulphate (3kPZS; Li et al., 2002), attracts females to a male's nest over a wide range of concentrations and distances (Johnson et al., 2009). Signaling with 3kPZS is likely shaped by inter-sexual selection as females prefer the more-concentrated of adjacent pheromone plumes (Johnson et al., 2009), males exhibit high variation in 3kPZS release rates (Buchinger et al., 2017), and females navigating through lek-like aggregations of males encounter pheromone signals from multiple potential mates (Applegate, 1950). Notably, males will also encounter pheromones from competing males but any effect of perceived competitors on signaling remains unknown.

We hypothesized that male sea lamprey increase their pheromone signaling intensity to better compete with nearby males. To test our hypothesis, we 1) quantified release of 3kPZS by males that were exposed to synthesized 3kPZS, 2) quantified 3kPZS and its biosynthetic precursor, petromyzonol sulfate (PZS), in tissue samples responsible for pheromone production, transport, and release, and 3) determined female preference between males exposed to synthesized 3kPZS versus a negative control.

Materials and Methods

Experimental Animals

Sea lamprey were captured by U.S Fish and Wildlife Service and Fisheries and Oceans Canada. Lamprey were then transported to U.S. Geological Survey Hammond Bay Biological Station, Millersburg, Michigan, and held in 200-1000 L aerated tanks fed with ambient temperature Lake Huron water. Males and females sexually matured after being held in cages (~25 individuals per cage) in the Ocqueoc River, Millersburg, Michigan. They were checked daily for expression of gametes with gentle pressure to the abdomen (Siefkes et al., 2003a) and once sexually mature, returned to Hammond Bay Biological Station for experiments and held in 200 L tanks. All experimental procedures followed protocols approved by the Michigan State University Institutional Animal Care and Use Committee (AUF#s 03/14-054-00, 02/17-031-00).

Pheromone Release into Water

Water holding individual sexually mature males was collected to determine 3kPZS release rates. Prior to experiments, sexually mature males were held for 12-14 h in aerated 200 L Bonar tanks fed with Lake Huron Water (16-18°C). Individual males were netted from their holding tank and put into a five-gallon bucket containing aeration and a replenishing supply of Lake Huron water held at 5 L to remove any trace odors from other males. After the 1 h acclimation, water was drained via a hole at the bottom of the bucket and replaced with 3 L of deionized water. After 30 min, a 50 mL baseline water sample was taken and immediately spiked with a 5-deuterated 3kPZS standard ([$^{2}H_{5}$] 3kPZS, 5d3kPZS) (Bridge Organics Inc., Vicksburg, MI, USA) to reach a concentration of 1 µg L^{-1}.

Immediately after baseline sampling, males were exposed to a randomly assigned treatment, 5d3kPZS (applied to reach an in-bucket concentration of $5x10^{-10}$ M, n=20) or a 1 mL

vehicle control (methanol: water, 1:1, n=20) to determine the effect of a perceived competitor on 3kPZS release rates. Treatments were poured into the bucket and gently mixed. Subsequent water samples were taken 10, 30, and 60 min after treatment application. Control samples were spiked with a 5d3kPZS internal standard, and 5d3kPZS treatment samples were spiked with 11-deuterated 3kPZS ([$^{2}H_{11}$] 3kPZS, 11d3kPZS) (Bridge Organics Inc.) internal standard to reach a concentration of 1 µg L^{-1}. Ten mL subsamples were taken from each 50 mL sample, and both were immediately stored at -20°C. Male sizes ranged from: control treatment (length = 458.50 ± 10.68 mm, mass = 210.86 ± 14.67 g, mean ± S.E.M) and 5d3kPZS treatment (length = 451.85 ± 9.06 mm, mass = 202.47 ± 13.11 g, mean ± S.E.M). All treatments were randomly assigned to each bucket. Individuals conducting water sampling experiments were not blind to treatment application and sample preparation for chemical analyses was not blind; however, all chemical analyses were conducted without any knowledge as to the treatment applied, fish identity, or sampling time.

