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Experimental determination of the function, production and release
of a sea lamprey male pheromone

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CHAPTER 1

A novel bile acid as a sex pheromone in sea lamprey

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We show that reproductively mature male sea lampreys release a potent sex pheromone that induces preference and searching behavior in ovulated females. We have identified it, by activity-directed fractionation and structural analysis, as 7α, 12α, 24-trihydroxy-5α-cholan-3-one 24-sulfate. This pheromone has several remarkable features: it is the first bile acid identified as a sex pheromone; it is released in much higher relative amounts than other known vertebrate pheromones; and, its release occurs not, as one might expect, via the urine or feces, but likely from the gills. It appears to be capable of signaling both reproductive status and location of mature males over long distances.
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The sea lamprey, *Petromyzon marinus*, is an ancestral jawless fish and an invasive parasite of fishes in the Great Lakes of North America. It migrates into streams to spawn in spring. The males arrive earlier than the females (*1*) and build nests in areas where flow rates are 0.5 to 1.5 m s^{-1} (*1, 2*). It has long been suspected that the males release a pheromone to guide the females to their nests (*3, 4*). This type of sex pheromone, capable of inducing spatial orientation of conspecifics 'downwind', is well-established in insects (*5*), but not so in vertebrates where identified sex pheromones tend to have a small 'active space' (e.g. *6-12*). In fish, the known sex pheromones are gonadal steroids or prostaglandins and have been identified from *a priori* knowledge of their structures (*9-12*). Little is known of the hormones produced by lamprey gonads. We have therefore set out to confirm the release and large active space of the male lamprey sex pheromone, to identify its structure, and to test the axiom that all fish sex pheromones are of hormonal origin.

Behavioral tests confirmed that water conditioned by spermiating males influences the distribution and locomotor activities of ripe females. When tested in a two-choice maze (*13*), ovulated females (but not males or pre-ovulatory females; data not shown) spent more time in the compartment conditioned with washings from spermiating males (Table 1). There was no preference of ovulated females for washings of pre-spermiating males or females (data not shown). Further, the ovulated females showed dramatic increases in

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search behavior in the chamber with water conditioned by spermiating males (Table 1). At a natural spawning site, ovulated females tagged with radio transmitters (14), and placed 65 m downstream (15), showed a similar response (P<0.02; data not shown) - indicating a large active space for the male pheromone.

Lamprey washings were passed through C-18 solid phase extraction (SPE) cartridges (16). Previous electro-olfactogram (EOG) experiments had shown that virtually 100% of odorant could be extracted by this means (17). In our maze (13), ovulated females spent more time and showed increased search behavior in the side scented with extracts from spermiating males (Table 1).

SPE extracts were subjected to Fast Atom Bombardment Mass Spectrometry (FABMS) and thin-layer chromatography (TLC) to detect the compounds being released by spermiating males, and then to reverse-phase HPLC to isolate them. FABMS identified an abundant ion with an MH^+ at m/z 473 in extracts from spermiating males. In the negative mode, a corresponding strong $[M-H]^-$ ion at m/z 471 was observed, suggesting the presence of an acidic moiety in the molecule (Fig. 1). Tandem analysis of this peak showed it lost 98 mass units, suggesting that the compound was phosphorylated or sulfated. Similar ions were not present in detectable amounts in extracts from pre-spermiating males (Fig. 1; inset), or females (data not shown). TLC of extracts (18) displayed a relatively large amount of a few major compounds in spermiating, as opposed to pre-spermiating, male washings (Fig. 2; inset). The material separated into three bands on TLC; the one at the origin being established, by dilution, as the most abundant. The HPLC (19) fractions eluting at 46 and 47 minutes contained the 472 Dalton molecule by FABMS analyses, stained strongly by PBA when spotted on TLC plates, remained at the origin when run on TLC, and had the highest olfactory potency by EOG (Fig. 2) (20). HPLC fractions of extracts of pre-spermiating male washings at 46 and 47 minutes did not contain the 472 Dalton molecule and did not show EOG potency (data not shown). Fractions 64 and 71 are unidentified.

The chemical structure of the 472 Dalton molecule was determined by magnetic resonance spectrometry (21). The one-dimensional ^{13}C spectrum (Fig. 3A) showed one peak at 210.9 ppm and no other peaks above 80 ppm, suggesting the presence of a carbonyl group and absence of double bonds between carbon atoms. The ^1H-^{13}C HSQC (Fig. 3B) showed three intense cross peaks characteristic of CH_3 groups. Two of them were singlet peaks, suggesting they were bonded to quarternary carbons. CH and CH_2 groups were distinguished by ^{13}C-editing. The cross peaks with ^1H chemical shifts >3.0 ppm and ^{13}C chemical shifts > 60 ppm were assigned to CH_2 or CH groups linked to an oxygen via a single bond. These chemical groups were then linked together via through-bond correlations obtained from two-dimensional ^1H-^1H COSY and TOCSY and ^1H-^{13}C HSQC-TOCSY and HMBC spectra. The stereochemistry of 7-H and 12-H was determined on the basis of their narrow multiplets (<10 Hz), and that of 5-H on the basis of the chemical shift of C-19 (9.7 ppm) (22, 23). The formula based on this structure, $C_{24}H_{40}O_7S$, was confirmed by an exact mass measurement (MH^+ calculated, 473.2573, observed, 473.2578, error 1.1 ppm), indicating the compound contained a sulfate rather than a phosphate group. We concluded that the structure was 7α,12α,24-trihydroxy-5α-cholan-3-one 24-sulfate.

