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Multiple functions of a multi-component mating pheromone in sea lamprey *Petromyzon marinus*

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The role of the C24 sulphate in the mating pheromone component, $7\alpha,12\alpha,24$ -trihydroxy- 5α -cholan-3-one 24-sulphate (3kPZS), to specifically induce upstream movement in ovulated female sea lampreys *Petromyzon marinus* was investigated. $7\alpha,12\alpha$ -dihydroxy- 5α -cholan-3-one 24-oic acid (3kACA), a structurally similar bile acid released by spermated males, but lacking the C24 sulphate ester, was tested in bioassays at concentrations between 10^{-11} and 10^{-14} molar (M). 3kACA did not induce upstream movement in females or additional reproductive behaviours. In contrast, spermated male washings induced upstream movement, prolonged retention on a nest and induced an array of nesting behaviours. Differential extraction and elution by solid-phase extraction resins showed that components other than 3kPZS + 3kACA are necessary to retain females on nests and induce nest cleaning behaviours. All pheromone components, including components in addition to 3kPZS + 3kACA that retain females and induce nest cleaning behaviours were released from the anterior region of the males, as had been reported for 3kPZS. It is concluded that the sea lamprey male mating pheromone has multiple functions and is composed of multiple components.

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Key words: 3kACA; 3kPZS; agnathan; bioassay; extraction; mating behaviour.

INTRODUCTION

Chemical communication in the sea lamprey *Petromyzon marinus* L. 1758 is critical for directing their migration into spawning streams (Vrieze *et al.*, 2010) and for locating a mate in stream segments with high water velocity and gravel substratum (Johnson *et al.*, 2006). *Petromyzon marinus* larvae residing in natal streams excrete migratory pheromones that direct the migration of spawning-phase adults from expansive ocean or lake environments to suitable habitats within streams (Sorensen & Vrieze, 2003). Spermated males build nests and secrete mating pheromones that are highly attractive to ovulated females, luring them to spawning nests (Li, 2005).

A mating pheromone released by spermated male *P. marinus* may have multiple functions as females display multiple behaviours during spawning including migration

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to the spawning grounds, landing on a nest, nest cleaning and synchronization of gamete release (Applegate, 1950). In moths, the multiple functions of female pheromones such as direct attraction, landing and mating of males are elicited by multiple components, and these have been shown to function independently (Bradshaw *et al.*, 1983) or synergistically (Linn *et al.*, 1987). In *P. marinus*, two bile acids are released specifically by spermiated males, $7\alpha,12\alpha,24$ -trihydroxy- 5α -cholan-3-one 24-sulphate (3kPZS; Li *et al.*, 2002) and $7\alpha,12\alpha$ -dihydroxy- 5α -cholan-3-one 24-oic acid (3kACA; Yun *et al.*, 2003). 3kPZS and 3kACA have identical steroidal skeletons but differ in chemical structure at C24; 3kACA has a carboxyl, as opposed to a sulphate ester in 3kPZS. In electro-olfactogram recordings, both 3kPZS and 3kACA are potent odorants in female *P. marinus*, probably by stimulating different olfactory receptor mechanisms (Siefkes & Li, 2004). While there is increasing evidence that 3kPZS induces overt upstream movement in females (Johnson *et al.*, 2009), it remains to be determined whether 3kACA induces behavioural responses in females (Luehring *et al.*, 2010). The first objective, therefore, was to determine if the C24 sulphate is critical for 3kPZS to induce upstream movement by determining if 3kACA modifies ovulated female behaviour in streams.

The second objective was to determine if there are unidentified male pheromone components that include cleaning behaviours in nesting females. Previous in-stream bioassays have shown that synthesized 3kPZS and spermiated male washings induce the same robust upstream movement of females to the odorant release location (Siefkes *et al.*, 2005; Johnson *et al.*, 2009). There was a discrepancy, however, in that male washings induced several additional behaviours in ovulated females and retained them much longer on spawning nests in comparison to 3kPZS alone. Here, two extraction methods are coupled to in-stream behavioural assays to determine whether these additional behaviours are induced by compounds other than 3kPZS and 3kACA.

The third and final objective was to determine whether all pheromone components are excreted from the anterior region of male adults. 3kPZS is released at high rates from the gills of spermiated males, probably through specialized glandular cells (Siefkes *et al.*, 2003), first identified in *Lampetra fluviatilis* (L. 1758) by Pickering & Morris (1977). This specialized mechanism seems to take advantage of the large surface area of gills to facilitate efficient release of 3kPZS at high rates, which is necessary for it to signal to ovulated females hundreds of metres downstream. This contrasts to the urinary release of steroidal mating pheromones documented in goldfish *Carassius auratus* (L. 1758) (Kobayashi *et al.*, 2002; Sorensen & Vrieze, 2003) where pheromone active spaces encompass a few square metres. If an additional pheromone component or components function to retain female *P. marinus* on nests, their active space would probably be smaller and thus their mode of release may be different (Johnson & Li, 2010).

Fish pheromones have been identified by screening compounds for olfactory potency and behavioural activity (Dulka *et al.*, 1987; Sorensen *et al.*, 1989) or through activity-directed fractionation of extracts from water in which the emitter was held (Li *et al.*, 2002; Yambe *et al.*, 2006). In *P. marinus*, where natural products are less understood, activity-directed fractionation has yielded novel compounds (Li *et al.*, 2002; Sorensen *et al.*, 2005). Here, experimentation with a known compound (3kACA) and activity-directed extraction of spermiated male washings is employed to further examine the chemical nature of *P. marinus* mating

pheromones. Interference with *P. marinus* chemical communication may offer unique and environmentally benign ways of managing *P. marinus* (Li, 2005), a destructive invader of the Laurentian Great Lakes (Smith & Tibbles, 1980), and may guide the development of other pheromone-based control methods for invasive fishes (Elkins *et al.*, 2009).

MATERIALS AND METHODS

GENERAL METHODS

Experimental animals

The use of *P. marinus* was approved under Michigan State University Institutional Animal Use and Care Committee permit 05/06-066-00. *Petromyzon marinus* were captured in mechanical traps from Great Lakes tributaries from May to July during 2005, 2006 and 2008 by the United States Fish and Wildlife Service, Marquette, MI, U.S.A., and Canadian Department of Fisheries and Oceans, Sault Ste Marie, ON, Canada. Females were identified by their soft abdomen (Vladykov, 1949) and classified as ovulated if eggs were expressed upon manual pressure to the abdomen. Pre-ovulatory females were maintained at United States Geological Survey, Hammond Bay Biological Station, Millersburg, MI, U.S.A., in 1000 l capacity flow-through tanks supplied with Lake Huron water at ambient temperatures that ranged from 4 to 18° C. Groups of pre-ovulatory females were moved to cages (c. 1 m³) located in Lake Huron tributaries to mature sexually.