For chemical analysis, samples were freeze-dried and dissolved in 1 mL of methanol. Extracts were then centrifuged at 12,000 rpm for 10 min at 4° C (accuSpin Micro 17R, Fisher Scientific, Hampton, NH, USA). Supernatants were then collected, freeze-dried completely, reconstituted in 100 µL methanol/water (1:1, v/v) for analysis by ultra-high- performance liquid chromatography tandem mass spectrometry (LC–MS/MS). Samples were analyzed adapting the method developed by (Xi et al., 2011). Briefly, the LC–MS/MS system consisted of a Waters ACQUITY H-Class UPLC™ (Waters Corporation, Milford, MA, USA) coupled to a Water Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation). A Waters BEH C18 column (2.1 x 100 mm, 1.7 µm particle size, 130 Å) (Waters Corporation) was used with mobile phase A: 10mM trimethylamine (TEA) in water and B: methanol. The injection volume was 10 µL, and the HPLC flow rate was 0.25 mL min^{-1}. Mass spectra were acquired using electrospray ionization in negative ion mode and Multiple Reaction Monitoring. Data were acquired with Waters MassLynx 4.1 (Waters Corporation) and processed for calibration and for quantification of the analytes with Waters QuanLynx software (Waters Corporation).

Pheromone Synthesis and Transport

To determine if pheromone exposure influenced hepatic synthesis and circulatory transport, liver (n=20, control and 5d3kPZS treatments), gill (n=20, control and 5d3kPZS

treatments), and plasma (control n=18, 5d3kPZS n=20) samples were collected from each treatment group for 3kPZS and PZS quantification. Immediately after taking the 60 min water samples, sea lamprey were euthanized with an overdose of tricaine methanesulfonate (MS222; Sigma-Aldrich, St. Louis, MO, USA). Liver and gill samples were taken and snap frozen in liquid nitrogen. Blood was sampled via cardiac puncture and centrifuged to collect plasma (1000 x g at 4˚C for 20 min; Eppendorf 5804, Hamburg, Germany). Tissue and plasma samples were held at -80°C until analysis. For bile acid extraction, liver and gill tissue were weighed and then homogenized in 500 uL 75% ethanol by finely chopping with scissors, and 400 µL of plasma was mixed with 800 µL of 100% ethanol. Following bile acid extraction, 5d3kPZS internal standard was added to reach a concentration of 100 ng mL^{-1}. All samples were incubated overnight at ~21˚C while being rotated at 70 rpm. Samples were then centrifuged at 13000 x g at 4˚C for 10 min (accuSpin Micro 17R) and the supernatant was removed, freeze dried, and reconstituted in 50% methanol for biochemical analysis via LC–MS/MS using the same methods for quantifying 3kPZS in water samples.

Behavioral Assays

In-stream assays were used to evaluate behavioral responses of ovulated females to odors of 5d3kPZS treated vs control treated males. Assays were conducted in the upper Ocqueoc River from 28-July-2019 to 31-July-2019 and from 2230 h to 0100 h. The upper Ocqueoc River is a historic spawning site (Applegate, 1950) but currently, sea lamprey are restricted from the upper stretches by an electric barrier. This ensures no background pheromone odor is present from naturally spawning populations.

Sexually mature female responses were monitored using a passive integrated transponder (PIT) array (Oregon RFID, Portland, OR, USA). Each female was fitted with a 23 mm half-duplex PIT tag (Oregon RFID), and a single trial contained 10-18 lamprey depending upon availability. Groups of females were held in 0.5 m^2 acclimation cages 50 m downstream of two 1 m^2 PIT nests for approximately 2 h prior to trial start time. Using a peristaltic pump (Masterflex L/S, item # EW-07554-90, Cole Parmer, Vernon Hills, IL, USA), 7.5 L of odorant was applied at 167 ml min^{-1} in each of the two adjacent 1 m^2 PIT nests set 0.9 m apart. After 10 min of odor application, a group of females was released and observed via PIT array for 35 min. The first PIT nest each female entered (first odor choice) and the duration of time females spent in each nest

were recorded (retention time). PIT data was used to calculate retention time, and fish that were not detected for greater than 15 s were considered to have left the nest. Females remained in stream after trials, but unique PIT tag numbers allowed lamprey to be analyzed only for their respective trial.

To determine female preference for males with increased pheromone signals, we compared female behavioral responses to the odor of males treated with 5d3kPZS against odor of males treated with the control. Odors for experimental trials were collected as described above (Pheromone Release into Water) with slight modification. Males were exposed to $5x10^{-10}$ M 5d3kPZS (n=16) or 1 mL control (methanol: water, 1:1) (n=16). To determine 3kPZS release rates for each individual, baseline and 10 min samples were collected as previously described. Immediately after 10 min sampling, males were removed from the bucket, and the remaining water (2.9 L) was collected in 1 L Nalgene bottles and stored on ice. After all sampling was completed, water collected from each treatment was pooled, thoroughly mixed, and re-aliquoted into 1 L Nalgene bottles for use in behavioral experiments. To quantify 3kPZS concentrations in odors used during experiments, three 50 mL samples of pooled water from each treatment were taken and analyzed via LC–MS/MS as previously described. Male sizes ranged from: control treatment (length = 435.25 ± 10.21 mm, mass = 196.21 ± 14.48 g, mean ± S.E.M) and 5d3kPZS treatment (length = 445.56 ± 9.45 mm, mass = 201.42 ± 13.41 g, mean ± S.E.M).

