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RESEARCH ARTICLE

Evidence for partial overlap of male olfactory cues in lampreys

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ABSTRACT

Animals rely on a mosaic of complex information to find and evaluate mates. Pheromones, often consisting of multiple components, are considered to be particularly important for species-recognition in many species. Although the evolution of species-specific pheromone blends is well described in many insects, very few vertebrate pheromones have been studied in a macro-evolutionary context. Here, we report a phylogenetic comparison of multi-component male odours that guide reproduction in lampreys. Chemical profiling of sexually mature males from eleven species of lamprey, representing six of ten genera and two of three families, indicated that the chemical profiles of sexually mature male odours are partially shared among species. Behavioural assays conducted with four species sympatric in the Laurentian Great Lakes indicated asymmetric female responses to heterospecific odours, where *Petromyzon marinus* were attracted to male odour collected from all species tested, but other species generally preferred only the odour of conspecifics. Electro-olfactogram recordings from *P. marinus* indicated that although *P. marinus* exhibited behavioural responses to odours from males of all species, at least some of the compounds that elicited olfactory responses were different in conspecific male odours compared with heterospecific male odours. We conclude that some of the compounds released by sexually mature males are shared among species and elicit olfactory and behavioural responses in *P. marinus*, and suggest that our results provide evidence for partial overlap of male olfactory cues among lampreys. Further characterization of the chemical identities of odour components is needed to confirm shared pheromones among species.

KEY WORDS: Petromyzontiformes, Species specificity, Chemical communication, Complex signals, Pheromone

INTRODUCTION

Animals search for and assess mates using a mosaic of multi-modal and multi-component information originating from potential mates (Andersson, 1994; Candolin, 2003; Bradbury and Vehrencamp, 2011). Individuals evaluate mates using multiple traits (cues), some of which are specifically expressed for the purpose of communication (signals). Individual cues within complex signals can provide redundant or distinct information (Bradbury and Vehrencamp, 2011). Multiple sources of information can be important for species-recognition, as shared ancestry and selective pressures can result in particular traits being important for mate choice across species (Pfennig, 1998; Candolin, 2003). For example, size is an important trait for mate choice in several swordtail species (*Xiphophorus phygmaeus* and *X. nigrensis*), but olfactory cues guide species-recognition (Crapon de Caprona and Ryan, 1990; Hankison and Morris, 2003). Across animal taxa, complex sexual signals that mediate species-recognition are particularly important for closely related sympatric species (Gerhardt, 1994; Höbel and Gerhardt, 2003). However, even partial overlap in the traits involved in mate search and choice can result in potentially lower fitness through reproductive interference (Crapon de Caprona and Ryan, 1990; Gröning and Hochkirch, 2008).

Olfactory communication is often considered to employ signals that mediate species-recognition (Endler, 1993; Wyatt, 2014). Pheromones, defined as chemicals that elicit an innate and specific reaction when detected by conspecifics (Karlson and Lüscher, 1959), often consist of species-specific blends of multiple components (Wyatt, 2014). Species-specific pheromone blends are hypothesized to evolve through either gradual transitions or major 'saltational' shifts (Symonds and Elgar, 2008), which will result in different degrees of pheromone overlap between species. For example, the major component of the pheromone blend in scarab beetles (*Anomala albopilosa* and *A. cuprea)* is shared between species, while minor components are species specific (Leal et al., 1996). In contrast, pheromone blends used by bark beetles (*Dendroctonus* and *Ips* species) are equally as different in distantly and closely related species (Symonds and Elgar, 2004). Although the evolution of species-specific pheromone blends in insects is increasingly well described (Symonds and Elgar, 2008; Steiger et al., 2011), similar macro-evolutionary studies of vertebrate pheromones are under-represented (Symonds and Elgar, 2008).

Petromyzon marinus is a jawless vertebrate that uses a multi-component pheromone during reproduction (Teeter, 1980; Buchinger et al., 2015). Parasitic lampreys reside in streams as juveniles for several years, emigrate downstream into lakes or oceans to feed on fish, and return to streams to spawn. The odours of stream-resident larvae guide adult *P. marinus* during the migration into spawning streams (Teeter, 1980; Vrieze and Sorensen, 2001). Upon reaching the final stages of sexual maturation, males construct nests and signal with an odour that elicits upstream movement and nesting behaviours in females (Li et al., 2002; Siefkes et al., 2005; Johnson et al., 2012). The major component 7α, 12α, 24-trihydroxy-5α-cholan-3-one-24-sulfate (3-keto petromyzonol sulfate, 3kPZS) guides female movement over long distances to the nest (Li et al., 2002; Siefkes et al., 2005; Johnson et al., 2009). Additional minor components that retain females in the area of the nest and elicit nesting behaviours remain unidentified (Johnson et al., 2012), but may include 3,12-diketo-4,6-petromyzonene-24-sulfate (DkPES; Li et al., 2013; Brant et al., 2016a) and petromyzestrosterol (Li et al., 2012).

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The olfactory cues and pheromones of *P. marinus* appear to be partially conserved among Petromyzontiformes. Many of the 41 species of lamprey occur in sympatry with one or more other species (Potter et al., 2015). The larval-released migratory cue appears to consist of unspecialized metabolites that are conserved among lamprey species (Yun et al., 2003; Fine et al., 2004; Robinson et al., 2009; Yun et al., 2011; Buchinger et al., 2013). Similar habitat preferences for rearing and spawning conceivably preclude strong selective pressure for a species-specific migratory cue (Dawson et al., 2015; Johnson et al., 2015). Frequent observations of heterospecific spawning indicate that unknown components of the male mating pheromone blend may be partially shared among species (Cochran et al., 2008; Johnson et al., 2015), but the species specificity of the mating pheromone remains unknown.

Here, we report a phylogenetic comparison of male olfactory cues across lampreys. Based upon observations of heterospecific spawning, we hypothesized that male olfactory cues exhibit partial overlap among species. According to our hypothesis, we predicted (1) overlap in the compounds released by sexually mature males, (2) behavioural responses of females to the odour of both conspecific and heterospecific males, and (3) olfactory sensitivity to conspecific and heterospecific male odours. To test our hypothesis, we determined (1) chemical profiles of compounds released by sexually mature males in eleven species of lamprey, (2) female responses to conspecific and heterospecific male odours in species sympatric in the Laurentian Great Lakes, and (3) the electrophysiological responses of *P. marinus* to male odours of species sympatric in the Laurentian Great Lakes. Our results offer a rare phylogenetic comparison of multi-component olfactory cues in a vertebrate.