Ovulated females were fitted with two 10 cm polyethylene streamer tags (Hallprint; www.hallprint.com) to increase visibility in streams. In 70 m 3kACA trapping experiments, ovulated females were also fitted with external radio telemetry tags (Model 393, Advanced Telemetry System; www.atstrack.com) and tracked with a directional radio antenna and receiver (Lotek Engineering Incorporated; www.lotek.com). In all other experiments, ovulated females were externally tagged with 23 mm glass encapsulated passive integrated transponder tags (PIT, Oregon RFID; www.oregonrfid.com). Distribution of PIT-tagged females in stream channels was monitored using PIT tag antennas connected to a multiplexer (Oregon RFID). Female tagging occurred at least 12 h prior to experimentation. Tagged females were placed in an acclimation cage (1 m³) in the experimental stream at least 8 h prior to experimentation. Sexually mature *P. marinus* spawn during the day and night (Applegate, 1950), but all experiments were conducted during the day to allow observation. Females were released in groups of three to 11. The number released was dependent on the experiment and availability of ovulated females. Each *P. marinus* released in an experiment was considered an individual sample (Siefkes *et al.*, 2005; Johnson *et al.*, 2009). Trial date was evaluated as a random effect in all statistical analyses to test if females released on a given date behaved differently than females released on other dates. The random effect of trial date never explained sufficient variation in the data to merit its inclusion in any statistical model as determined by likelihood ratio tests. All statistical tests were conducted in R version 2.9.2 (R Development Core Team; www.r-project.org).

Experimental stream

Experiments were conducted in bifurcated and single channel reaches of the upper Ocqueoc River, MI, U.S.A., characterized by water <1 m deep and water velocities between 0.2 and 1.0 m s⁻¹ with gravel and boulder substratum (Applegate, 1950; Johnson *et al.*, 2009). The upper Ocqueoc River was historically infested with *P. marinus* (Applegate, 1950), but a barrier built several km downstream currently prevents infestation. Accordingly, it was assumed that natural populations of *P. marinus* and their pheromones were not present. Discharge was estimated using a flow meter (Flow Mate, Marsh-McBirney Incorporated; www.marsh-mcBirney.com; McMahon *et al.*, 1996) at least once weekly or after rainfall >1 cm. During experimentation, discharge ranged from 0.70 to 1.34 m³ s⁻¹.

Test odorants

Application of synthesized 3kPZS and 3kACA (Bridge Organics; www.bridgeorganics.com) and pheromone-related bioactive components was approved by the Michigan Department of Environmental Quality and United States Environmental Protection Agency through experimental user permit 75437-EUP-2. Purity of 3kPZS and 3kACA was >95% and dissolved in methanol. Water conditioned with spermated males was termed spermated male washings (SMW). Washings were aliquoted into 1 l bottles and frozen at -20° C. All SMW and extracted odorants were analysed for 3kPZS concentration using liquid chromatography coupled with mass spectrometry (LC-MS/MS; Xi *et al.*, 2011). Test odorants were applied to reach target molar (M) concentrations, calculated assuming full mixing with stream discharge. The amount of the odorant needed was diluted in river water (to make a working solution) and then introduced into the stream with peristaltic pumps (Cole-Parmer; www.coleparmer.com). In all experiments, females were pre-exposed to the odorants for 30 min prior to release.

OBJECTIVE 1: FUNCTION OF C24 SULPHATE OF 3kPZS

General approach

The goal of experiments in this subsection was to determine if the C24 sulphate in 3kPZS specifically induces upstream movement by determining if 3kACA modifies ovulated female behaviour in streams. A set of experiments was specifically designed to evaluate whether 3kACA alone or when added to 3kPZS influences (1) long-distance upstream movement to a spawning ground, (2) the ability of females to locate a nest on a spawning ground and (3) female nesting behaviour.

Does 3kACA direct long-distance upstream movement?

A *P. marinus* trap was set in each channel of the bifurcated stream described in Johnson *et al.* (2009) where an island divides the Ocqueoc River into two nearly identical channels with similar depth, width and water velocity. The traps were 0.359 m^3 , had two funnels and were set so that the funnels were parallel to current direction to create an odorant plume originating from the downstream trap entrance. Females were released 70 m downstream of the traps and the capture rates of females were observed 2 h after release when (1) both traps were baited with control vehicle (1 ml methanol), (2) when one trap was baited with 3kACA at 10^{-11} , 10^{-12} , 10^{-13} or 10^{-14} M and the other trap was baited with control vehicle and (3) when one trap was baited with a mixture of 3kPZS + 3kACA at a 1:0.1 ratio with 3kPZS equal to 10^{-11} , 10^{-12} , 10^{-13} or 10^{-14} M and the other trap was baited with control vehicle. Trials were conducted from 2 to 30 July 2005 and treatments were randomized without replacement. Differences in capture rates among traps baited with different odorants were evaluated with generalized linear models (GLM) assuming a binomial data distribution. Results from all GLMs reported in this article showed no evidence of overdispersion.

In a second experiment, upstream movement and capture rate of females over a 650 m distance were observed when (1) both traps were baited with control vehicle, (2) when one trap was baited with 3kPZS 10^{-12} M and the other trap baited with control vehicle and (3) when one trap was baited with a mixture of 3kPZS 10^{-12} M + 3kACA 10^{-13} M and the other trap was baited with control vehicle. Trials were conducted randomly without replacement from 31 May to 9 June 2006. Females were tracked for 8 h after release and the number of females that moved upstream into the trapping array, the number captured in traps, and the time it took to capture the *P. marinus* were recorded. Differences in the proportion of females moving upstream and the proportion captured in traps baited with different odorant treatments were evaluated with logistic regression models, where the probability of upstream movement or capture was explained by odorant treatment. Differences in the time to capture were evaluated with a GLM, where time to capture was explained by odorant treatment.

Does 3kACA influence short-range preference for spawning nests?

Female preference and retention on spawning nests baited with different ratios of 3kPZS + 3kACA were directly compared. Four spawning nests with dimensions of natural nests

(Applegate, 1950) were constructed perpendicular to stream flow and spaced *c.* 1 m apart in a section of the river with a single channel. Four ratios of 3kPZS + 3kACA were applied to each nest: (1) 3kPZS 10^{-12} M (1:0), (2) 3kPZS 10^{-12} M + 3kACA 10^{-12} M (1:1), (3) 3kPZS 10^{-12} M + 3kACA 10^{-13} M (1:0.1) and (4) 3kACA 10^{-12} M (0:1). The 3kPZS:3kACA ratio in SMW has been reported at *c.* 1:0.05 (Yun *et al.*, 2003) but varied within the range of ratios applied. Application of each ratio to a spawning nest was randomized. Trials were conducted from 13 to 28 June 2006. Females were released 250 m downstream of the odorants. Females were tracked for 2 h after release and the first nest visited by females and retention at that nest were recorded. The distribution of females visiting the odorant treatment was evaluated with a logistic regression model where entry into a nest was explained by odorant treatment. Difference in retention time at the first odorant treatment visited was evaluated with a GLM where retention at the nest was described by the odorant treatment.