Experiments directly compared these odorants by applying them into adjacent 1 m^2 PIT nests (n trials = 7, n females = 98). Odor treatment was randomly assigned to a nest for the first trial and then alternated for subsequent trials. Control or 5d3kPZS male odor (3.26 L) was combined with river water (4.24 L) to reach a final volume of 7.5L and applied to one nest during the 45 min experiment at a pump rate of 167 ml min^{-1}. Male odors were collected in 2.9 L of water for 40 min, therefore 3.26 L applied over 45 min matches an individual male's pheromone release over the experiment's duration

Statistical Analysis

The 3kPZS concentrations in water samples were used to calculate release rates for time intervals between sampling events. A sample (x) and the subsequent time point sample (x+1) were discarded if internal standard was not detected (n=4), 3kPZS concentration was not greater in x+1 than x (3kPZS not accumulating due to sampling or analysis error, n=8), or a sample was

lost (n=2) (Raw Data). These criteria were not pre-established; however, these instances did not allow an accurate release rate to be calculated for corresponding sampling times.

All statistical analyses were done using R v3.5.1 (R Core Team, 2018). We analyzed 3kPZS release rates during experimental washings using non-parametric longitudinal analysis (F1-LD-FI models, α=0.05) in the nparLD package (Noguchi et al., 2012) . Models included a whole plot factor (treatment) and one sub-plot factor (time). Post-hoc comparisons using separate non-parametric F1-LD-F1 models were used to determine treatment effects between the following release rates: baseline to 10 min, baseline to 30 min, and baseline to 60 min, and p values were adjusted for multiple comparisons with the Bonferroni correction (α = 0.017). Due to only sampling at baseline and 10 min when collecting odor for behavioral trials, multiple comparisons were not needed for analysis. Additionally, a Mann-Whitney U test was used to compare baseline 3kPZS release rates between treatments for odors used in behavioral trials. For liver, gill, and plasma samples, Levene's tests indicated the group variances were not homogenous so Welch's t-tests for unequal variances (α=0.05) were used to compare 3kPZS and PZS concentrations across treatments.

For behavioral experiments, a mixed effect logistic regression model with a binomial distribution was used to evaluate the effect of treatment on first odor choice and a mixed effect linear model to evaluate the effect of treatment on retention time. The model for first odor choice included odor treatment and nest bias as covariates, and the model for nest retention included odor treatment. Each model included a random effect of trial, and the mixed effect linear model contained an additional random effect of fish identity for a paired analysis of nest retention. Retention time data were square root transformed to meet model assumptions and all analyses used the *lmer* and *glmer* functions in R package lme4 (Bates et al., 2015) and the *Anova* function with type III sums of squares, α=0.05 in R package car (Fox and Weisberg, 2019).

Results

Males increase 3kPZS release after detecting a simulated competitor

Exposure to 5d3kPZS increased pheromone release when compared to the control, and males increased 3kPZS release rates 92 ± 19.36% (mean increase ± S.E.M) within 10 min following treatment application. The overall F1-LD-F1 model indicated a significant treatment and time interaction for 3kPZS release rates (ANOVA-type statistic (ATS) = 3.79, df = 2.41 p =

0.016) (Figure 1). Post-hoc tests indicated release rates were significantly higher than the baseline for 5d3kPZS treated males when compared to the control at 10 min (ATS = 22.12, df = 1, adjusted $p < 0.001$), but no different than the baseline at 30 min (ATS = 0.88, df = 1, adjusted $p = 1$) or 60 min (ATS = 0.62, df = 1, adjusted $p = 1$).