The deduced structure differs from that of petromyzonol sulfate ($3\alpha,7\alpha,12\alpha,24$-tetrahydroxy-$5\alpha$-cholan 24-sulfate; PS) by its 3-keto, as opposed to a 3α-hydroxyl, group. PS is a lamprey larvae bile acid (*20, 23*) and a component of a pheromone which influences behaviors of migrating, but not ripe, adults (*20, 24*). We converted the 3α-OH of synthetic PS into 3-keto (*25*) and acquired its ^{1}H-^{13}C HSQC (*21*). The chemical shifts and intensity of cross peaks were virtually identical between the converted compound and purified male pheromone (Fig. 3B), suggesting that both molecules had an identical chemical structure and purity. Further, these two compounds co-migrated on TLC, co-eluted on HPLC, and showed the same fragmentation patterns under FABMS.

We confirmed that the purified compound (that showed virtually identical ^{1}H-^{13}C HSQC to the synthetic compound; Fig. 3B) replicated the pheromonal activity of washings of spermiating males. Approximately 30 mg pheromone was isolated from 4 h washings of approximately 30 spermiating males, suggesting a rate of release of about 250 μg male^{-1} h^{-1}. From this we estimated that in the experiments with live males (*13*) the pheromone reached a concentration between 0.1 and 0.2 nM. We therefore tested it in our maze (*13*) at a final concentration of 0.17 nM. Ovulated females spent a longer time and showed increased search behavior in the scented side (Table 1).

In order to determine the site of release of the pheromone, we tested washings from bisected (*26*) male lampreys. Water conditioned by the head region induced a large EOG (*20*) response at 10, 000 times dilution whereas the water conditioned by the posterior region did not induce a detectable response (data not shown). Further, only water conditioned by the head region was attractive to ovulated females ($P<0.01$) and, by FABMS, contained the [M-H]$^{-}$ ion at m/z 471 (data not shown).

Preliminary tests indicate that $7\alpha,12\alpha,24$-trihydroxy-5α-cholan-3-one 24-sulfate is present in the liver of spermiating males (data not shown), suggesting that this is where it is synthesized. It is unlikely that this bile acid is needed for lipid digestion since adult lampreys do not feed, nor do they have bile ducts or gall bladders (*27*). Its delivery to the gills must be via the bloodstream. Because the hepatic veins carry blood directly to the heart – and because all the blood from the heart goes through the gills – its excretion is potentially very efficient. However, at the level of the gills, there would appear to be a problem. In elasmobranchs and teleosts (*26, 28*), the passive transfer of sulfated compounds across gills is negligible. How have lampreys overcome this problem? Interestingly, concomitant with spermiation, profuse glandular cells with secretory papillae (that have actually been proposed to excrete "sex substances") appear in the gills of spermiating males (*29*). Females do not develop these cells at any stage. It seems probable that these cells are responsible for the active excretion of the identified pheromone. If so, this suggest that male lampreys are 'active signalers' rather than the females being 'chemical spies', the current leading hypothesis concerning the evolution of fish sex pheromones (*30*).

We conclude that ripe male lampreys release a large amount of $7\alpha,12\alpha,24$-trihydroxy-5α-cholan-3-one 24-sulfate, probably via the gills, to signal the location of their nests to

ovulated females at a long distance downstream. The selection pressure favoring the evolution of a bile acid derivative, rather than a steroid or prostaglandin, as a sex attractant may have been the necessity to cover a large active space. Bile acids, in particular sulfated ones, are more water-soluble and can be produced in larger quantities than steroids. A spermiating male lamprey (ca. 250 g) releases sufficient of this pheromone in 4 h to be detectable by females when diluted in 10^7 l of water (*17, 30*). This volume is about 10^5 times greater than that (130 l) of the main gonadal (steroid) pheromone released by a 25 g female goldfish (*31*).

This pheromone influences distribution and oriented locomotion of female lampreys in their natural habitat. Interference with this pheromone system offers an attractive target for selective and environmentally benign control of the sea lamprey, whose invasion of the Great Lakes represents arguably the worst ecological disaster ever to befall a large watershed (*32*).

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13. Animals were classified as *spermiating males* and *ovulated females* if milt and eggs, respectively, could be expressed by manual pressure, or otherwise as *pre-spermiating males* and *pre-ovulatory females*, and used as either test subjects or odorant donors in a flow through (0.07 m s^{-1}) maze (L 4.6 m x W 1.2 m) with plywood bottom, sides, and a partition in the middle which extended 2.4 m from the upstream end, and with plastic meshes blocking fish movement at both the upstream and downstream ends. Odorant donors were held above the upstream mesh. Between 0700 and 1700 h, a single test subject was acclimated for 10 min in the maze, and its behavior video recorded for 20 min. Then, 5 lampreys (all of one sex and maturity) were introduced into the mesh chamber on a randomly chosen side, and behavior of test subject recorded for another 20 min. When washings were used, a spermiating male was held in 10 l of water for four hours and the water introduced into the odor chamber at 75 ml min^{-1}. Naïve

observers scored videotapes for the total time spent in each side before (Be and Bc) and after (Ae and Ac) odorant introduction. To measure attraction of test subjects to conditioned side of the maze, the scores were used to calculate an index of preference: I = (Ae/(Ae + Be)) − (Ac/(Ac + Bc)). A similar index was computed for search behaviors which involved pacing back and forth across the upstream barrier, increased swimming speed, and rapid beating of the tail by the test subject.