Does 3kACA influence female nest cleaning behaviours?

The role of 3kACA in the display of nesting behaviours on spawning nests was tested. Five confined stream channels, 1.2 m wide and 5.0 m long, were constructed parallel to flow on the Ocqueoc River using plywood to separate flow in each channel and sandbags to prevent water from mixing between channels (Fig. 1). The confinement of water in each channel was confirmed with rhodamine dye tests (Turner Designs; www.turnerdesigns.com), where dye was applied to one channel, and water was sampled in neighbouring channels and analysed with a luminescence spectrometer (Perkin Elmer LSS55; www.perkinelmer.com). Block nets were placed at the upstream and downstream ends of each channel to prevent females from switching channels. In each channel, water depth ranged from 0.20 to 0.25 m, water velocity ranged from 0.20 to 0.28 m s⁻¹, and the substratum was gravel. A *P. marinus* nest was constructed 1 m downstream of the upstream block net in each channel (Applegate, 1950). The nest in each channel received one of the following odorants during a trial: (1) SMW with 3kPZS concentration of 10^{-12} M, (2) 3kPZS 10^{-12} M + 3kACA 10^{-13} M, (3) 3kPZS 10^{-12} M, (4) 3kACA 10^{-12} M and (5) control vehicle (1 ml methanol). Odorants were randomly applied to each nest during a trial as described in the above experiment. Trials were conducted from 20 to 24 July 2006. Three females were released in each channel per trial. Upon release, female entries into nests were recorded for 4 h. While in the nest, rock movements and tail fans were recorded. A rock movement was defined as when a female used its oral disc to move a rock in the nest. A tail fan was defined as a rapid tail movement that expelled silt from the nest. Differences in the number of females that visited nests baited with different

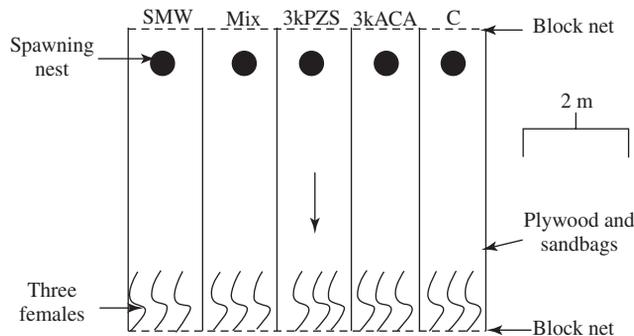


FIG. 1. Experimental design used to determine if 3kACA induced nest cleaning behaviours in ovulated female *Petromyzon marinus*. Five stream channels were created using plywood and sandbags to divide the flow of the Ocqueoc River, MI. Test odorants (SMW, spermated male washings; mix, 3kPZS 10^{-12} M + 3kACA 10^{-13} M; C, control vehicle) were randomly applied to a spawning nest at the upstream end of each channel. Three ovulated females were released in each channel. Females were retained in each channel by block nets. The arrow in the 3kPZS channel indicates direction of water flow.

test odorants were evaluated with a logistic regression model, where entry into a nest was explained by odorant treatment. Differences in the frequency of rock movements and tail fans in nests baited with different test odorants were evaluated with GLM, where the number of nest cleaning behaviours exhibited was explained by odorant treatment.

OBJECTIVES 2 AND 3: EXTRACTION EFFICIENCY AND RELEASE LOCATION OF MALE MATING PHEROMONES

Extraction methods and collection of extraction products

SMW were extracted for behavioural assays using macrotetacular aliphatic acrylic polymer resin (Amberlite XAD7HP, Sigma-Aldrich; www.sigma-aldrich.com; XAD). XAD extract was subjected to reversed-phase octadecyl resin (C18, Waters Cooperation; www.waters.com) to further concentrate the extract. Samples collected for behavioural tests included SMW, XAD extract, water that passed through XAD extraction (XAD waste water), C18 extract and water that passed through the C18 extraction (C18 waste) as described in Fig. 2. Technical details of extraction techniques are described in supporting information Appendix SI.

Anterior and posterior washings collection

A plexiglass washing tank, with a dry chamber separating anterior and posterior regions, was used to collect anterior and posterior washings of spermiated males (see Siefkes *et al.*, 2003 for details). Deionized water (7 l) was put in each compartment and aerated. One male was placed in the tank for 1 h, then removed and the anterior and posterior washings were frozen at -20° C. Anterior and posterior washings from 20 different males were thawed, combined according to their source (140 l each) and refrozen at -20° C.

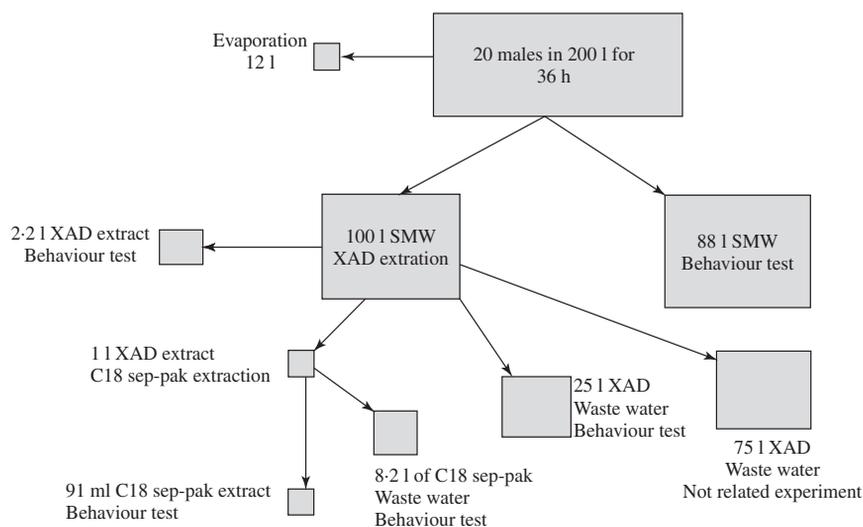


FIG. 2. Extraction of spermiated male *Petromyzon marinus* washings (SMW) for testing in-stream bioassays. Twenty spermiated males were placed in 200 l of water for 36 h. Eighty-eight litres of washings was frozen for the behaviour test. Hundred litres of the SMW was extracted with macrotetacular aliphatic acrylic polymer (XAD) resin. Twenty-five litres of the water that passed through the XAD resin was frozen for the behaviour test. Elution of the XAD resin yielded 3.2 l of methanol extract and 2.2 l of the extract was frozen for the behaviour test. One litre of the XAD extract was further extracted with 100 g of C18 resin in a sep-pak. Ninety-one millilitres of C18 extract was collected for behaviour test and 8.2 l of water that passed through C18 sep-pak was frozen for the behaviour test.