Males increase hepatic synthesis and circulatory transport of 3kPZS and PZS after detecting a simulated competitor

Exposure to 5d3kPZS increased production and transport of 3kPZS and its biosynthetic precursor, PZS, in sexually mature male sea lamprey. These compounds are produced in the liver, transported via blood to the gills where PZS is converted to 3kPZS (Brant et al., 2013) and then released into the water via specialized gill cells (Siefkes et al., 2003b). Males exposed to the pheromone treatment had elevated concentrations of 3kPZS (M=47.51 ng mg^{-1} tissue, S.D. = 35.28) and PZS (M=4454.37 ng mg^{-1} tissue, S.D.=2671.87) in liver samples compared to control males (3kPZS, M=5.00 ng mg^{-1} tissue, S.D.=4.94; PZS, M=2280.67 ng mg^{-1} tissue, S.D.=1543.84), $t_{(19.74)} = -5.34$, $p < 0.001$ and $t_{(30.42)} = -3.15$, $p = 0.004$ (Figure 2). Additionally, males exposed to the pheromone treatment had increased plasma concentrations of 3kPZS (M=26369.37 ng mL^{-1} plasma, S.D.=19428.84) and PZS (M=465679.59 ng mL^{-1} plasma, S.D.=232059.17) compared to control males (3kPZS, M=3110.77 ng mL^{-1} plasma, S.D.=1861.08; PZS, M=270444.91 ng mL^{-1} plasma, S.D.=148945.90), $t_{(19.39)} = -5.33$, $p < 0.001$ and $t_{(32.71)} = -3.12$, $p = 0.004$ (Figure 2). In gill samples, pheromone treatment resulted in elevated levels of 3kPZS (M=52.89 ng mg^{-1} tissue, S.D.=41.55) compared to control males (M=7.25 ng mg^{-1} tissue, S.D.=4.20), $t_{(19.59)} = -4.87$, $p < 0.001$, but resulted in decreased PZS concentrations (M=267.99 ng mg^{-1} tissue, S.D.=152.88) compared to control males (M=471.55 ng mg^{-1} tissue, S.D.=125.38), $t_{(27.10)} = 2.55$, $p = 0.017$ (Figure 2).

Increasing pheromone release in response to a simulated competitor attracts more females

Consistent with the other experimental washing, males exposed to 5d3kPZS increased 3kPZS release by 205 ± 12.32% (mean increase ± S.E.M) over a 10 min period (ATS = 81.49, df =1, $p < 0.001$). Release rates were not different between treatment and vehicle at baseline sampling (control median = 0.216 mg hr^{-1}, 5d3kPZS median = 0.214 mg hr^{-1}, Mann-Whitney U = 145, $n_1=16$, $n_2=16$, $p = 0.539$), indicating that differences in 3kPZS concentrations in pooled

treatment samples (control, 42.21 ± 1.90 ng ml^{-1}, mean ± S.E.M), (5d3kPZS, 71.80 ± 1.47 ng ml^{-1}, mean ± S.E.M), were due to the treatment and not differences in basal 3kPZS release between treatment groups.

Behavioral experiments confirmed that females preferred the odor of males exposed to 5d3kPZS. Only females that swam upstream and entered a nest were included in statistical analyses (n=50) and remaining sea lamprey died (n=10) or did not enter a nest during experiments (n=38). Females preferred nests treated with exposed male odor over control male odor (χ^2 (1) = 4.18, P = 0.041, Figure 3). A side bias was apparent, but it was not significant (χ^2 (1) = 3.10, P = 0.078). Females tended to spend more time on nests baited with exposed male odor, but this trend was not significant (χ^2 (1) = 3.18, P = 0.075, Figure 3).

Discussion

Our results support the hypothesis that male sea lamprey increase their pheromone signal upon perceiving competitors in order to better compete for mates. Biochemical analyses indicated male sea lamprey increase pheromone production, transport and release when exposed to a major pheromone component, 3kPZS. Across all experiments, 94% of males increased 3kPZS release, and the increases in two separate experiments averaged 92% and 205% resulting in concentration changes that females are likely to perceive (Johnson et al., 2009). Behavioral assays confirmed females prefer the odor of males that had been exposed to 3kPZS. The ability to rapidly increase pheromone release conceivably increases male fitness given the sea lamprey reproductive ecology, in which males compete for females in lek-like aggregations (Applegate, 1950) and females prefer the higher of adjacent pheromone concentrations (Buchinger et al., 2017; Johnson et al., 2009). Alternatively, our results could be explained by 1) a positive feedback loop in which male sea lamprey detect their own pheromone signal and subsequently upregulate pheromone production and release or 2) male cooperation that increases the aggregate pheromone signal and attracts additional females to a spawning area. The experimental design indicates a positive feedback loop is unlikely as males in the control group, despite being constantly exposed to their own pheromone signal, show no increases in pheromone production or release. Additionally, the sea lamprey naris is located anterior to gill epithelia and individuals on a nest typically orient facing upstream, making it unlikely that males detect their own pheromone signal consistently. Male cooperation is also unlikely given the reproductive ecology