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15. Field study was conducted in a 65 m segment of the Ocqueoc River, Presque Isle County, Michigan USA, a tributary to Lake Huron - with a barrier to prevent lamprey migration from the lake. The average discharge was 2.3 m^3 s^{-1}. Upstream, an island divided the streams into two channels. Cages (1 m^3) of plastic mesh (~1.5 cm mesh size) containing 5 male lampreys (spermiating or pre-spermiating) were randomly placed in the two channels. A female fitted with an external radio transmitter (*14*) was acclimated in a cage for two h, released 65 m downstream, and its location recorded every 5 min. Tests were conducted between 0700 and 1700 h in water temperatures ranging from 12 to 24°C.

16. A lamprey was placed in 10 l of aerated water for 4 h, and removed. The water was drawn through a filter paper (Whatman No. 3) and then SPE cartridges ('Sep-Pak'; Waters Chromatography, Millipore, Milford, MA, USA; pre-washed with 5 ml methanol, followed by 5 ml distilled water) at a rate of up to 20 ml min^{-1}. 1 l was pumped through each cartridge, which was then washed with 5 ml distilled water and eluted with 5 ml methanol.

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18. Samples were loaded in 50 μl ethanol on silica gel plates (Whatman type LK6DF) which were developed with chloroform/methanol (50/6, v/v) for 45 min., sprayed with 5% phosphomolybdic acid (PBA) in methanol, placed on a hot-plate at 100 °C for 3 to 5 min to develop the color, and photocopied.

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21. The samples were dissolved in perdeuterated methanol or dimethyl sulfoxide (DMSO) and subjected to a Varian INOVA 600 spectrometer at 25°C for two-dimensional homonuclear ^{1}H COSY and TOCSY spectra, and heteronuclear ^{1}H-^{13}C HSQC, HSQC-TOCSY and HMBC spectra. The one-dimensional ^{13}C spectrum was acquired on a Varian VXR 500 spectrometer. Standard pulse sequences were used. Suitable window functions were applied to the time domain data before Fourier transformation for resolution or sensitivity enhancement. Both ^{1}H and ^{13}C chemical shifts were referenced to the solvent resonances.

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sodium phosphate buffer at pH 7.6. A further 20 mg NAD and 10 units of enzyme were added at 1 h. The products of the reaction were extracted with SPE cartridges (*16*) and purified by HPLC (*19*).

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33. Mr. Roger Bergestedt, USGS Lake Huron Biological Station, provided space and advice for behavioral experiments. Dr. Dan Gallaher advised on conversion of petromyzonol sulfate into the male pheromone. Dr. John Kelso advised on radio-telemetry tracking of sea lamprey. Mike Twohey and Rod MacDonald supplied lampreys for this study. Ms. Beverly Chambers assisted with MS analysis. Ms. Dolly Trump and Ms. Lydia Lorenz provided their private land as a field study site. The Great Lakes Fishery Commission financed this study.

Figure Legends

Figure 1   Fast atom bombardment (10 KV) mass spectra of an extract of washings from a spermiating male sea lamprey, and of an extract of washings from a pre-spermiating male (inset). Matrix: glycerol.

Figure 2   Electro-olfactograghic potency of fractions derived from reverse-phase HPLC separation *(19)* of water extracted from five spermiating male lampreys. The response magnitude is in millivolts (mV). The spots produced by staining of 5 µl of each fraction with phosphomolybdic acid (PMA) *(18)* are shown just below the ordinate. The insert shows thin-layer chromatography (TLC) of equal amounts of extract from spermiating (S) and pre-spermiating (N) male lampreys; followed by staining with PMA. The three TLC bands had the following correspondence to the HPLC fractions: origin = 46/47; slow-moving band = 71; fast-moving band = 64. NB the staining method is not quantitative; thus the relative size of the TLC bands is not a true reflection of their relative abundance.

Figure 3   One-dimensional ^{13}C spectrum of the isolated male sea lamprey sex pheromone (A) and overlay of two-dimensional ^{1}H-^{13}C HSQC spectra (B) of the isolated (red) and synthetic (green) male sea lamprey sex pheromone *(20)*. The unlabeled cross peaks in the ^{1}H-^{13}C HSQC spectrum (B) are due to impurities or the solvent.