Behavioural assay

Behavioural responses of ovulated females to extraction and washing products were evaluated in the bifurcated stream segment described above (Johnson *et al.*, 2009). In each stream channel, two *P. marinus* nests were constructed 1.25 m apart. Odorants were directly compared by applying a test odorant to each nest. Test odorants were switched between nests for each trial. Both channels of the river were used simultaneously to conduct independent experiments. Sandbags were used to fortify sections of the island to prevent mixing of water between channels. Females were held in a 1 m³ acclimation cage located 45 m downstream of the baited nests. PIT-tagged ovulated females were released for 2 h and PIT tag antennas recorded which nests females entered and how long they remained. The number of rock movements and tail fans in each nest per trial were recorded. Differences in the first odorant visited were evaluated with logistic regression models, where the first nest visited was explained by odorant treatment. Differences in retention, rock movements and tail fans were evaluated with GLMs, where the behaviour was explained by odorant treatment. Exceptions to this statistical framework are stated when individual comparisons are described.

Direct comparisons of test odorants

Ten pairs of odorants were directly compared using the in-stream bioassay to accomplish objectives 2 and 3. Odorant release rates were standardized to reach a final in-channel 3kPZS concentration of 5×10^{-13} M. Direct comparisons of test odorants were categorized into three groups: XAD extraction comparisons, C18 extraction comparisons and anterior and posterior washing comparisons. Each comparison of an odorant pair was subjected to a separate statistical analysis because it was not expected that all 10 comparisons would be completed in one field season, and thus, comparisons were not randomized through time.

The first group of direct comparisons ($n = 4$) evaluated products of XAD extraction. (1) SMW were compared to XAD extract of the same SMW in the right channel (determined by looking upstream) from 1 to 7 July 2008. If XAD resin extracted and eluted all behaviourally active pheromone components in SMW, female attraction, retention and cleaning behaviours in nests baited with XAD extract and SMW should be equal. (2) SMW were compared to XAD extract + XAD waste water in the left channel from 1 to 7 July 2008. If XAD extraction captured, but failed to elute all behaviourally active pheromone components with the same experimental efficiency, female attraction, retention and cleaning behaviours in nests baited with SMW should be greater than nests baited with XAD extract + XAD waste. (3) XAD extract was compared to synthesized 3kPZS in the right channel from 8 to 18 July 2008. If XAD captured and eluted 3kPZS plus additional unidentified pheromone components, female attraction, retention and cleaning behaviours in the nest baited with XAD extract would be greater than nests baited with synthesized 3kPZS. (4) Synthesized 3kPZS was compared to synthesized 3kPZS + XAD waste in the left channel from 8 to 18 July 2008. If behaviourally active pheromone components passed through the XAD resin, female attraction, retention and cleaning behaviours in the nest baited with 3kPZS + XAD waste would be greater than nests baited with synthesized 3kPZS. In this comparison, the logistic regression model, which evaluated data describing the first nest visited, included the explanatory variables of pheromone treatment and nest location because females preferred to enter the right nest first regardless of pheromone treatment ($\chi^2 = 9.49$, d.f. = 1, $P = 0.01$).

The second group of comparisons ($n = 4$) evaluated products of C18 extraction. (1) C18 extract (of XAD extract of SMW) was compared to SMW in the right channel from 20 to 25 July 2008. (2) SMW were compared to C18 extract + C18 waste in the right channel from 26 to 30 July 2008. In this comparison, the logistic regression model, which evaluated data describing the first nest visited, included the explanatory variables of pheromone treatment and nest location because females preferred to enter the right nest first regardless of pheromone treatment ($\chi^2 = 4.94$, d.f. = 1, $P < 0.05$). Also, the general linear model evaluating rock movements included the explanatory variables of pheromone treatment and nest location because females exhibited more rock movements in the right nest regardless of pheromone treatment ($t = -3.11$, d.f. = 9, $P < 0.05$). (3) C18 extract was compared to synthesized 3kPZS in the left channel from 20 to 25 July 2008. In this comparison, the

logistic regression model, which evaluated data describing first nest visited, included the explanatory variables of pheromone treatment and nest location because females preferred to enter the right nest first regardless of pheromone treatment ($\chi^2 = 5.73$, d.f. = 1, $P < 0.05$). (4) Synthesized 3kPZS was compared to 3kPZS + C18 waste in the left channel from 26 to 30 July 2008. The comparisons above were selected based on the same logic as comparisons for XAD extraction products. See supporting information Appendix SI for details concerning the volume of extraction waste water applied to a nest when mixed with XAD extract, C18 extract or synthesized 3kPZS.

The third group of comparisons ($n = 2$) evaluated anterior and posterior washings from permeated males. For comparisons of SMW from the anterior and posterior regions, an equal volume of washings from each region were applied to each nest. (1) Anterior washings were compared to posterior washings in the left channel from 31 July to 5 August 2008. (2) Anterior washings were compared to posterior washings + synthesized 3kPZS in the right channel from 31 July to 7 August 2008. Posterior washings were spiked with 3kPZS to ensure some females would visit the posterior washings nest.

RESULTS

C24 SULPHATE IS CRITICAL FOR 3kPZS IN DIRECTING UPSTREAM MOVEMENT OF OVULATED FEMALE *P. MARINUS*

3kPZS specifically directs upstream movement

Capture rates of females in traps baited with a mixture of 3kPZS + 3kACA at a 1:0.1 ratio with 3kPZS at 10^{-11} , 10^{-12} or 10^{-13} M were greater than traps baited with control vehicle and averaged 64% (Table I). Females were only caught in the trap baited with a 3kPZS + 3kACA mixture. Capture rates of females in traps baited with mixtures of 3kPZS + 3kACA at 10^{-11} , 10^{-12} or 10^{-13} M did not differ. Females did not swim upstream and enter traps baited with 3kACA when applied at concentrations ranging from 10^{-11} to 10^{-14} M. Capture rates of females in 3kACA-baited and control traps did not differ and were $<10\%$.

Adding 3kACA to 3kPZS did not increase the likelihood that females moved upstream and entered the pheromone-baited trap over a 650 m stream distance. Traps baited with 3kPZS alone and a mixture of 3kPZS + 3kACA did not differ in capture rate and lured *c.* 36% of females upstream and 33% of females into traps (Table II). The time taken for females to enter traps baited with 3kPZS and a mixture of 3kPZS + 3kACA did not differ (mean \pm s.d. = 207 ± 110 min). During control trials only 3% of females moved upstream, and no females were captured.