and behavior of sea lamprey (Johnson et al., 2015), where males are competitive and fight with each other over access to mates. Our results are consistent with male ability in other species to modify signals or signaling strategies in competitive scenarios (Bee and Bowling, 2002; Breithaupt and Atema, 2000; Reichert and Gerhardt, 2013) and with female preference for signaling modifications (Bosch and Márquez, 1996; Fisher et al., 2003; Johnston et al., 1997), but to our knowledge, provide the first direct evidence that perceived competition increases signaling with a specific pheromone component and results in better access to mates.

We observed males increase pheromone release within 10 min after detecting a simulated competitor. It is possible that the actual peak rate in 3kPZS release is higher than what we observed and calculated as the average release rate over 10 min, especially if increased pheromone release happens quickly during the beginning of this timeframe. While our methods prevented us from observing a larger peak, our results indicate increases in pheromone release likely do not occur after 10 min because release rates returned to baseline levels by 30 min. In a similar study, lesser wax moths, *Achroia grisella*, also increased ultrasonic signaling for a short duration of time (Jia et al., 2001). Signals such as these are presumably costly, otherwise males would maintain the same signaling rate across social contexts. Maintaining a higher 3kPZS release rate for extended durations may be too metabolically costly considering the limited energy stores available during sea lamprey reproduction (William and Beamish, 1979). Likewise, females orient towards 3kPZS at concentrations as low as 10^{-14} M (Johnson et al., 2009); therefore, males likely gain little benefit by signaling at maximum rates when competition is low. Males likely release a pheromone reserve after detecting a nearby competitor, which may be signaled by a rapid change in local pheromone concentration; however, upregulation of the bile acid synthetic pathway, secretion into circulation, and transport to the gills for release into the water in 10 min is unlikely (Brant et al., 2013; Yeh et al., 2012). Interestingly, quantification of 3kPZS and PZS in tissues showed males exposed to 3kPZS had higher concentrations of both compounds in the liver and plasma and more 3kPZS but less PZS in the gills. These results indicate 1) increased hepatic synthesis and circulatory transport of both compounds and 2) increased conversion of PZS to 3kPZS in the gills (Brant et al., 2013), and support physiological compensation to replenish pheromone rapidly released into water. While reductions of PZS in gill tissue appear inconsistent with all other tissue results, it is expected given our current understanding that PZS is likely converted to 3kPZS in gill tissue prior to release (Brant et al.,

2013). Sea lamprey gills do contain a reserve of pheromone (Siefkes et al., 2003b), and the physiological mechanisms responsible for such rapid modulation of pheromone release across gill epithelia should be investigated in future studies.

Whether perceiving competitors stimulates male sea lamprey to alter release of all components of their pheromone or only the major component 3kPZS remains unclear. Many signals consist of multiple components that can have distinct functions and be shaped by different selective forces (Candolin, 2003). Evidence suggests the male pheromone in sea lamprey likely consists of multiple components that induce different behaviors rather than acting as a singular blend that collectively induces one response (Johnson et al., 2012; Johnson et al., 2009). Although 3kPZS replicates natural male pheromone in attracting females to males nest over long distances, additional minor components retain females in close proximity and elicit nesting behaviors (Johnson et al., 2012; Johnson et al., 2009). Our data clearly indicate males exposed to 3kPZS increase their own release of 3kPZS and females respond to exposed male odor with behaviors usually elicited by 3kPZS (nest entry). Interestingly, female retention was noticeably but not significantly ($p = 0.078$) higher in nests treated with exposed male odor. This observation may indicate that 1) males increase release of minor components, but the resulting effect on female retention was too subtle to conclusively observe in our behavioral assay or 2) males specifically adjust release of 3kPZS but not minor components, and the small increase in female retention was random variation or due to the higher concentration of 3kPZS in exposed male odor. Notably, how retention time is impacted by different concentrations of 3kPZS has yet to be directly tested. Changes in female behavior appear to be the result solely of changes in pheromone release by males, and not due to the additional 5d3kPZS present in exposed male odor, which only increased 3kPZS concentration in the behavioral assay treatment by 0.0035%. Additional research designed to evaluate the specificity with which males increase pheromone release would be especially interesting as it may implicate the balance of costs and benefits to males across different pheromone components and the responses they evoke in females.