MATERIALS AND METHODS

Experimental animals

Use of experimental animals and approaches were approved by the Michigan State University's Animal Use and Care Committee (approval numbers 4/10-043-00 and 02-13-040-00). Lampreys were collected using traps, backpack electrofishing, fyke nets, or by hand (Table 1). Classification of species followed Renaud (2011). Sexual maturation was evaluated by the expression of eggs (ovulation) or milt (spermiation) upon gentle manual pressure (Siefkes et al., 2003). Adult lampreys that did not express gametes were deemed sexually immature. Chemical profiling was completed in 11 species; behavioural and electrophysiological assays were only completed in species sympatric in the Laurentian Great Lakes, because the release of chemicals as possible odorants was most important to test our hypothesis and more logistically feasible than behavioural and electrophysiological assays. Notably, the sympatric species tested in behavioural and electrophysiological assays included *P. marinus*, a species that recently became sympatric with *Ichthyomyzon unicuspis*, *I. fossor*, *I. castaneus* and *Lethenteron appendix* in the upper Laurentian Great Lakes, but which also co-occurs naturally with the same species in some areas (i.e. St Lawrence River Basin; Table 1).

Male chemical profiles

Chemical profiles of sexually mature males were determined for eleven species of lamprey from six genera and two families (Table 1). Odours were sampled by collecting the holding waters from individual sexually mature males (Buchinger et al., 2013). Samples were also collected from sexually immature males for comparison, except in *P. marinus*, *I. castaneus* and *Lampetra aeryptera*, due to difficulty in obtaining experimental animals. A single male was held in 5 litres of aerated water for 2 h, after which 10 ml of water was sampled, and stored below −20°C for subsequent analysis. Although male lampreys exhibit inter- and intraspecific variation in size, we collected odours in the same volume of water regardless of size following previous reports (Siefkes et al., 2003; Buchinger et al., 2013; Brant et al., 2013). Six replicates were sampled, except for sexually mature *Geotria australis* (N=4), *I. castaneus* (N=5), and sexually immature *Entosphenus tridentatus* (N=3; Table 1). Admittedly, chemical profiling does not directly implicate species similarities or differences in the compounds that are behaviourally active pheromones. However, chemical profiling reveals which compounds are in the water and available to the female olfactory system. Furthermore, our comparison was limited to compounds released by sexually mature males by contrasting profiles of sexually mature males against immature males, and hence the observed chemical profiles include likely candidates for olfactory cues.

Chemical profiles were determined using ultra-high performance liquid chromatography (UHPLC) and high resolution mass spectrometry (HR-MS). Water samples were evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco, Kansas City, MO, USA) and reconstituted in 50% HPLC-grade methanol:water (v:v). Aliquots (10 µl) of concentrated water samples were injected into a Waters Acquity UPLC coupled to a Xevo G2-S™ Q-Tof system (Waters Corporation, Milford, MA, USA). Metabolites were separated using an ACQUITY C_{18} BEH UPLC column (2.1×100 mm, 1.7 µm particle size; Waters Corporation; 30°C), with a mobile phase of acetonitrile (A) and water (B). The gradient elution used a flow rate of 300 µl min^{-1} for 10 min and the following gradient programme: 20% A for 1.0 min; increased to 100% A from 1.0 to 7.0 min; maintained at 100% A from 7.01 to 9.0 min; decreased to 20% A at 9.01 min and maintained for 10 min until column equilibrium. The needle was washed with 80% methanol twice after each injection to prevent cross-contamination of samples. Mass spectrometry was performed in negative electrospray ionization mode. A full scan MS analysis of samples was conducted by recording spectra with mass to charge ratios (m/z) between 100 and 1000, and with a resolution of ±0.05 Da. Nitrogen gas was used as the desolvation gas (600 l h^{-1}) and the cone gas (50 l h^{-1}). Argon gas was used as the collision gas at a pressure of 5.3×10^{-5} Torr. The source and desolvation temperatures were 102 and 400°C, respectively. The cone voltage and capillary voltage were set to 30 V and 2.8 kV, respectively. The collision energies for collision-induced dissociation were 5 and 40 eV for the MS spectrum and MS/MS spectrum, respectively. The scan time was set at 0.2 s, with an interscan delay of 0.5 s. The LockSpray™ dual electrospray ion source with internal references used for these experiments was leucine enkephalin at a concentration of 100 ng ml^{-1}. Lock-mass calibrations at m/z 554.2615 in negative ion mode were used for the complete analysis. UHPLC HR-MS yielded a list of intensities of the detected peaks identified by the corresponding retention times and mass data pairs. The ion intensities for each peak detected were then normalized within each sample by the sum of the peak intensities in that sample, with a total intensity of 10,000. Hence the end metric for each peak is a magnitude relative to the other peaks in the sample, out of 10,000.

The chemical profiles of males were filtered by eliminating peaks that had a normalized peak intensity less than 10 (<0.1% of the total peak intensity). The remaining peaks were compared against a control group. Controls were samples collected from sexually

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Table 1. Pheromone sampling from males from eleven species of Petromyzontiformes

Species	Maturity	Location	Mass (g)	Length (mm)	Collection	Distribution
Geotria australis	m	Canterbury, NZ	164.36±22.21	488.25±26.21	h	Drainages of southern Australia, New Zealand, Chile, Argentina
	i	Southland, NZ	149.10±3.07	564.50±4.29	n	
Ichthyomyzon unicuspis	m	Michigan, USA	41.22±4.72	248.67±7.94	t	Drainages of Hudson Bay, Great Lakes, St Lawrence River and Mississippi River
	i	Michigan, USA	47.76±4.21	270.00±10.61	t	
I. fossor	m	Michigan, USA	2.90±0.22	113.50±1.73	ef	Same as *I. unicuspis*
	i	Michigan, USA	5.43±0.73	126.17±4.11	ef	
I. castaneus	m	Michigan, USA	33.26±3.00	239.4±9.74	h/t	Drainages of Hudson Bay, Great Lakes, St Lawrence River, Mississippi River, Gulf of Mexico
Petromyzon marinus	m	Michigan, USA	177.83±19.15	443.83±10.98	t	Drainages of the North Atlantic
Entosphenus tridentatus	m	Oregon, USA	336.83±18.05	554.33±11.16	n	Drainages of the North Pacific
	i	Oregon, USA	392.33±20.63	578.33±9.21	n	
Lethenteron appendix	m	Michigan, USA	3.94±0.17	137.17±1.19	h	Drainages of the Great Lakes, eastern USA, St Lawrence River and Mississippi River
	i	Michigan, USA	4.59±0.59	149.00±6.57	h	
L. camtschaticum	m	Jilin, China	101.09±12.03	390.00±12.96	n	Drainages of Arctic and North Pacific
L. reisneri	m	Liaoning, China	7.04±0.82	167.83±7.25	ef	Drainages of Amur River, Sakhalin Island and Kamchatka Peninsula, Russia and in South Korea and Japan
L. morii	m	Liaoning, China	28.49±1.13	261.83±3.85	n	Drainages of the Yalu River
	i	Liaoning, China	37.16±3.65	288.00±6.05	n	
Lampetra aeryptera	m	Indiana, USA	7.51±0.68	156.50±4.04	ef	Drainages of northwestern Atlantic and Gulf of Mexico