3kACA does not influence female preference for spawning nests

Nests baited with 3kPZS and mixtures of 3kPZS + 3kACA were visited by ovulated females. Nests baited with 3kACA (0:1) did not attract ovulated females (Table III). The proportion of females attracted to nests baited with 1:0, 1:1 and 1:0.1 3kPZS to 3kACA did not differ. Retention on the first nest visited did not differ among the three nests baited with 3kPZS (mean \pm s.d. = 407 ± 534 s).

3kACA does not influence female nest cleaning behaviours

About 50% of released females were lured to nests baited with SMW, a mixture of 3kPZS + 3kACA and 3kPZS, which was more than nests baited with 3kACA or

TABLE I. Number of ovulated female *Petromyzon marinus* captured when both traps were baited with control vehicle (control), when one trap was baited with control vehicle and the other trap was baited with 3kACA and when one trap was baited with control vehicle and the other was baited with a mixture of 3kPZS + 3kACA at 1:0.1 (mix; M = molar). When 3kACA or a mixture of 3kPZS + 3kACA was applied to a trap, the reported captures [captured (*n*)] are *P. marinus* that entered the pheromone-baited trap. Females were not captured in control traps when 3kACA or a mixture of 3kPZS + 3kACA was applied to a trap. Data were evaluated with logistic regression. Statistics reported in the furthest right column are contrasts between control treatments and mix treatments (e.g. control *v.* mix 10^{-11}). Statistics below the raw data report the overall significance of the model. Treatments with the same uppercase letter are not significantly different ($P = 0.05$)

Trap bait (M)	<i>n</i>	Captured (<i>n</i>)	Trap bait (M)	<i>n</i>	Captured (<i>n</i>)	<i>P</i> (d.f., χ^2)
Control	24	2 A	Control	24	2 A	NA
3kACA 10^{-11}	24	2 A	Mix 10^{-11}	24	15 B	0.001 (1, 11.70)
3kACA 10^{-12}	25	0 A	Mix 10^{-12}	24	18 B	<0.001 (1, 15.93)
3kACA 10^{-13}	19	0 A	Mix 10^{-13}	19	10 B	0.01 (1, 8.28)
3kACA 10^{-14}	12	1 A	Mix 10^{-14}	25	0 A	>0.05 (1, 0.00)
	χ^2	4.94		χ^2	43.87	
	d.f.	4		d.f.	4	
	<i>P</i>	>0.05		<i>P</i>	<0.001	

NA, not applicable.

control vehicle (Table IV). 3kACA alone did not lure females into nests. Females in nests baited with SMW exhibited a total of 118 rock movements and 34 tail fans, which was greater than all other test odorant treatments ($P < 0.05$ for all comparisons). Females averaged three rock movements and zero tail fans when in nests baited with 3kPZS, 3kPZS + 3kACA, 3kACA and control vehicle.

TABLE II. Number of ovulated female *Petromyzon marinus* that moved upstream 650 m and were captured when both traps were baited with control vehicle (control), when one trap was baited with control vehicle and the other trap was baited with 3kPZS and when one trap was baited with control vehicle and the other was baited with a mixture of 3kPZS (M) + 3kACA at a 1:0.1 ratio. Time indicates the average time for captured females to swim upstream and enter the baited trap. All females were captured in the pheromone-baited trap. Upstream movement and capture data were evaluated with logistic regression. Time to capture was evaluated with a general linear model. Treatments with the same uppercase letter are not significantly different ($P = 0.05$)

Treatment (M)	<i>n</i>	Upstream (<i>n</i>)	Captured (<i>n</i>)	Time (min)
Control	33	1 A	0 A	NA
3kPZS 10^{-12}	40	13 B	12 B	195 A
Mix 10^{-12}	41	16 B	15 B	217 A
	χ^2	19.59	22.09	<i>F</i> -stat = 0.30
	d.f.	2	2	NDF/DDF = 1/28
	<i>P</i>	<0.001	<0.001	<i>P</i> > 0.05

NA, not applicable; NDF, numerator d.f.; DDF, denominator d.f.

TABLE III. The first pheromone treatment visited by ovulated female *Petromyzon marinus* when presented with four nests baited with different pheromone treatments: 3kPZS alone, 3kPZS + 3kACA mixed 1:0.1, 3kPZS + 3kACA mixed 1:1 and 3kACA. Retention is the average time females spent on the first nest visited. The first compound listed in the treatment column was applied to reach an in-stream concentration of 10^{-12} M. Nest visit data were evaluated with logistic regression. Retention time was evaluated with a general linear model.

Treatments that share an uppercase letter were not significantly different ($P = 0.05$)

Treatment	Visit (<i>n</i>)	Retention (s)
3kPZS	17 A	448 A
3kPZS (1) + 3kACA (0.1)	16 A	415 A
3kPZS (1) + 3kACA (1)	14 A	347 A
3kACA	0 B	NA
χ^2	32.39	$F = 0.138$
d.f.	3	NDF/DDF = 2/44
<i>P</i>	<0.001	$P > 0.05$

NA, not applicable.

ADDITIONAL PHEROMONE COMPONENTS INDUCE NEST CLEANING BEHAVIOURS AND ARE EXTRACTED BY XAD RESIN

XAD extract contained pheromone components in addition to 3kPZS that induced rock movements and tail fans. Equal proportions of females visited nests baited with XAD extract and SMW. Females on nests baited with XAD extract and SMW exhibited equal numbers of rock movements and tail fans (Tables V and VI). Further, XAD extract, when compared to synthesized 3kPZS, attracted more females and

TABLE IV. Number of ovulated female *Petromyzon marinus* that were released (*n*) in channels baited with an odorant treatment and the number of females that moved upstream into the baited nest. Rock and fan indicate the total number of rock movements and tail fans exhibited by females in the baited nest. Spermiated male washings (SMW) and 3kPZS were applied to reach a 3kPZS concentration of 10^{-12} M. 3kACA, when applied alone, reached a concentration of 10^{-12} M. Mix = 3kPZS + 3kACA at 1:0.1. Nest entry data were evaluated with logistic regression and number of rock movements and tail fans evaluated with general linear models. Treatments that share an uppercase letter were not significantly different ($P = 0.05$)

Treatment	<i>n</i>	Nest (<i>n</i>)	Rock	Fan
SMW	15	9 A	118 A	34 A
3kPZS	15	9 A	5 B	0 B
Mix	15	6 A	5 B	0 B
3kACA	15	0 B	0 B	0 B
Control	15	3 B	2 B	1 B
χ^2		22.43	F	2.18
d.f.		4	NDF/DDF	4/20
<i>P</i>		<0.001	P	0.108
				0.126