Our results highlight the dynamic evolution of pheromone communication systems (Steiger et al., 2011). Preference for 3kPZS likely originated in a migratory context, and was later exploited by males releasing 3kPZS at high rates during mating (Buchinger et al., 2013). Females then optimized their response to 3kPZS to guide mate search and because of their preference for higher concentrations of 3kPZS, exerted strong positive sexual selection on

3kPZS release by males (Buchinger et al., 2019). Here, we report evidence that 3kPZS signaling was subsequently shaped by intra-sexual selection. Our results provide insight into the importance of communication networks on the evolution of pheromone signals, which, especially in vertebrates (Symonds and Elgar, 2008), are less studied than other types of signal modalities.

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Competing Interests

Ugo Bussy is currently employed by Mars Incorporated (Mars Inc., McLean, VA, USA).

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Data Availability

The datasets along with the R code and outputs supporting this article have been uploaded to Dryad.

Author Contributions

SDF performed experiments, carried out statistical analyses, and drafted the manuscript. UB and BH ran chemical analyses on water samples. TJB assisted with drafting and revising the manuscript. SDF, TJB, and WL designed and coordinated the study. WL conceived the overall study.

Ethical Statement

All procedures involving sea lamprey were approved by the Michigan State University Institutional Animal Care and Use Committee (AUF#s 03/14-054-00, 02/17-031-00).

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Figures

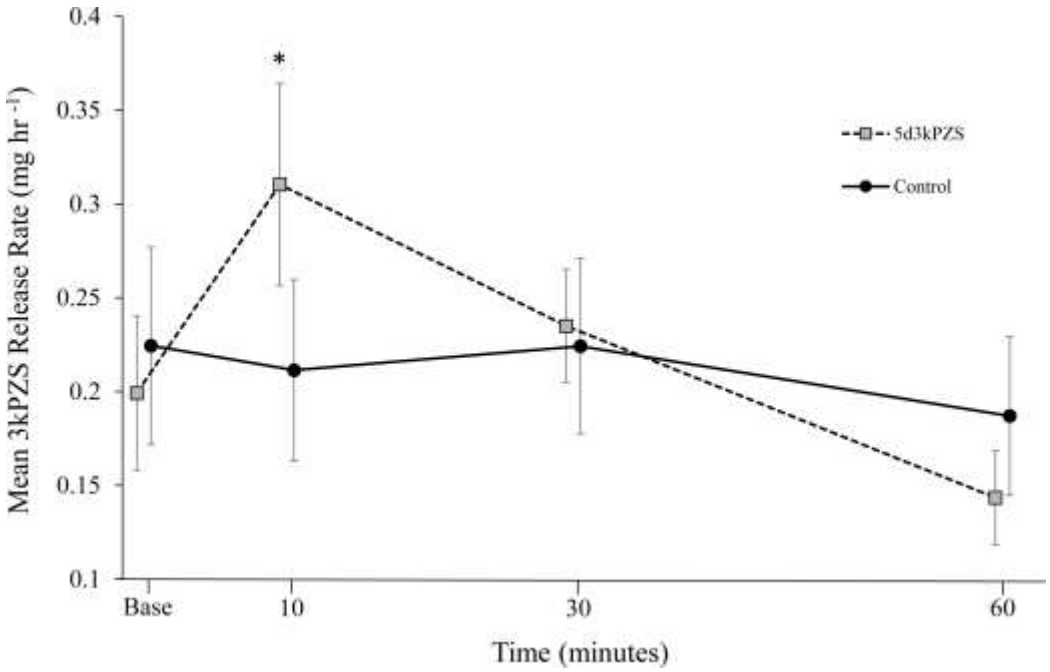

Figure 1. Summary of mean 3kPZS release rates from males exposed to a 5d3kPS or control treatment. For each male, water samples were collected once prior to treatment ("Base", 5d3kPZS n=19, control n=20) and three times post treatment at 10 min (5dk3PZS n=18, control n=20), 30 min (5dk3PZS n=18, control n=18), and 60 min (5dk3PZS n=17, control n=15). High performance liquid chromatography and tandem mass spectrometry were used to quantify 3kPZS concentrations in water samples. Males exposed to 5d3kPZS at $5x10^{-10}$ M increased 3kPZS release compared to males exposed to a control, 1 mL methanol:water 1:1 ($p = 0.016$, overall model, treatment:time interaction). Post-hoc tests indicated a significant effect was only observed within 10 min of treatment application (adjusted $p < 0.001$, treatment:time interaction) but not at 30 min (adjusted $p=1$, treatment:time interaction) or 60 min (adjusted $p=1$, treatment:time interaction). All data and post-hoc tests were analyzed using non-parametric longitudinal analysis. Points are offset on the x-axis to avoid crowding, * indicates $p<0.05$ for time point comparisons, and error bars represent ± 1 S.E.M.