Maturity: sexually immature (i) or mature (m). Mean±s.e.m. values for mass and total length are given. Collection method: hand (h), net (n), trap (t) or electrofishing (ef). Reported native distribution is given according to Potter et al. (2015) and Renaud (2011).

immature males of each species if available, but *P. marinus*, and *L. aeryptera* were compared with blank water, *I. castaneus* was compared with sexually immature male *I. unicuspis*, and *L. camtschaticum* and *L. reisneri* were compared with sexually immature males of the closely related *L. morii*. We estimated the effect of this discrepancy by repeating our analysis for *P. marinus* using sexually immature male *I. unicuspis* as the control instead of blank water. *Ichthyomyzon unicuspis* is closely related to *P. marinus* and was sampled at the same laboratory, and was a logical alternative control. The proportional intensities of each peak were arcsine square root transformed to meet assumptions of the distribution and differences between the peaks in male samples and control samples were evaluated using one-way *t*-tests (α=0.1). We did not control for multiple comparisons with a *post hoc* adjustment because our goal was a conservative removal of peaks that were detected in control and mature male samples. A multivariate factor analysis was conducted to determine if species could explain variation in the detected peaks. The *factanal* () function in R was used to reduce peaks to factors (http://www.R-project.org/). The number of factors to extract was determined using a scree plot. A multivariate analysis of variance (MANOVA; α=0.05) was used to determine if there was a difference in each factor across species and *post hoc t*-tests with a Benjamini and Hochberg (1995) adjustment were used to evaluate differences between species (α=0.05).

UHPLC HR-MS provides the relative intensity of peaks with a given retention time and mass–charge (*m/z*) ratio, but does not provide structural information about the peak or allow a particular peak to be attributed to specific compounds. Hence UHPLC-HRMS allows us to test our hypothesis that species exhibit overlap in male chemical profiles, but does not allow conclusions regarding the specific structures of compounds released by males.

Behavioural responses of females to male odours

Two-choice behavioural assays were used to evaluate female responses to conspecific and heterospecific male odours when compared with no odour (Siefkes et al., 2005; Buchinger et al., 2013). Behavioural responses were evaluated for sympatric *I. unicuspis*, *I. fossor*, *P. marinus*, and *L. appendix* based upon availability. Experimental apparatus were constructed adjacent to the Little Oqcueoc River, Presque Isle County, MI, USA in June and July 2012 and 2013, and the upper Oqcueoc River Presque Isle County, MI, USA in July 2014, and supplied with river water. The Oqcueoc River system was selected for use during behavioural assays because a barrier prevents colonization by lampreys, which allows for water void of lamprey pheromones. Experimental apparatus for each species were based upon the design used for *P. marinus* (Siefkes et al., 2005) but approximately scaled for size differences between species (Fig. 1; Buchinger et al., 2013).

Odours were collected from sexually mature conspecific and heterospecific male lampreys. Immediately prior to an experiment, donor males were held in 3 litres of aerated river water for 15 min. Conspecific odours were collected from a group of four males for all species. Females do not exhibit a preference for the odour of several males versus the odour of a single male at a similar concentration (Luehring, 2007). The number of heterospecific donor males was adjusted proportionally based upon the experimental species and hence the size of the apparatus. *Petromyzon marinus* were exposed

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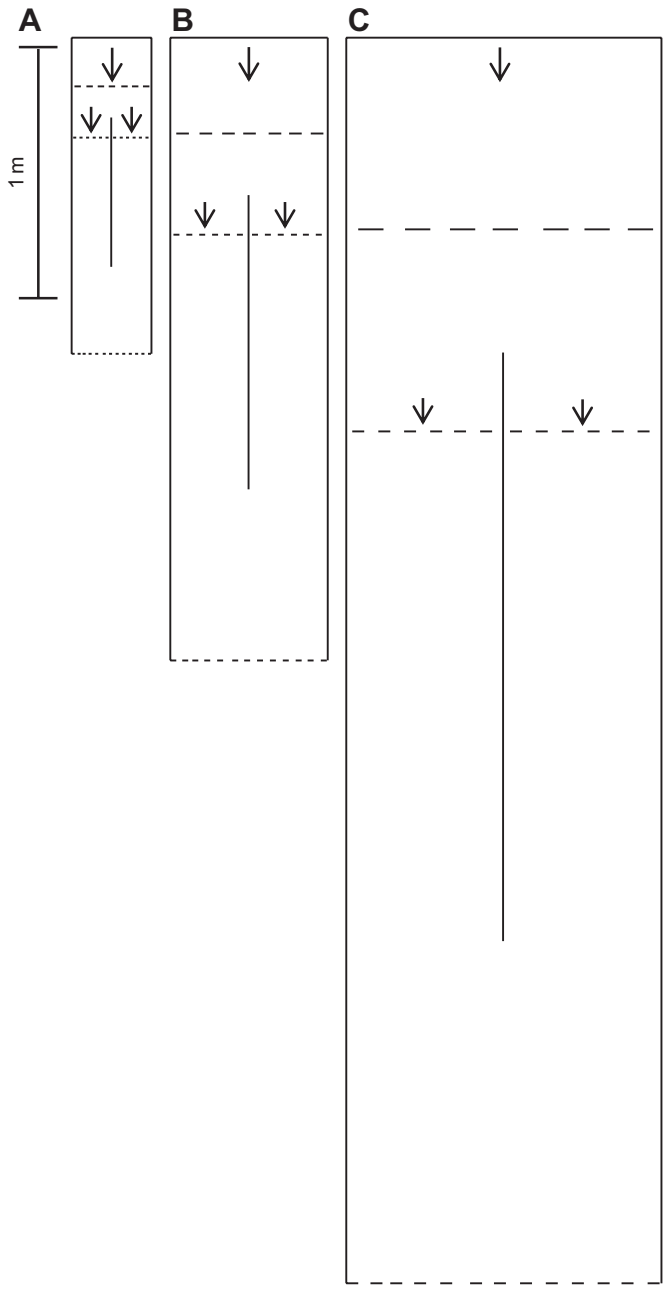

Fig. 1. Behavioural assays used to evaluate female responses to conspecific and heterospecific odours. The design and methods are similar to Li et al. (2002), but dimensions were adjusted based upon the size of the test species (Buchinger et al., 2013). (A) Assay used to evaluate responses of *I. fossor* and *L. appendix*. (B) Assay used to evaluate responses of *I. unicuspis*. (C) Assay used to evaluate responses of *P. marinus*. Arrows denote the direction of flow.

to odours collected from 16 *L. appendix* and *I. fossor,* and eight *I. unicuspis* and *I. castaneus*. *Ichthyomyzon unicuspis* were exposed to odours collected from eight *L. appendix* and *I. fossor*, four *I. castaneus*, and two *P. marinus*. *Ichthyomyzon fossor* and *L. appendix* were exposed to odours collected from four *L. appendix* and *I. fossor*, respectively, two *I. unicuspis* and *I. castaneus*, and one *P. marinus*. Standardizing heterospecific odours by weight, a common approach in chemical ecology, may not be meaningful because of the large differences in weight between species. For example, the equivalent weight of four *P. marinus* requires an ecologically irrelevant 260 *I. fossor* or *L. appendix*. Hence the odour concentrations used may differ in concentration by some indeterminable amount, but no other method of standardization was appropriate, and the method used creates ecologically relevant concentrations.