Table 1   Influence of male odorants on distribution and search behavior of ovulated female sea lampreys in a two-choice maze

Stimuli	Attraction			Searching Behavior		
	Unconditioned	Conditioned	P-value	Unconditioned	Conditioned	P-value
SM	0	22	0.01	0	8	0.01
PSM	12	12	NS	3	3	NS
SMW	3	12	0.01	0	7	0.01
SME	3	11	0.01	2	7	0.05
PP	0	8	0.01	0	6	0.03

"Conditioned" = side of maze into which caged fish or test substances were placed; "unconditioned" = control side.   The numbers refer to the number of ovulated females which were tested and which spent more of their time (attraction) or showed more activity (search behavior) in either the "conditioned" or "unconditioned" sides *(13)*. Abbreviations: SM, spermiating male; PSM, pre-spermiating male; SMW, washings collected from spermiating males; SME, C-18 SPE extracts of spermiating male washings; PP, purified pheromone ($7\alpha,12\alpha,24$-trihydroxy-5α-cholan-3-one 24-sulfate).   *P*-values were determined with a Wilcoxon Signed Ranks Test (2-tailed) using indices of preference described in method section *(13)*.
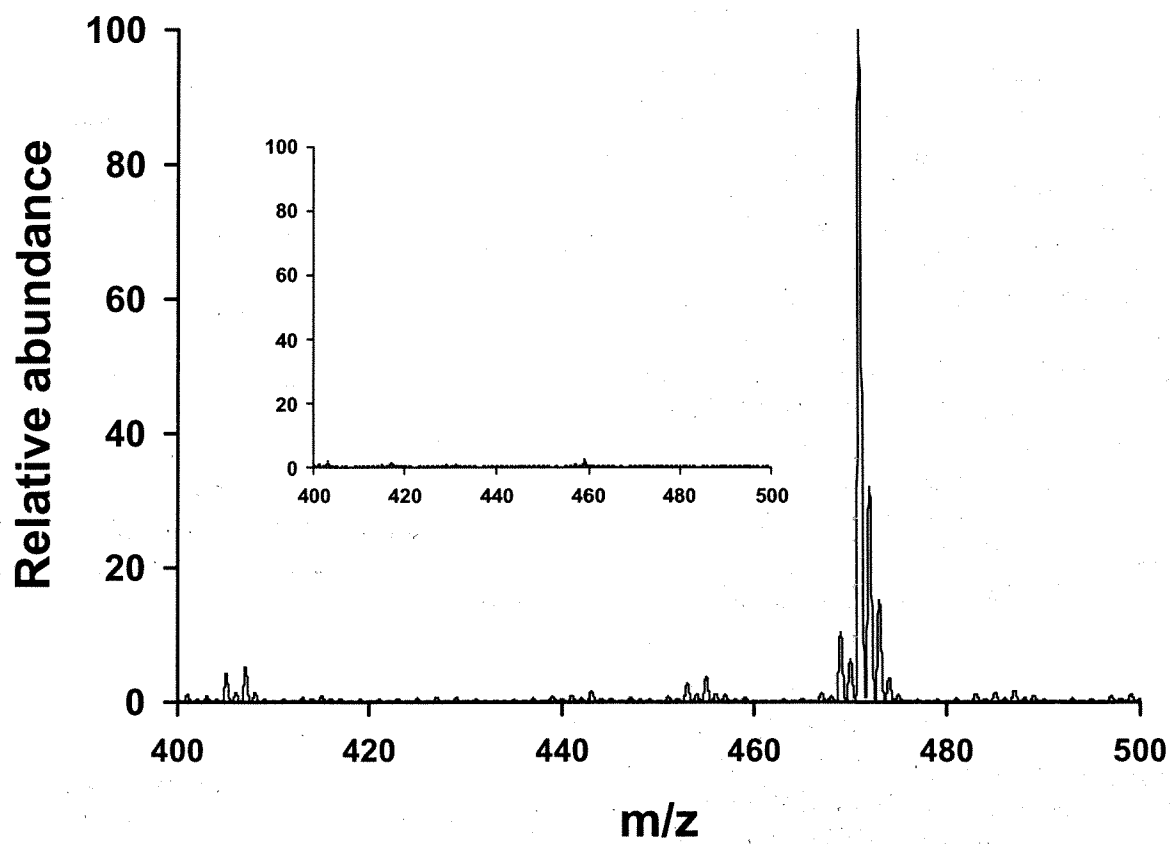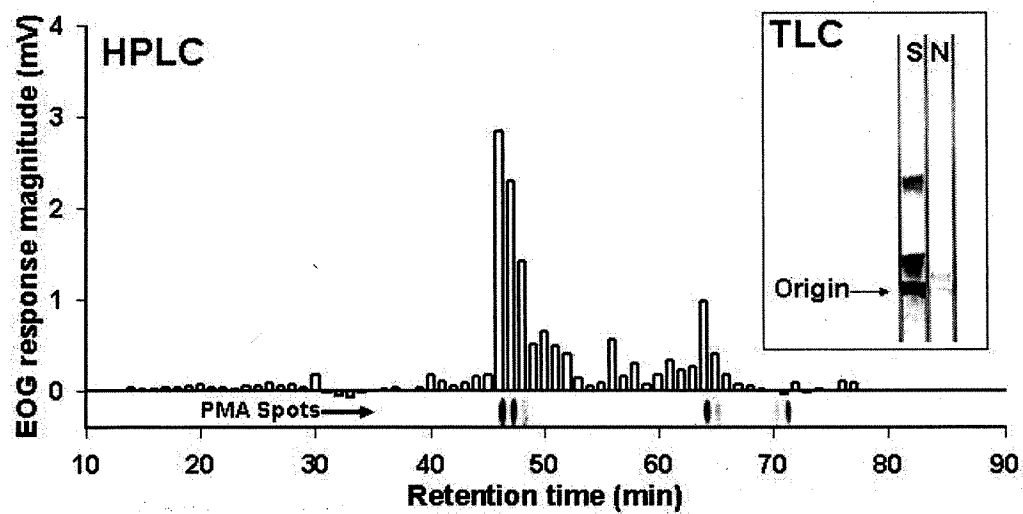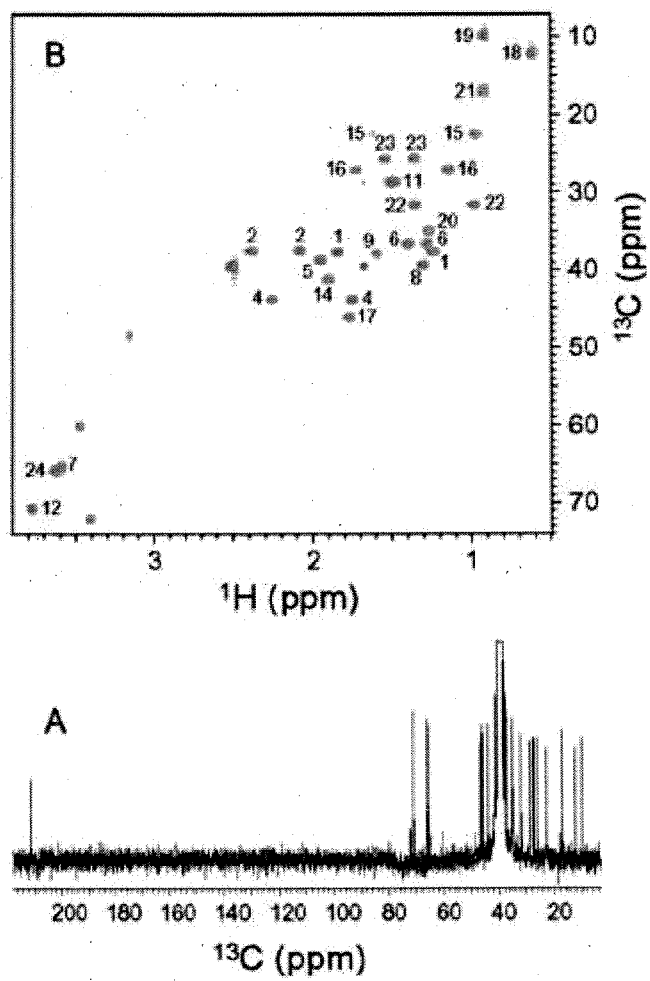
Figure 1