TABLE V. Distribution of ovulated female *Petromyzon marinus* when presented with two nests baited with spermated male washings (SMW), extraction products from a non-ionic macroreticular aliphatic acrylic polymer (XAD) resin and a reversed-phase octadecyl (C18) resin. Retention is the average time spent within 0.5 m of the first nest visited. Distribution data were evaluated with logistic regression and retention data were evaluated with a general linear model

Treatment	Trials	n	Distribution		P (d.f., χ^2)	Retention		P (NDF/DDF, F)
			A (n)	B (n)		A (s)	B (s)	
SMW v. XAD	6	46	10	12	>0.05 (1, -0.19)	1508	796	0.05 (1/20, 4.69)
SMW v. XAD + waste	6	51	10	17	>0.05 (1, -1.80)	1017	1265	>0.05 (1/18, -0.40)
XAD v. 3kPZS	9	69	24	3	0.001 (1, 18.34)	645	175	>0.05 (1/25, 1.86)
3kPZS + waste v. 3kPZS	8	67	27	15	0.01 (1, 5.53)	1252	88	0.001 (1/40, 21.06)
SMW v. C18	6	48	20	10	>0.05 (1, 3.27)	1698	367	0.01 (1/23, 11.44)
SMW v. C18 + waste	6	54	25	14	0.05 (1, 4.36)	1030	316	0.01 (1/31, 11.91)
C18 v. 3kPZS	6	49	16	8	0.05 (1, 3.80)	749	81	0.05 (1/15, 4.44)
3kPZS + waste v. 3kPZS	6	54	10	12	>0.05 (1, -0.02)	62	238	>0.05 (1/18, -3.43)
Anterior v. posterior	4	38	15	0	0.001 (1, 20.19)	1444	N/A	NA
Anterior v. posterior + 3kPZS	8	69	14	15	>0.05 (1, -0.04)	926	207	0.01 (1/26, 11.31)

n, number of females released; A (n) and B (n), number of females that entered nest A or B first; A, the first odorant listed in the direct comparison column; B, the second odorant listed in the direct comparison column; NA, statistical test not applicable; waste, water that passed through an extraction resin; anterior, anterior washings; posterior, posterior washings.

TABLE VI. Total number (n) of rock movements and tail fans ovulated female *Petromyzon marinus* exhibited in two nests baited with spermated male washings (SMW), extraction products from a non-ionic macrotetacular aliphatic acrylic polymer (XAD) resin and a reversed-phase octadecyl (C18) resin. Data were evaluated with a general linear model

Treatment	Rock		Statistics P (NDF/DDF, F)	Fan		Statistics P (NDF/DDF, F)
	A (n)	B (n)		A (n)	B (n)	
Direct comparison						
SMW <i>v.</i> XAD	50	42	>0.05 (1/10, 0.04)	31	25	>0.05 (1/10, 0.60)
SMW <i>v.</i> XAD + waste	44	64	>0.05 (1/10, -0.19)	33	39	>0.05 (1/10, -0.03)
XAD <i>v.</i> 3kPZS	27	0	>0.05 (1/16, 3.47)	15	0	>0.05 (1/16, 3.23)
3kPZS + waste <i>v.</i> 3kPZS	78	0	0.05 (1/14, 4.61)	15	0	>0.05 (1/14, 2.94)
SMW <i>v.</i> C18	91	4	0.05 (2/9, 4.07)	39	0	0.05 (2/9, 5.68)
SMW <i>v.</i> C18 + waste	95	11	0.01 (2/9, 8.26)	59	3	>0.05 (1/10, 2.56)
C18 <i>v.</i> 3kPZS	14	0	>0.05 (1/10, 3.06)	3	0	>0.05 (1/10, 1.00)
3kPZS + waste <i>v.</i> 3kPZS	0	1	NA	0	0	NA
Anterior <i>v.</i> posterior	85	0	NA	29	0	NA
Anterior <i>v.</i> posterior + 3kPZS	61	8	>0.05 (1/14, 2.63)	19	0	NA

A, first odorant listed in the direct comparison column; B, the second odorant listed in the direct comparison column; NA, statistical test not applicable; waste, water that passed through an extraction technique; anterior, anterior washings; posterior, posterior washings.

XAD extract elicited rock movements and tail fans, while 3kPZS did not elicit nest cleaning behaviours.

The efficiency of XAD resin to extract and elute 3kPZS was low (supporting information Appendix SI and Tables SI and SII). An unknown quantity of unidentified pheromone components also passed through the XAD resin. Synthesized 3kPZS spiked with XAD waste water was more attractive, retained females longer and induced more nest cleaning behaviours than 3kPZS alone. Unidentified pheromone components that retained females on nests may not have been extracted by XAD resin, as SMW retained females longer than XAD extract. Retention on nests baited with XAD extract and 3kPZS did not differ, and 3kPZS + XAD waste showed higher retention than just 3kPZS.

In contrast to XAD extracts, C18 extract did not retain females longer or induce more nest cleaning behaviours when compared to synthesized 3kPZS. Nests baited with SMW attracted more females, retained females longer and induced more rock movements and tail fans than C18 extract of the XAD extract (Tables V and VI). These data demonstrated that C18 extract only contained 3kPZS at behaviourally active quantities. Even when C18 waste water was added to C18 extract, SMW were more attractive and induced more nest cleaning behaviours demonstrating that unidentified pheromone components did not pass through the C18 resin but probably remained on the resin after elution. Confirming these results are comparisons of 3kPZS and 3kPZS spiked with C18 waste water, where each test odorant equally attracted and retained females, but neither odorant induced nesting behaviours. Taken together, C18 sep-pak extraction captured 3kPZS with very high efficiency (supporting information Table SII), but unidentified pheromone components were not eluted from C18 resin with methanol.

ALL BEHAVIOURALLY ACTIVE MATING PHEROMONE COMPONENTS ARE RELEASED THROUGH THE ANTERIOR REGION

3kPZS was only present at measurable concentrations in anterior washings (supporting information Table SI). When anterior and posterior washings were directly compared, females only visited the nest baited with anterior washings (Tables V and VI). Females in nests baited with anterior washings were retained and exhibited mating behaviours at levels comparable to females in nests baited with whole SMW. Females showed equal attraction for nests baited with anterior washings and posterior washings spiked with 3kPZS, but anterior washings retained females longer and induced more mating behaviours than posterior washings spiked with 3kPZS.

DISCUSSION

The results confirm that 3kPZS is a specific and potent mating pheromone component that lures ovulated female *P. marinus* upstream to spawning nests. 3kACA, a potent olfactory stimulant and nearly identical in structure to 3kPZS, did not influence any female mating behaviours when applied over a 1000-fold range of concentrations. Replacing the C24 sulphate ester of 3kPZS with a carboxyl, while not diminishing its EOG potency, abolishes the function of 3kPZS in triggering upstream movement in ovulated females. These results further support that 3kACA targets a different olfactory receptor mechanism than 3kPZS (Siefkes & Li, 2004).