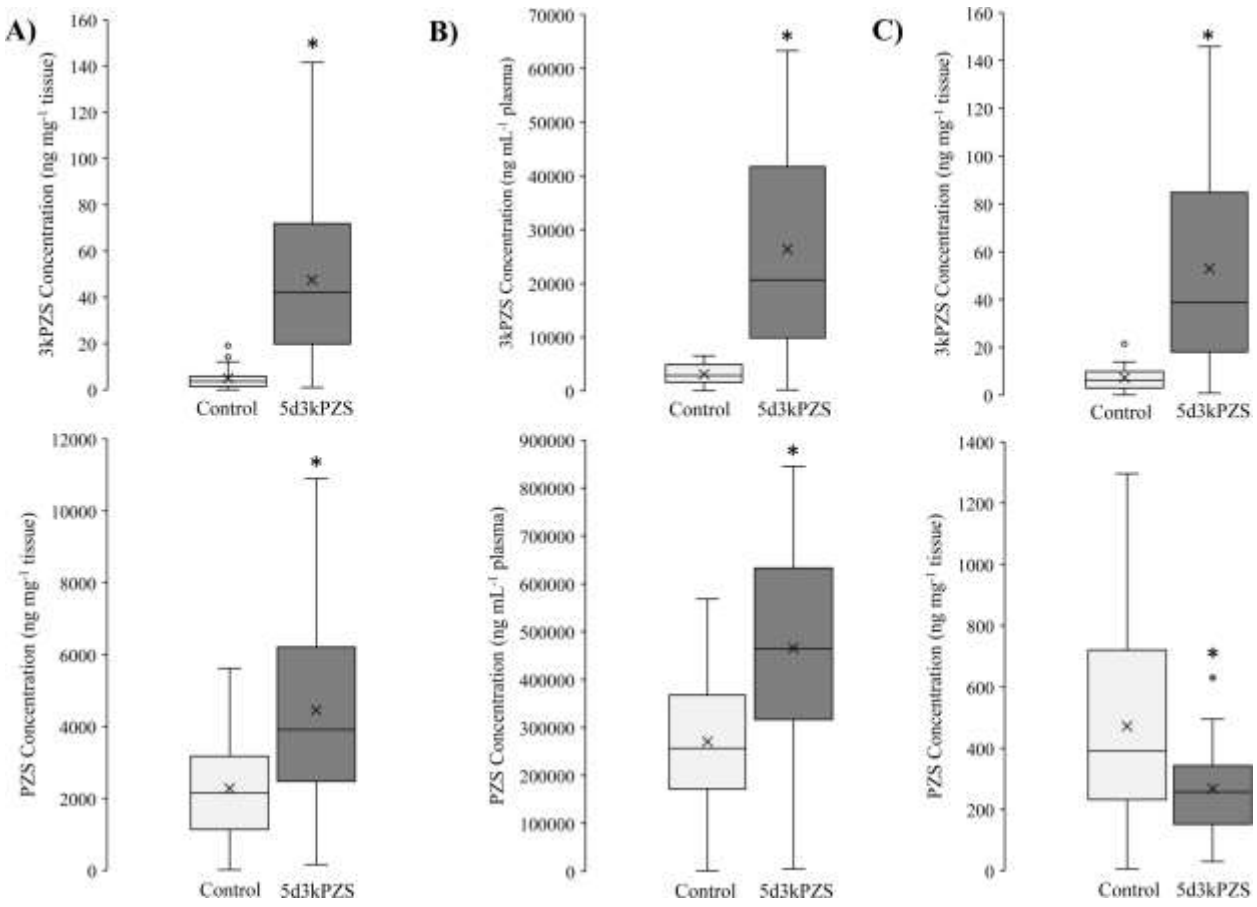

Figure 2. Pheromone exposure results in 3kPZS and PZS concentration changes in tissues responsible for synthesis, transport, and release into water. Boxplots indicate 3kPZS and PZS concentrations in **A)** liver (n=20 each treatment), **B)** plasma (control n=18, 5d3kPZS n=20), and **C)** gill samples (n=20 each treatment), of males exposed to 5d3kPZS ($5x10^{-10}$ M) or control (1 mL methanol:water, 1:1). The bile acid pheromone, 3kPZS, and its precursor PZS are produced in the liver, transported via blood to the gills, and then released into the water via specialized gill cells (Brant et al., 2013; Siefkes et al., 2003b). Males exposed to 5d3kPZS had elevated 3kPZS concentrations in liver, plasma, and gill samples. They also had had elevated concentrations of PZS in liver and plasma samples, but reduced PZS concentrations in the gill, where PZS is likely converted to 3kPZS prior to release into the water (Brant et al., 2013). Analyses were conducted using two-tailed Welch's t-tests and an * indicates $p<0.05$. All boxplots display the minimum value (lower whisker), maximum value (upper whisker), interquartile range (box), median line, mean (x), and any outliers (dots).