An experiment began when a single lamprey was placed into the downstream end of the flume. Following a 5 min acclimation, the time a lamprey spent in each channel was recorded while no odour was applied to either side. After 10 min of recording, an odour was introduced to one channel for 5 min without recording the lamprey's behaviour. Lastly, the time spent in each channel was recorded for 10 min while an odour was applied to one channel. After recording the time spent in the control and experimental channels before odour application (*bc*, *be*), and after odour application (*ac*, *ae*), an index of preference (*i*) was calculated for each test $\{i=[ae/(ae+be)-ac/(ac+bc)]\}$. The indices of preference were evaluated using a Wilcoxon signed-rank test ($\alpha=0.05$; Li et al., 2002).

Electro-olfactogram responses to male odours

Electro-olfactogram (EOG) recordings from *P. marinus* were used to determine if male odours from sympatric lampreys elicited olfactory responses, and if the olfactory mechanisms used were the same for odours from different species (Li et al., 1995). EOG recordings and data analysis were conducted following established methods (Brant et al., 2016a). Responses to male odours from *P. marinus*, *I. unicuspis*, *I. fossor*, *I. castaneus* and *L. appendix* were recorded. For EOG recordings, a sexually immature adult male or female lamprey was anesthetized using 3-aminobenzoic acid ethyl ester methanesulphonate (50 mg l^{-1}; MS222; Sigma), immobilized with an intramuscular injection of gallamine triethiodide (30 mg kg^{-1}; Sigma), and secured in a Plexiglas trough while their gills were continuously perfused with aerated water containing anaesthetic. Immature lamprey were used because measurement of olfactory sensitivity with EOG recordings becomes less robust as lampreys become sexually mature (Li, 1994), probably a result of lampreys nearing the end of their life. A recording electrode placed between olfactory lamellae recorded olfactory responses relative to a reference electrode placed on the skin near the naris. Electrical signals were filtered and amplified using a NeuroLog filter and pre-amplifier (Digitimer Ltd, Hertfordshire, England), integrated using an Axon Instruments Digidata system 1550 (Molecular Devices, CA, USA), and stored and processed on a PC with Axon Instruments Axoscope software 10.5.

Male odours collected with the same methods as chemical profiling were used to determine olfactory responses of *P. marinus* to heterospecifics. After holding individual males in 5 litres for 2 h, 1 litre of water was collected and stored below −20°C. Samples were freeze-dried using a FreeZone Plus freeze dry system with a bulk tray dryer (Labconco), the bottle rinsed with 20 ml methanol, evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco), and reconstituted in 1 ml 50% methanol (v:v). Samples were pooled across three males within each species before use in experiments.

Sensitivity to conspecific and heterospecific male odours was evaluated by determining dilution–response curves to pooled samples. The dilution of male odour in the original 1 litre sample was recreated by diluting 10 µl of the 1 ml concentrated sample in 10 ml water, which was then serially diluted down to a $1{:}10^7$ dilution. The lowest dilution at which responses to a stimulus were significantly larger than those to the control (paired *t*-test, $\alpha=0.05$) was considered to be the threshold of detection (Siefkes and Li, 2004). Dilution–response curves were determined for six

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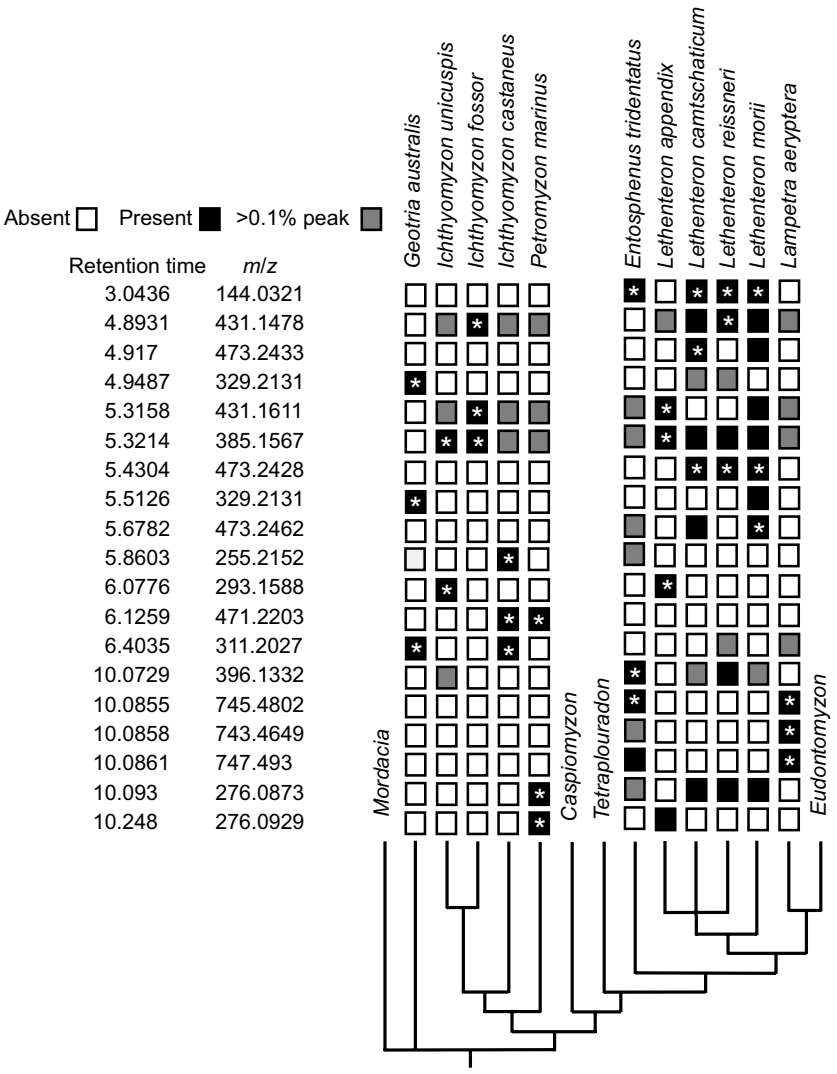

Fig. 2. Phylogenetic tree (Potter et al., 2015) illustrating the distribution of peaks across species. The three most abundant peaks for each species different between mature male samples and control samples are included, and indicated with asterisks. Retention time and the mass to charge ratio (*m*/*z*) are unique identifiers of a signal, but cannot be used to conclusively identify a compound as our method does not provide information on chemical structure. Black boxes represent peaks greater than 0.1% of the total peak intensity and significantly higher than the control (one-way *t*-test, α=0.1, except for *E. tridentatus* where α=0.15). Grey boxes represent peaks that had intensities that were not significantly higher than the control, but had intensities higher than 0.1% of the total peak intensity in more than one individual of a species. White boxes represent peaks that were not significantly different from the control or were detected at an average intensity less than 0.1% of the total peak intensity.

individuals. The order in which lampreys were exposed to the odours of each species was randomized. Responses were normalized by the response to L-arginine at 1×10^{-5} mol l^{-1} (Siefkes and Li, 2004).