Figure2

Figure 3

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CHAPTER 2

Bile acids produced by conspecifics are uniquely and independently stimulatory to the olfactory organ of female sea lampreys, *Petromyzon marinus*

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Abstract

Sea lampreys at different life stages release at least two bile acid pheromones. Larval sea lampreys release petromyzonol sulfate (PZS) and allocholic acid (ACA), which are potent olfactory stimulants and function to attract migratory adults searching for suitable spawning streams. Recently, spermiating males have been found to produce and release two bile acids, 3-keto petromyzonol sulfate (3kPZS) which functions to attract ovulating females and 3-keto allocholic acid (3kACA) whose function is unknown, however the olfactory potency of 3kPZS and 3kACA has not been demonstrated directly by electrophysiological recording. Also, the ability of adult sea lampreys to distinguish between larval and spermiating male bile acids has not been demonstrated, which would be challenging but critical because of the similar chemical structure and different functions of the bile acids that are both present in water during spawning times. Electro-olfactogram recordings were used to determine the olfactory potency of 3kPZS and 3kACA and whether adult female sea lampreys are specifically sensitive to bile acids produced by conspecifics. Synthetic and natural 3kPZS showed nearly identical concentration-response curves. 3kPZS and PZS were the most stimulatory of the bile acids tested. Cross adaptation showed that 3kPZS and PZS represented different odor qualities, but 3kACA and ACA represented the same odor qualities to adult females. These experiments show that 3kPZS and 3kACA are highly stimulatory to and demonstrate that 3kPZS and PZS are discriminated by the olfactory epithelium of adult female sea lampreys, supporting their function as two independent pheromones.

Introduction

Bile acids are potent chemosensory stimulants for many fish species (Hara 1994, Li et al. 1995; Michael and Lubomudrov 1995). In teleost species, they have been demonstrated to stimulate both the gustatory and olfactory systems at concentrations as low as 10^{-11} M (Hara et al. 1984). Initially, bile acids were thought to be good candidates for pheromones in salmonids, mainly because of their diversity in structure and stability in aquatic environments (Doving et al. 1980; Stabell 1987). More recently, it has been suggested that adult rainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*) use bile acids as sex pheromones (Vermeirssen and Scott 2001; Zhang et al.