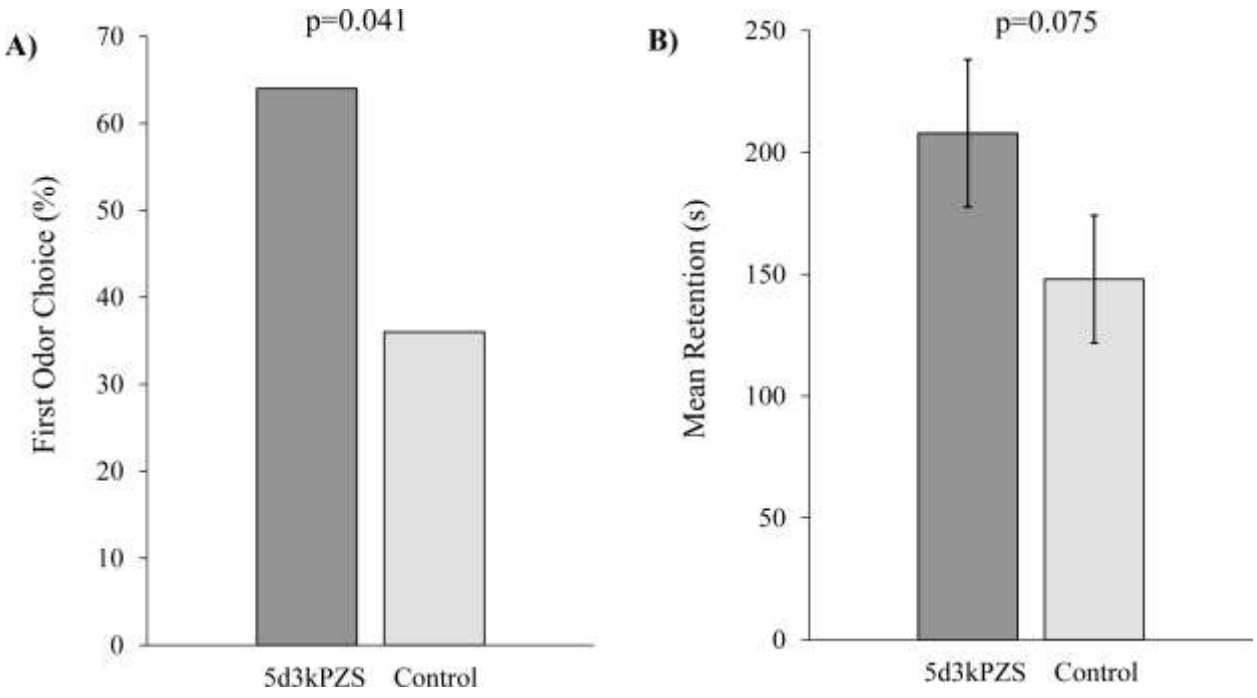

Figure 3. Sexually mature females were more likely to enter and stayed longer at passive integrated transponder (PIT) nests containing the odor of males with increased 3kPZS release rates. A) The percentage of females choosing a treatment odor first during behavioral trials (5d3kPZS, n=32), (Control, n=18). Treatment odors were the pooled water from sexually mature males exposed to the 5d3kPZS treatment or control, and females preferred the odor of 5d3kPZS treated males ($p = 0.041$, mixed effect logistic regression with binomial distribution). **B)** The mean retention time (s) ± S.E.M. females spent within each 1 m^2 PIT nest baited with pooled odor from males exposed to the 5d3kPZS treatment or control (n=50 for each treatment). Females tended to stay longer at the odor of 5d3kPZS treated males, but this result was not significant ($p = 0.075$, mixed effect linear model). Error bars represent ± 1 S.E.M.