The specificity with which *P. marinus* detected conspecific and heterospecific odours was determined using cross-adaptation experiments (Caprio and Byrd, 1984). Cross-adaptation experiments record the responses to a stimulus while the epithelium is saturated with a second stimulus (the adapting stimulus). Experiments were conducted with dilutions that were equipotent across stimuli, as determined in preliminary experiments (*P. marinus*, $1:10^2$; *I. castaneus*, 1:10; *L. appendix*, 1:5; *I. unicuspis*, 1:1; *I. fossor*, 1:1). The experiment began by recording the response to the adapting stimulus. While saturated with the odour of *P. marinus*, the olfactory epithelium of a fish was exposed to 2× the odour of *P. marinus* (self-adapted control; SAC), and binary mixtures (1×:1×; Mix) of *P. marinus* odour+*I. unicuspis* odour, *I. fossor* odour, *I. castaneus* odour, or *L. appendix* odour. Secondly, while saturated with the odour of each individual heterospecific species, the olfactory epithelium of a fish was exposed to 2× the heterospecific odour (SAC) and 1× the heterospecific odour+1× *P. marinus* odour (Mix). Cross-adaptation experiments were conducted on five individuals. The responses to the SAC and the Mix were normalized by the response to the adapting stimulus, and were evaluated for differences using an ANOVA followed by paired *t*-tests with Benjamini and Hochberg (1995) adjustments for multiple comparisons (when adapted with *P. marinus* odour) or paired *t*-tests (when adapted with heterospecific odour; α=0.05). A difference between the response to the SAC and the Mix indicates that the odours are detected by distinct olfactory mechanisms.

RESULTS

Male chemical profiles

The chemical profiles of compounds released by sexually mature males were partially shared among species (Fig. 2). Chromatograms yielded 317 peaks in male odours across all species. Of the 317 peaks, 67 were detected at a relative concentration greater than 0.1% in more than one individual in at least one species. The 67 peaks were further filtered by a conservative removal of peaks detected at similar or higher magnitudes in control samples (one-way *t*-test, α=0.1). Additional peaks possibly detected at a magnitude greater than in the control (one-way *t*-test, α=0.15) were retained in *E. tridentatus* due to the small sample size for immature males (N=3) and the resultant low power. The results of our re-analysis for *P. marinus* were no different after using sexually immature male *I. unicuspis* as a control instead of blank water. In total, 48 peaks were detected at a relative concentration greater than 0.1% in more than one individual in at least one species and were significantly

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Table 2. Results from the factor analysis on 48 peaks found to have higher intensities in sexually mature male water samples compared with controls

	Loading				Species effect		Species overlap			
Factor	Retention time	*m/z*	Loading	Variance (%)	*F* (ndf,ddf)	*P*-value	Group a	Group b	Group c	Group d
1	5.5126	487.21	0.964	11.2	27.70 (10,52)	<0.01	Iu, If, Ic, Pm, Et, Lap, Lc, Lr, Lm, Lae	Ga		
	4.9487	329.213	0.946							
	5.1033	329.215	0.911							
2	3.0436	144.032	0.746	7.0	19.18 (10,52)	<0.01	Ga, Iu, If, Ic, Pm, Et, Lae	Ga, If, Lap	Lc, Lm	Lc, Lr
	5.4304	473.243	0.547							
	3.6865	206.068	0.537							
3	5.3214	385.157	0.741	5.9	4.44 (10,52)	<0.01	Ga, Ic, Pm, Lae, Lr, Et	Ga, Iu, If, Ic, Lc, Lm, Lr,	Ga, Iu, Ic, Pm, Lc, Lm, Lr,	Ga, Iu, If, Lc, Lm, Lr, Lap
	4.8931	431.148	0.718							
	5.3158	431.161	0.481							
4	4.917	473.243	0.857	5.3	2.10 (10,52)	0.04	Ga, Iu, If, Ic, Pm, Et, Lap, Lc, Lr, Lm, Lae			
	5.1018	473.244	0.656							
	5.4304	473.243	0.631							
5	5.8603	255.215	0.994	4.6	1.21 (10,52)	0.31	Ga, Iu, If, Ic, Pm, Et, Lap, Lc, Lr, Lm, Lae			
	6.1286	255.213	0.596							
	6.3176	255.217	0.393							

Loading: the three peaks with the most influence (Loading) on each factor. Variance: the proportion of variance explained by each factor. Species effect: significance of species effects on factor scores as determined using a multivariate analysis of variance (MANOVA). Species overlap: grouping of species-based up factor scores as determined using pairwise *t*-tests followed by Benjamini and Hochberg adjustments for multiple comparisons. Ga, *Geotria australis*; Iu, *Ichthyomyzon unicuspis*; If, *I. fossor*; Ic, *I. castaneus*; Pm, *Petromyzon marinus*; Et, *Entosphenus tridentatus*; Lap, *Lethenteron appendix*; Lc, *L. camtschaticum*; Lr, *L. resneri*; Lm, *L. morii*; Lae, *Lampetra aeryptera*.

higher in sexually mature male samples compared with controls. The most abundant three peaks within each species were detected at a relative concentration of 0.1% or higher in at least one other species (Fig. 2). The factor analysis reduced the 48 peaks to five factors that explained a total of 34.0% of the variance among species (Table 2, Fig. 3). Factors 1–4 were significantly different among species (MANOVA, $P<0.05$; Table 2), while factor 5 was not (MANOVA, $P>0.05$; Table 2). Between-species comparisons (*t*-tests, Benjamini and Hochberg adjustments) indicated that factor 1 differentiated *G. australis* from all other species, but differences in other factors were not clearly differentiated based upon phylogenetic relationships (Table 2; Fig. 3).