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2001). However, no specific bile acids have been identified as either sex or migratory pheromones in teleost fish so far. The only species in which specific bile acids have been linked to specific pheromone functions is a jawless fish, the sea lamprey (*Petromyzon marinus*).

Sea lampreys at different life stages are found to release at least two pheromones, both of which are comprised of bile acids, that induce relevant behavioral responses in conspecifics. This anadromous species experiences three stages through its life history. Its larvae inhabit tributary streams, enter the Atlantic Ocean to feed after metamorphosis, and return as adults to spawn (Hardisty and Potter 1971). The land-locked sea lampreys in the Laurentian Great Lakes have a similar life history (Smith and Tibbles 1980). Larval sea lampreys produce and release two bile acids, petromyzonol sulfate (PZS) and allocholic acid (ACA) (Haslewood and Tokes 1969; Li et al. 1995; Polkinghorne et al. 2001), that are potent olfactory stimulants and induce relevant behavioral responses in migratory adults searching for suitable spawning streams (Bjerselius et al. 2001; Li et al. 1995; Li and Sorensen 1997). Furthermore, adult males, after onset of spermiation, produce and release two bile acids, 3-keto petromyzonol sulfate (3kPZS; Li et al. 2002) and 3 keto allocholic acid (3kACA; Yun et al. in press). These two bile acids differ from their larval counterparts by possessing a carbonyl, as opposed to a hydroxyl, at C-3. 3kPZS has been shown to function as a sex pheromone that induces search and preference behaviors in ovulated females (Li et al. 2002). The function of 3kACA has not been determined but is likely a minor component of the male sex pheromone (Yun et al. in press). The olfactory potency of 3kPZS and 3kACA has not been demonstrated directly by electrophysiological recording.

While it seems efficient for the sea lamprey to produce two types of pheromones in two different life stages with a common biosynthetic pathway (Li et al. 2002), it would be remarkably challenging for their olfactory system to distinguish between adult and larval bile acids, which have very similar structures but totally different functions. Yet it is critical for adult sea lampreys to overcome this challenge because their conspecific larvae inhabit spawning streams year round (Moore and Schleen 1980), and release PZS and ACA into the same stream where spermiating males release 3kPZS and 3kACA to attract ovulated females (Li et al. 2002; Polkinghorne et al. 2001).

To further demonstrate that 3kPZS and 3kACA function as a male pheromone, we reasoned that the olfactory epithelium of adult females should be highly sensitive to these two bile acids, and be able to distinguish them from larval bile acids. In particular, we attempted to address three questions in this study. First, do naturally produced 3kPZS and chemically synthesized 3kPZS represent the same odor quality to females? Second, are 3kPZS and 3kACA highly stimulatory for the olfactory epithelium of female adults? Third, can female olfactory organs distinguish 3kPZS from 3kACA, PZS and ACA? Our EOG data from this study provide further evidence that 3kPZS and 3kACA function as a male sex pheromone, and offer insights on contributions of specific functional groups to odor quality.

Materials and Methods
Experimental Fish
Adult sea lampreys were collected from tributaries to lakes Huron and Michigan between April and July, 2000-2002 by the staff of the U.S. Fish and Wildlife Service,

Marquette Biological Station, Marquette, Michigan, USA. Lampreys were transported to the main laboratory at the U.S. Geological Survey, Hammond Bay Biological Station, Millersburg, Michigan USA. Females were separated from males and held in flow-through tanks (1000 L) with chilled Lake Huron water at temperatures ranging from 6°C to 8°C to preserve there responsiveness throughout the spawning season. Males were held in flow-through tanks (200 L) at temperatures ranging from 15°C to 18°C with no more than 15 males per tank. Males were checked for spermiation according to the criteria set forth by Siefkes et al. (in press). Any spermiating males were separated into another tank. Spermiating males served as odor donors for electro-olfactogram recordings.

Collection and estimate of naturally produced bile acids

Water was conditioned with spermiating male sea lampreys by holding individual males for 4 h in polyethylene buckets filled with 10 L of Lake Huron water. These buckets were aerated and kept in a water bath of 18°C. The conditioned water was used immediately or stored at -80°C for electro-olfactographic analyses. Conditioned water was measured for naturally produced 3kPZS using an enzyme linked immunosorbent assay (ELISA; Yun et al. in press).

Test Stimuli

The olfactory epithelium of female sea lampreys were exposed to solutions of L-arginine, water conditioned with spermiating males, and four bile acids (ACA, PZS, 3kACA, 3kPZS; Figure 1). These bile acids were chosen because they are known to be produced and released by sea lampreys (3kPZS and 3kACA by spermiating males and PZS and ACA by larvae) and have been demonstrated to have entirely different functions (Polkinghorne et al. 2001; Li et al. 2002). Testing these compounds will address the functional significance of olfactory specificity.