Results of the behavioural tests suggest that 3kACA does not interact synergistically or antagonistically with 3kPZS, even though these two compounds target different olfactory receptor mechanisms (Siefkes & Li, 2004). 3kACA did not function to retain females on nests or induce cleaning behaviours. A nest baited with 3kPZS alone lured and retained females as well as mixtures of 3kPZS + 3kACA at 1:1 and 1:0.1. Females in nests baited with a mixture of 3kPZS + 3kACA did not exhibit more rock movements or tail fans than 3kPZS-baited nests. Over a 70 m distance, traps baited with a mixture of 3kPZS + 3kACA captured 64% of females, which was higher than the 46% female capture rate in traps baited with 3kPZS alone observed in Johnson *et al.* (2009). During 650 m trapping experiments reported here, however, the proportion of females swimming upstream during 3kPZS and 3kPZS + 3kACA treatments did not differ and the number captured in traps baited with the two test odorants did not differ. These results are consistent with Luehring *et al.* (2010), who found that traps baited with mixtures of 3kPZS + 3kACA did not capture more females than traps baited with 3kPZS. The function of 3kACA, if one exists, has yet to be defined. Future work should determine if 3kACA facilitates synchronization of maturation in *P. marinus*.

Several lines of evidence from the chemical extraction coupled with spawning channel experiments (Fig. 1) demonstrate that SMW contain pheromone components in addition to 3kPZS + 3kACA that not only retain females on nests (Siefkes *et al.*, 2005; Johnson *et al.*, 2009), but induce rock movements and tail fanning. Activity-directed extraction of SMW showed that male mating pheromone components that induced rock movements and tail fans were captured by XAD resin and eluted from the resin with methanol; however, components retaining females on the nest were not captured by XAD. Although XAD resin extracted 3kPZS and unidentified pheromone

components, it did so with low efficiency (25% for 3kPZS). In the study by Fine *et al.* (2006), 3kPZS was extracted with XAD with 81% efficiency using same equipment, although the solution used in this study contained higher concentrations of 3kPZS. LC-MS/MS analysis indicated that 25% of the 3kPZS in the SMW passed through the XAD resin and was in the waste water, suggesting that extraction was incomplete. Bioassay comparisons determined that unidentified pheromone components were also in the waste water at high enough quantities to elicit behavioural responses. Using more XAD resin, slower flow rates, and solutions with lower 3kPZS concentrations would probably improve capture efficiency. It was evident, however, that 50% of 3kPZS was probably retained on the resin and not eluted when washed with 100% methanol because it was not present in the extract or waste water (supporting information Table SII). Rinsing the resin with >3 l of methanol may improve elution efficiency. Alternatively, stronger reversed-phase solvents such as acetone or hexane may increase elution of 3kPZS and unidentified pheromone components from XAD resin.

C18 sep-paks extracted and eluted 3kPZS from XAD extract with high efficiency; however, the in-stream bioassay showed that C18 resin captured unidentified pheromone components that induced mating behaviours, but did not release them when the resin was rinsed with 100 ml of 100% methanol. The success of Li *et al.* (2002) in isolating and purifying 3kPZS from SMW was probably attributed to selective elution of 3kPZS from C18 with methanol. Future investigations aimed at identifying additional behaviourally active pheromone components that could benefit from the differential extraction and elution characteristics of non-ionic macrotetacular aliphatic acrylic polymer resin and reversed-phase octadecyl resin.

Bioassay comparisons demonstrated that all behaviourally active pheromone components were excreted through the anterior region. Results of anterior and posterior washing experiments confirmed that 3kPZS is excreted from the anterior region and suggest that unidentified pheromone components may also be released across the gills in a similar manner as 3kPZS (Siefkes *et al.*, 2003). It is interesting that unidentified pheromone components that influence near-source behaviours may be actively released across the gills during respiration, whereas steroidal mating pheromones released by female *C. auratus* are passively excreted in the urine (Sorensen, 1992) and in male peacock blenny *Salaria pavo* (Risso 1810) pheromones are released by the anal gland (Barata *et al.*, 2008). Interactions of nesting *P. marinus* involve extensive touching with the oral disc and spawning occurs when a male attaches to a female's head (Manion & Hanson, 1980). Given the relative positions of *P. marinus* during courtship, it may be advantageous for mating pheromones with small active spaces to be released through the gills, whereas urinary release of pheromones would render pheromone detection difficult because the female naris is typically upstream of the male urogenital pore. As the pheromone components studied here are released through the anterior region, compounds in the urine or semen can be eliminated as potential releaser pheromones for the behaviours observed in this study.

In conclusion, the C24 sulphate ester of 3kPZS directly contributes to its specificity as a potent mating pheromone component that lures ovulated female *P. marinus* upstream to spawning nests. The multiple functions of the male mating pheromone are supported by multiple compounds. All pheromone components are excreted by males through the anterior region, which may be advantageous for efficient transmission between nesting *P. marinus*. Identification of additional components

of the *P. marinus* mating pheromone would enhance understanding of fish chemical communication and advance the use of pheromones in *P. marinus* control in the Great Lakes (Twohey *et al.*, 2003).

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SUPPORTING INFORMATION

Supporting Information may be found in the online version of this paper:

Appendix SI. Technical extraction methods, results and references.

Table SI. Volume, 3kPZS concentration and total 3kPZS in odorants generated for bioassay.

Table SII. Accounting of 3kPZS that originated through extraction

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References

- Applegate, V. C. (1950). *The Natural History of the Sea Lamprey in Michigan*. Washington, DC: U. S. Department of Interior Fish & Wildlife Service.
- Barata, E. N., Serrano, R. M., Miranda, A., Nogueira, R., Hubbard, P. C. & Canari, A. V. M. (2008). Putative pheromones from the anal glands of male blennies attract females and enhance reproductive success. *Animal Behaviour* **75**, 379–389.
- Bradshaw, J. W. S., Baker, R. & Lisk, J. C. (1983). Separate orientation and releaser components in a sex pheromone. *Nature* **304**, 265–267. doi: 10.1038/304265a0
- Dulka, J. G., Stacey, N. E., Sorensen, P. W. & Vanderkraak, G. J. (1987). A steroid sex-pheromone synchronizes male-female spawning readiness in goldfish. *Nature* **325**, 251–253. doi: 10.1038/325251a0
- Elkins, A., Barrow, R. & Rochfort, S. (2009). Carp chemical sensing and the potential of natural environmental attractants for control of carp: a review. *Environmental Chemistry* **6**, 357–368. doi: 10.1071/EN09032
- Fine, J. M., Sisler, S. P., Vrieze, L. A., Swink, W. D. & Sorensen, P. W. (2006). A practical method for obtaining useful quantities of pheromones from sea lamprey and other fishes for identification and control. *Journal of Great Lakes Research* **32**, 832–838. doi: 10.3394/0380(2006)32[832:APM Fou] 2.0.Co;2
- Johnson, N. S. & Li, W. (2010). Understanding behavioural responses of fish to pheromones in natural freshwater environments. *Journal of Comparative Physiology A* **196**, 701–711. doi: 10.1007/s00359-101-0523-7