Behavioural responses to male odours

Species-specificity of female responses to male odours varied among species (Fig. 4). Females of every species tested responded to conspecific male odours, indicating a common role of male-released mating pheromones (Wilcoxon signed-rank tests, $P<0.05$). Female *L. appendix* showed no response to heterospecific odours compared with no odour. Female *I. unicuspis* and *I. fossor* were attracted to the odour of males from both species, but not the odour of male *I. castaneus*, *P. marinus* or *L. appendix*. Female *P. marinus* were attracted to male odours from all species tested (Wilcoxon signed-rank tests, $P<0.05$; Fig. 4).

Olfactory responses to male odours

Olfactory responses of *P. marinus* differed depending upon the species of male odour donors (Figs 5 and 6). Responses to the L-arginine standard and the control were similar to previous reports (mean±s.e.m.: L-arginine, 2.25±0.41 mV; control, 0.18±0.03 mV; Siefkes and Li, 2004). The detection thresholds for male odours from *P. marinus* and *I. castaneus* were 1:10^4, and 1:10 for *I. unicuspis*, *I. fossor* and *L. appendix* (paired *t*-tests, $P<0.05$). Cross-adaptation experiments indicated that male odours from each species were detected as at least partially distinct odours. Adaptation to conspecific odours did not completely diminish the response to heterospecific male odours; responses to the SAC remained different from the Mix for odours from all species ($F_{4,20}=4.167$,

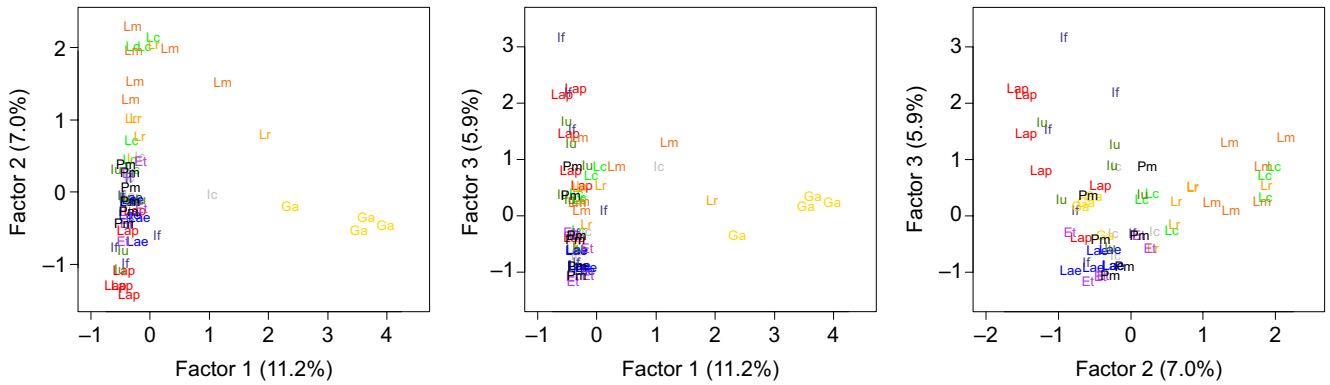

Fig. 3. Results from the factor analysis on 48 peaks found to have higher intensities in sexually mature male water samples compared with controls. A multivariate analysis of variance followed by pairwise *t*-tests indicated differences between species for factors 1, 2 and 3 ($P<0.05$). Species abbreviations: Ga, *G. australis*; Iu, *I. unicuspis*; If, *I. fossor*; Ic, *I. castaneus*; Pm, *P. marinus*; Et, *E. tridentatus*; Lap, *L. appendix*; Lc, *L. camtschaticum*; Lr, *L. resneri*; Lm, *L. morii*; Lae, *Lampetra aepyptera*.

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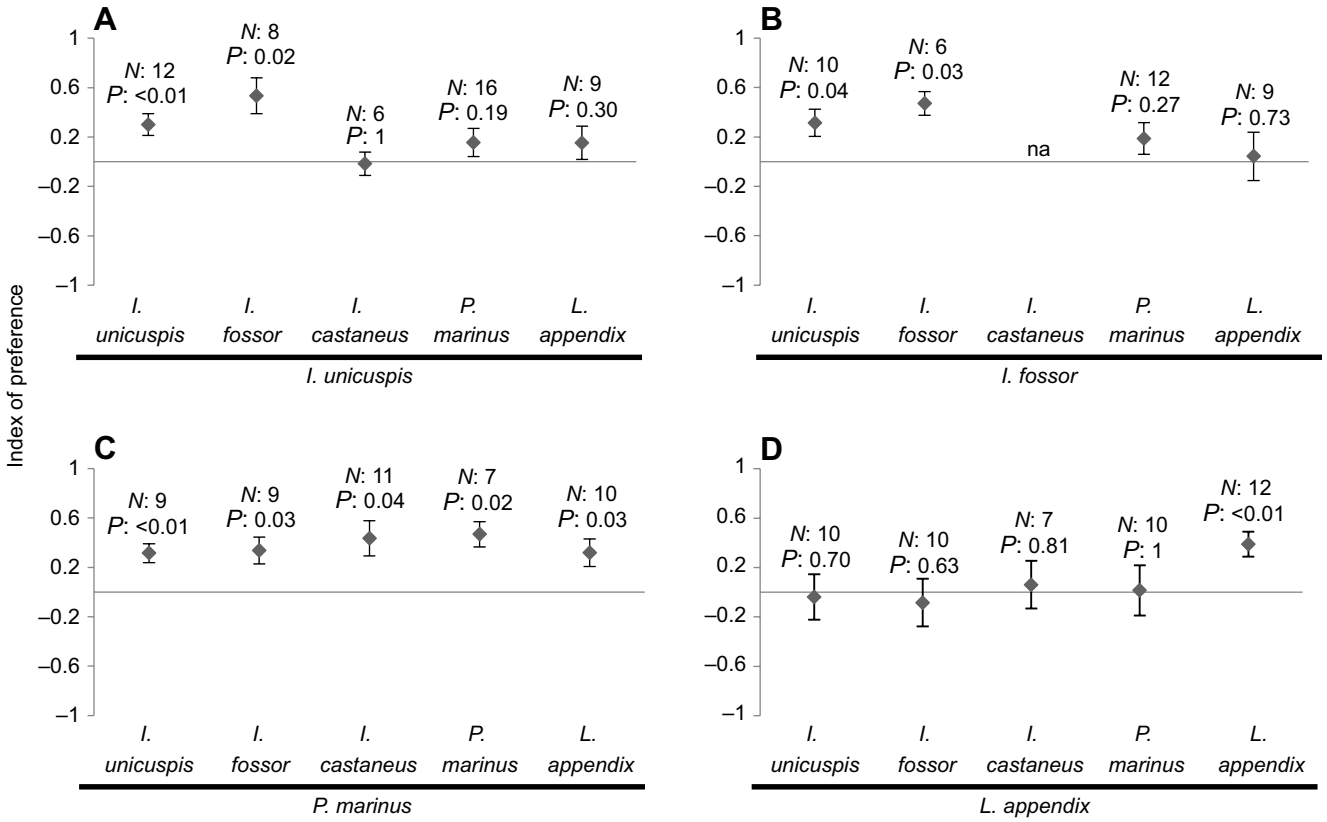