Electro-olfactogram recording

Female sea lampreys were tested for olfactory sensitivity to naturally produced sex pheromone and the four bile acids described above. EOG recording was performed as described by Li et al. (1995). Briefly, sea lampreys were anaesthetized with an intramuscular injection of metomidate hydrochloride (3 mg/kg body weight; Syndel, Vancouver, British Columbia, Canada), immobilized with an intramuscular injection of gallamine triethiodide (150 mg/kg body weight; Sigma, St Louis, Missouri, USA) and placed in a water-filled trough. The head of the female remained above the water and the gills were supplied with aerated Lake Huron water. The olfactory lamellae were then exposed and perfused with water from a source that could deliver either clean water or the same clean water containing chemical stimuli on demand. Differential electrical responses between the skin surface and the sensory epithelia in response to each chemical stimulus were recorded using two Ag/AgCl electrodes (type EH-1S, World Precision Instruments, Sarasota, Florida, USA) filled with 3 M potassium chloride and bridged with 8% gelatin:0.9% saline – filled glass capillary tubes. The recording electrode was placed between two lamellae and was adjusted to maximize the response to the L-arginine standard while minimizing the response to a blank water control. The reference electrode was placed on the skin near the naris. Electrical signals were amplified and digitized by power lab (ADI Instruments) and displayed on a PC computer.

Stock solutions of bile acids were made at concentrations of 10^{-3} M using deionized water or methanol and stored at $-20°C$. Conditioned water was collected directly from washing buckets and stored at $-80°C$. The 10^{-2} M L-arginine standard stock solution was made with deionized water every week and stored at $4°C$. These stock solutions were diluted in Lake Huron water immediately before testing. To determine concentration-response relationships, a 10^{-5} M L-arginine standard was pulsed into the olfactory epithelium of a female for 5 seconds and the electro-olfactgram response measured to establish a baseline of electrical activity. Next blank water control was introduced and the response measured to confirm the absence of response from the clean water supply used both to continually supply the olfactory organ and to dilute chemical stimuli for testing. Increasing concentrations of the test stimuli starting at 10^{-13} M for bile acids and 10^{6} times dilution for conditioned water were then introduced and the responses measured. Measuring the response to the L-arginine standard and blank water control again at the end of the dilution series concluded each trial. The epithelium of each female was allowed to recover at least 3 min between stimuli and each of the test stimuli concentrations were assayed at least twice. Female electro-olfactogram response magnitudes were measured in mV and expressed as a percentage of the response to the L-arginine standard.

Cross adaptation

Cross adaptation, described by Li and Sorensen (1997) was used to compare the EOG response to a test stimulus before and during adaptation to an adapting compound. In a given trial, baseline olfactory EOG responses of females to blank water control (Control A), a L-arginine standard and test stimuli (water conditioned with a spermiating male and all four bile acids; 'Initial Response') were recorded. The test stimuli were used at concentrations that elicited equipotent olfactory responses at about 100% of the L-arginine standard. During adaptation, the olfactory epithelium of a female was continually exposed to the adapting stimulus for 5 min after which a 5 second pulse of the same adapting stimulus was tested, first at the concentration used in the adaptation ('Control B') and then at twice the concentration used for the adaptation ('Self-adapted Control'). Then the other test stimuli were tested in the adapting solution ('Adapted Response'). Each test was interspersed with 5 second pulses of the L-arginine standard and Control B to confirm the responsiveness of the female. Switching the adapting stimulus back to clean water completed the trial. The epithelium of the female was allowed to recover for 30 min and then the female was tested again using another adapting stimulus. Cross adaptation data were expressed as Percent Initial Response (PIR) using the following formula adapted from Li and Sorensen (1997),

$$PIR = \frac{(\text{Adapted Response} - \text{Control B})}{(\text{Initail Response} - \text{Control A})} \times 100$$

Where a larger PIR indicates less cross-reactivity between olfactory receptor mechanisms or separate receptor sites and a low PIR indicates more cross reactivity or shared recpetor sites and/or a common signal transduction pathway.

Statistical analysis

In concentration-response experiments, responses were visually compared. The lowest concentration at which a stimulus elicited a response larger than the blank water

control was considered to be its detection threshold and was analyzed using a student t-test (SAS).

In cross adaptation experiments, PIR data were analyzed first to directly detect cross-reactivity by subjecting all PIR data to a two-way analysis of variance (ANOVA; SAS). If the comparison was found to be significant ($P<0.05$), the PIR data was divided into five groups according to adapting stimulus, each analyzed by a one-way ANOVA (SAS). When a difference was found within a group, the stimuli responsible were identified by comparing PIRs with the Self-adapted Control in that group using Dunnett's test (SAS).

Results

All odorants tested were stimulatory to the olfactory epithelium of female sea lampreys. Water conditioned with spermiating males was analyzed using ELISA to determine the concentration of 3kPZS. The concentration response relationship of the water conditioned with spermiating males had a steep slope with a detection threshold of 10-12 M (Figure 2A). When compared with the concentration response curve generated from synthetic 3kPZS, the curves were similar with the natural compounding causing slightly larger responses and the detection thresholds of both 10-12 M (Figure 2A). Of the four bile acids, ACA and 3kACA had similar concentration response curves with detection thresholds of 10-10M, while PZS and 3kPZS had similar curves with thresholds of 10-12M (Figure 2B). Curves for ACA and 3kACA were shallower than the curves for PZS and 3kPZS. The response to the L-arginine standard was 0.859 mV (0.149 SD) in these experiments.