- Johnson, N. S., Luehring, M. A., Siefkes, M. J. & Li, W. (2006). Mating pheromone reception and induced behaviour in ovulating female sea lampreys. *North American Journal of Fisheries Management* **26**, 88–96. doi: 10.1577/M05-018.1
- Johnson, N. S., Yun, S.-S., Thompson, H. T., Brant, C. B. & Li, W. (2009). A synthesized pheromone induces upstream movement in female sea lampreys and summons them into traps. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 1021–1026. doi: 10.1073/pnas.0808530106
- Kobayashi, M., Sorensen, P. W. & Stacey, N. E. (2002). Hormonal and pheromonal control of spawning behaviour in the goldfish. *Fish Physiology and Biochemistry* **26**, 71–84.
- Li, W. M. (2005). Potential multiple functions of a male sea lamprey pheromone. *Chemical Senses* **30**, I307–I308. doi: 10.1093/chemse/bjh237
- Li, W., Scott, A. P., Siefkes, M. J., Yan, H., Liu, Q., Yun, S. S. & Gage, D. A. (2002). Bile acid secreted by male sea lamprey that acts as a sex pheromone. *Science* **296**, 138–141. doi: 10.1126/science.1067797
- Linn, C. E., Campbell, M. G. & Roelofs, W. L. (1987). Pheromone components and active spaces: what do moths smell and where do they smell it? *Science* **237**, 650–652. doi: 10.1126/science.237.4815.650
- Luehring, M. A., Wagner, C. M. & Li, W. (2010). The efficacy of two synthesized sea lamprey sex pheromone components as a trap lure when placed in direct competition with natural male odors. *Biological Invasions* **13**, 1589–1597. doi: 10.1007/s10530-010-9916-3
- Manion, P. J. & Hanson, L. H. (1980). Spawning behaviour and fecundity of lampreys from the upper three Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences* **37**, 1635–1640. doi: 10.1139/f80-211
- McMahon, T. E., Zale, A. V. & Orth, D. J. (1996). Aquatic habitat measurements. In *Fisheries Techniques*, 2nd edn (Murphy, B. R. & Willis, D. W., eds), pp. 83–120. Bethesda, MD: American Fisheries Society.
- Pickering, A. D. & Morris, R. (1977). Sexual dimorphism in the gills of the spawning river lamprey, *Lampetra fluviatilis* L. *Cell and Tissue Research* **180**, 1–10. doi: 10.1007/BF00227026
- Siefkes, M. J. & Li, W. (2004). Electrophysiological evidence for detection and discrimination of pheromonal bile acids by the olfactory epithelium of female sea lampreys (*Petromyzon marinus*). *Journal of Comparative Physiology A* **190**, 193–199. doi: 10.1007/s00359-003-0484-1
- Siefkes, M. J., Scott, A. P., Zielinski, B., Yun, S. S. & Li, W. (2003). Male sea lampreys, *Petromyzon marinus* L., excrete a sex pheromone from gill epithelia. *Biology of Reproduction* **69**, 125–132. doi: 10.1095/biolreprod.102.014472
- Siefkes, M. J., Winterstein, S. R. & Li, W. M. (2005). Evidence that 3-keto petromyzonol sulphate specifically attracts ovulating female sea lamprey, *Petromyzon marinus*. *Animal Behaviour* **70**, 1037–1045. doi: 10.1016/j.anbehav.2005.01.024
- Smith, B. R. & Tibbles, J. J. (1980). Sea lamprey (*Petromyzon marinus*) in Lakes Huron, Michigan, and Superior - history of invasion and control, 1936-78. *Canadian Journal of Fisheries and Aquatic Sciences* **37**, 1780–1801. doi: 10.1139/f80-222
- Sorensen, P. W. (1992). Hormonally derived sex-pheromones in goldfish - a model for understanding the evolution of sex-pheromone systems in fish. *Biological Bulletin* **183**, 173–177. doi: 10.2307/1542420
- Sorensen, P. W. & Vrieze, L. A. (2003). The chemical ecology and potential application of the sea lamprey migratory pheromone. *Journal of Great Lakes Research* **29**, 66–84. doi: 10.1016/S0380(03)70478-X
- Sorensen, P. W., Stacey, N. E. & Chamberlain, K. J. (1989). Differing behavioural and endocrinological effects of two female sex pheromones on male goldfish. *Hormones and Behavior* **23**, 317–332.
- Sorensen, P. W., Fine, J. M., Dvornikovs, V., Jeffrey, C. S., Shao, F., Wang, J., Vrieze, L. A., Anderson, K. R. & Hoyer, T. R. (2005). Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey. *Nature Chemical Biology* **1**, 324–328. doi: 10.1038/nchembio739

- Twohey, M. B., Sorensen, P. W. & Li, W. M. (2003). Possible applications of pheromones in an integrated sea lamprey management program. *Journal of Great Lakes Research* **29**, 794–800. doi: 10.1016/S0380(03)70532-2
- Vladykov, V. D. (1949). *Quebec Lamprey. I.-List of Species and their Economic Importance*. Quebec: Province of Quebec Department of Fisheries.
- Vrieze, L. A., Bjerselius, R. & Sorensen, P. W. (2010). Importance of the olfactory sense to migratory sea lampreys *Petromyzon marinus* seeking riverine spawning habitat. *Journal of Fish Biology* **76**, 949–964.
- Yambe, H., Kitamura, S., Kamio, M., Yamada, M., Matsunaga, S., Fusetani, N. & Yamazaki, F. (2006). L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 15370–15374. doi: 10.1073/pnas.0604340103
- Yun, S. S., Scott, A. P. & Li, W. (2003). Pheromones of the male sea lamprey, *Petromyzon marinus* L.: structural studies on a new compound, 3-keto allocholic acid, and 3-keto petromyzonol sulphate. *Steroids* **68**, 297–304. doi: 10.1016/S0039-128X(02)00178-2
- Xi, X., Johnson, N. S., Brant, C. O., Yun, S.-S., Chambers, K. L., Jones, A. D. & Li, W. (2011). Quantification of a male sea lamprey pheromone in tributaries of the Laurentian Great Lakes by liquid chromatography-tandem mass spectrometry. *Environmental Science and Technology* **45**, 6437–6443. doi: 10.1021/es200416f