Fig. 4. Behavioural responses of female lampreys to conspecific and heterospecific male odours as determined using two-choice behavioural assays comparing male odour with no odour. Female *I. unicuspis* (A), *I. fossor* (B), *P. marinus* (C) and *L. appendix* (D) were tested for responses to the odours of male *I. unicuspis*, *I. fossor*, *I. castaneus*, *L. appendix* and *P. marinus*. Index of preference=[*ae*/(*ae*+*be*)–*ac*/(*ac*+*bc*)], where *bc* is the time spent in the control channel before odour was applied, *be* is the time spent in the experimental channel before odour was applied, *ac* is the time spent in the control channel after odour was applied and *ae* is the time spent in the experimental channel after odour was applied. A positive index of preference indicates attraction to an odour. *P*-values were determined using a Wilcoxon signed-rank test (α=0.05, two-tailed). *N* is the number of individuals tested for each experiment. na indicates data were not available.

P=0.013, paired *t*-tests *P*<0.05; Fig. 6). Likewise, adaptation to heterospecific odours did not completely diminish the responses to conspecific male odours (paired *t*-tests *P*<0.05; Fig. 6).

DISCUSSION

Our results demonstrate partial overlap in male-released compounds among lampreys and interspecific olfactory and behavioural responses to male-released compounds. Chemical profiling indicated that some of the major compounds released by males are species-specific, but the whole chemical profiles exhibit substantial overlap. In two-choice behavioural assays, females of all species responded to conspecific male odours, but only *P. marinus* responded to odours from males of all heterospecific species. Electro-olfactogram recordings indicated that *P. marinus* detect at least a subset of the odorants released by conspecific and heterospecific males with distinct receptor mechanisms despite the observed behavioural responses to male odours from all species. Our results provide evidence that male odours that guide reproduction in lampreys exhibit partial overlap among species. In particular, our results support shared release of *P. marinus* olfactory cues by males in other species.

The ecological context of our results should be considered bearing several caveats. First, chemical profiling and EOG recordings do not directly translate into evidence for pheromone activity. The peaks detected are likely to be specific to sexually mature males as chemical profiles were first compared with sexually immature males. However, release of a compound by a sexually mature male provides females the opportunity to detect a compound, but does not directly implicate pheromone function. Similarly, the physiological ability to detect an odour, such as that determined by EOG recordings, does not directly indicate that the odour elicits a behavioural response. For example, *P. marinus* sensitivity to 3-keto allocholic acid (Siefkes and Li, 2004) does not translate into a behavioural response (Johnson et al., 2012). Hence an alternative to the hypothesis that release of male pheromone components is partially shared among species is that commonly released compounds are not active pheromones in any species and that the compounds released by male *I. unicuspis*, *I. fossor*, *I. castaneus* and *L. appendix* that attracted female *P. marinus* in behavioural assays are not released by male *P. marinus*. Secondly, behavioural responses to odours in the laboratory can be different from those in natural environments (Johnson and Li, 2010). The observed responses or lack of responses to odours in our two-choice assays may not reflect female responses or lack of responses in a natural context. For example, our assays evaluated female responses in the absence of additional cues, such as the physical structure of the nest. Tactile cues from the structure of the nest (Johnson et al., 2015) or other lampreys combined with partial overlap in pheromone components may act together to elicit an association response to heterospecifics. Thirdly, the release of pheromones can also be context dependent. For example, several species of fish increase urinary release of pheromones when in the presence of mates or competitors (Appelt and Sorensen, 2007; Barata et al., 2007; Rosenthal et al., 2011).

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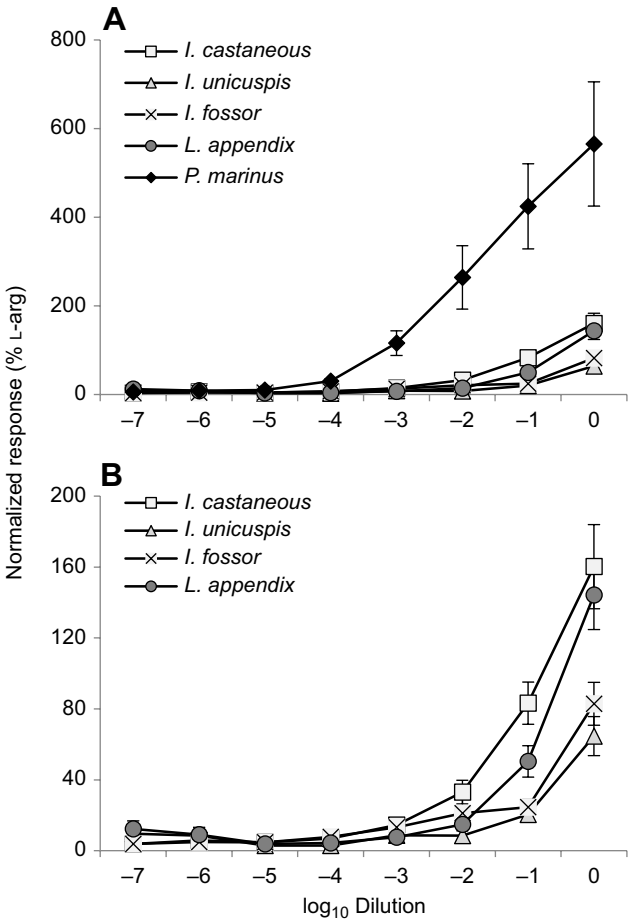

Fig. 5. Dilution–response curves of *P. marinus* to male odours collected from *P. marinus*, *I. unicuspis*, *I. fossor*, *I. castaneus* and *L. appendix*. Dilution–response relationships are presented as semi-logarithmic plots with responses presented as a percentage of L-arginine (L-arg) at 1×10^{-5} mol l^{-1}. (A) Dilution–response curves of all species tested. (B) Dilution–response curves of heterospecific male odours. Dilution–response curves were determined for six individuals. Error bars represent the standard error of the mean.

Release of some minor pheromone components may only be high enough to be detected when males are in the appropriate social context. Likewise, other potential differences in environmental or physiological context related to sampling of lampreys throughout the world should be considered when interpreting variation in male chemical profiles. Finally, the observed non-specific behavioural responses could mediate social interactions rather than reproduction. However, lampreys are semelparous and focused solely on reproduction during the terminal phase of their life, and are not known to have any non-reproductive social interactions with other species. In fact, the only known inter-specific interactions are shared spawning nests (Cochran et al., 2008), the observation of which led to the present research question. Regardless of the above caveats, our results offer support for partial overlap of sexually mature male odours among species.

Our results indicate that components of the pheromone blend in *P. marinus* may have evolved through distinct evolutionary mechanisms. Components of complex signals can have different underlying functions and be shaped by different selective pressures (Candolin, 2003). In *P. marinus*, the major pheromone component 3kPZS elicits long-distance mate search (Siefkes et al., 2005; Johnson et al., 2009), while minor components probably facilitate close-proximity courtship behaviours (Johnson et al., 2012). Most pheromones identified in fish are released at relatively low rates, are likely to guide close-proximity spawning synchronization rather than mate search, and are hypothesized to represent receiver adaptations (Stacey, 2015). Minor components of the pheromone blend used by *P. marinus* are similarly short distance cues involved in spawning synchronization, and conceivably represent an adaptation of receivers rather than the adaptation of signaller hypothesized for 3kPZS communication (Buchinger et al., 2013; Brant et al., 2016b). The observation that female *P. marinus* exhibit a behavioural response to male odours from all species tested, including at least one species that does not release 3kPZS (*I. unicuspis*; Buchinger et al., 2013), together with overlapping chemical profiles, indicates that release of some compounds that function as minor components in *P. marinus* may be conserved across species. Notably, pheromone release and response might not be strictly coupled in all species; *I. unicuspis* respond to but do not release 3kPZS (Buchinger et al., 2013). Likewise, compounds that are released commonly among species may function as pheromones in some but not others. Conserved release of minor components across lamprey species offers indirect evidence that the role of minor components in *P. marinus* pheromone communication evolved through receiver adaptations in contrast to a signaler adaptation hypothesized for 3kPZS (Buchinger et al., 2013).

The role of olfactory cues, including pheromones, in reproductive isolation among sympatric lampreys remains unclear. Sympatric lampreys potentially face substantial decreases in fitness as a result of reproductive interference (Gröning and Hochkirch, 2008; Johnson et al., 2015), as hybrids are not viable (Piavis et al., 1970). In many insects, reproductive isolation is partially maintained through species-specific components, component ratios, or antagonists in pheromone blends (Symonds and Elgar, 2008). The importance of ratios in species-specific pheromone blends in vertebrates is generally poorly understood, but female *P. marinus* respond to the male pheromone when the blend is incomplete and when components are presented at various ratios (Johnson et al., 2009, 2012), although some ratios appear to be more effective (Li et al., 2013; Brant et al., 2016a). Likewise, our results, together with field observations of heterospecific spawning (Cochran et al., 2008), indicate that even partial overlap in lamprey odours may result in attraction to heterospecific odours. Shifts in pheromone blends of lampreys may be the result of random processes, such as mutation and genetic drift, or differences in ecology more so than selective pressure for species-specificity (West-Eberhard, 1983). Reproductive isolation might be maintained by minor differences in the timing and location of spawning (Johnson et al., 2015), conspecific-directed courtship and gamete release on a nest, or species-specific sperm chemosensation (Miller, 1997; Eisenbach, 1999).

In conclusion, we present evidence for partial overlap of male odours that guide reproduction in lampreys. The odour of sexually mature male *P. marinus* is known to primarily consist of pheromones (Siefkes and Li, 2004), but chemical characterization of the compounds shared among species is required to conclusively determine overlap in pheromones. Our results can direct future research into pheromone identities across lampreys, which will provide additional insight into the evolution of pheromones in vertebrates and potential restoration tools for imperilled species throughout the world. Combined with evidence that commonly released hormones function as pheromones in many fishes (Stacey, 2015), our results raise questions regarding species-specificity as a

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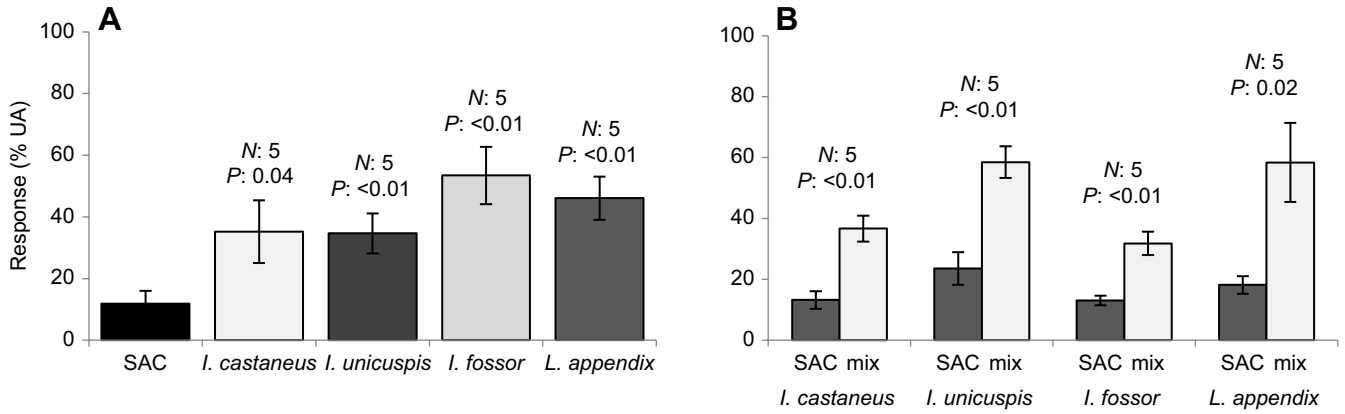

Fig. 6. Results from cross-adaptation experiments on *P. marinus* with odours collected from *P. marinus*, *I. unicuspis*, *I. fossor*, *I. castaneus* and *L. appendix*. Results are presented as a percentage of the unadapted response (UA). (A) SAC: self-adapted control; *I. castaneus*: *I. castaneus*+*P. marinus*; *I. unicuspis*: *I. unicuspis*+*P. marinus*; *I. fossor*: *I. fossor*+*P. marinus*; *L.appendix*: *L.appendix*+*P. marinus*. (B) SAC: self-adapted control; mix: adapting stimuli+*P. marinus*. The species names below the bars represent the adapting stimuli. Significant differences from the SAC were determined with paired *t*-tests (α=0.05, two-tailed). Error bars represent the standard error of the mean.

tenet of pheromone communication in vertebrates (Wyatt, 2014). Lastly, we suggest that *P. marinus* is a useful system for the study of how sexual signals function and evolve, which is less often studied from the perspective of chemical communication compared with other sensory modalities (Andersson, 1994; Coleman, 2009; Steiger et al., 2011), particularly in vertebrates (Johansson and Jones, 2007; Symonds and Elgar, 2008).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.J.B. devised and executed experiments and wrote the manuscript. K.L. conducted chemical analysis, M.C.H. contributed to electro-olfactogram experiments, C.F.B., L.J. and M.C.H. collected experimental animals and sampled male odours. W.L. and N.S.J. contributed to study design and writing the manuscript. All authors contributed to writing and approved the manuscript prior to submission.

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