Although the responses to L-arginine standard did not significantly change before and during cross-adaptation (Figure 3; t-test, $P>0.10$), there were significant differences among bile acid responses during adaptation (2-way ANOVA, $P<0.01$). When used as the adapting stimuli, all four bile acids significantly suppressed EOG responsiveness to themselves (Figure 3; Dunnett's, $P<0.01$, using Self-adapting Control; Control B not shown). The Dunnett's tests demonstrated two patterns of cross-reactivity among the four bile acids. First, ACA suppressed responsiveness to itself and 3kACA, and vice versa (Figure 3A&B). Second, PZS suppressed responsiveness to itself and partially suppressed the response to 3kPZS, and vice versa (Figure 3C&D). When conditioned water was used (SMW), all stimuli were significantly suppressed (Figure 3E; Dunnett's, $P<0.01$).

Discussion

Both concentration response and cross adaptation experiments demonstrated that natural 3kPZS (water conditioned with spermiating males) and synthetic 3kPZS possess similar odor qualities. Concentration response curves generated with female EOG responses to natural and synthetic 3kPZS were similar, lending support to the conclusion of Li et al. (2002) that the bile acid, 3kPZS produced by spermiating males acts as a sex pheromone. The natural 3kPZS curve was slightly larger than the synthetic, possibly do to other bile acids such as ACA present in conditioned water. Concentration response curves showed that both 3kPZS and 3kACA were highly stimulatory to female olfactory organs. 3kPZS showed a more robust curve with a detection threshold of 10-12 M, while 3kACA had a detection threshold of 10-10 M. Comparatively, 3kPZS and PZS and 3kACA and

ACA generated similar concentration response curves with the same detection threshold. These results support the hypothesis that 3kPZS is the sex pheromone in spermiating males, and 3kACA is a possible minor component (Li et al. 2002) and that PZS and ACA are the migratory pheromone from larvae (Polkinghorne et al. 2001).

Cross adaptation experiments demonstrated that sea lamprey bile acids represent different odor qualities to the olfactory epithelium of females. 3kPZS and PZS appear to represent different odorants because they do not significantly suppress the responses to each other. Also, both ACA and 3kACA appear to possess odor qualities different from both PZS or 3kPZS because when adapted to these bile acids, responses to ACA and 3kACA were not suppressed. However, ACA and 3kACA appear to possess the same odor qualities because both were able to suppress responses to each other.

The cause for the difference in odor qualities of PZS and 3kPZS appears to be related to the 3-OH/3-keto group, which is the only difference between the two compounds. If these two bile acids represent two independent pheromones, this difference in odor quality is essential for their function.. because of the similarity in structure of the two compounds, which have altogether different functions and are present in the stream at the same time.

3kACA and ACA possessed different odor qualities than either PZS or 3kPZS. This difference appears to be mediated through the carboxyl/sulfate group at C-24. This specificity has also been demonstrated by Li and Sorensen (1997) who provided evidence of sulfate and carboxyl-specific receptor sites. The difference in odor qualities may allow for a mixture of 3kPZS and 3kACA to function as the spermiating male sex pheromone with each component mediating a specific behavior within the total response.

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Figure Legends

Figure 1. Molecular structure of sea lamprey bile acids. (A) 3α, 7α, 12α-trihydroxy-5α-cholan-24-oic-acid; Allocholic acid; ACA, (B) 3α, 7α, 12α, 24-tetrahydroxy-5α-cholan-24-sulfate; Petromyzonol sulfate; PZS, (C) 7α, 12α-dihydroxy-5α-cholan-3-one-24-oic-acid; 3 keto-allocholic acid; 3kACA, and (D) 7α, 12α, 24-trihydroxy-5α-cholan-3-one-24-sulfate; 3 keto-petromyzonol sulfate; 3kPZS. ACA and 3kACA possess a carboxyl group on carbon 24, while PZS and 3kPZS possess a sulfate at the C-24 position. ACA and PZS posses a carbonyl group at carbon 3, while 3kACA and 3kPZS possess a keto group at the C-24 position.

Figure 2. (A) Concentration-response plot of natural and synthetic 3 keto-petromyzonol sulfate. The natural compound was from water conditioned with spermiating males and itscconcentrations were determined by measuring 3kPZS concentration in the conditioned water using an enzyme linked immunosorbant assay developed by Yun et al. (2002). Mean response magnitudes are presented as a percentage of the response elicited by a 10-5 M L-arginine standard solution. Vertical bars represent one standard error. (B) Concentration response plots of sea lamprey bile acids. Mean response magnitudes are presented as a percentage of the response elicited by a 10-5 M L-arginine standard solution. Vertical bars represent one standard error. Abbreviations are listed in the legend for Figure 1.

Figure 3A-E. Results of the five cross-adaptation experiments, by adapting stimulus. Mean percent initial responses (PIR) are shown with horizontal bars representing one standard error. Response to self-adapting controls (twice the concentration of the adapting stimulus) are underlined and are used as the control for each group. Asterisks signify that the PIR is significantly larger than its corresponding self-adapting control (Dunnett's test, $P<0.05$). Numbers indicate sample sizes. Abbreviations and concentrations tested are listed in the legend for Figure 1.
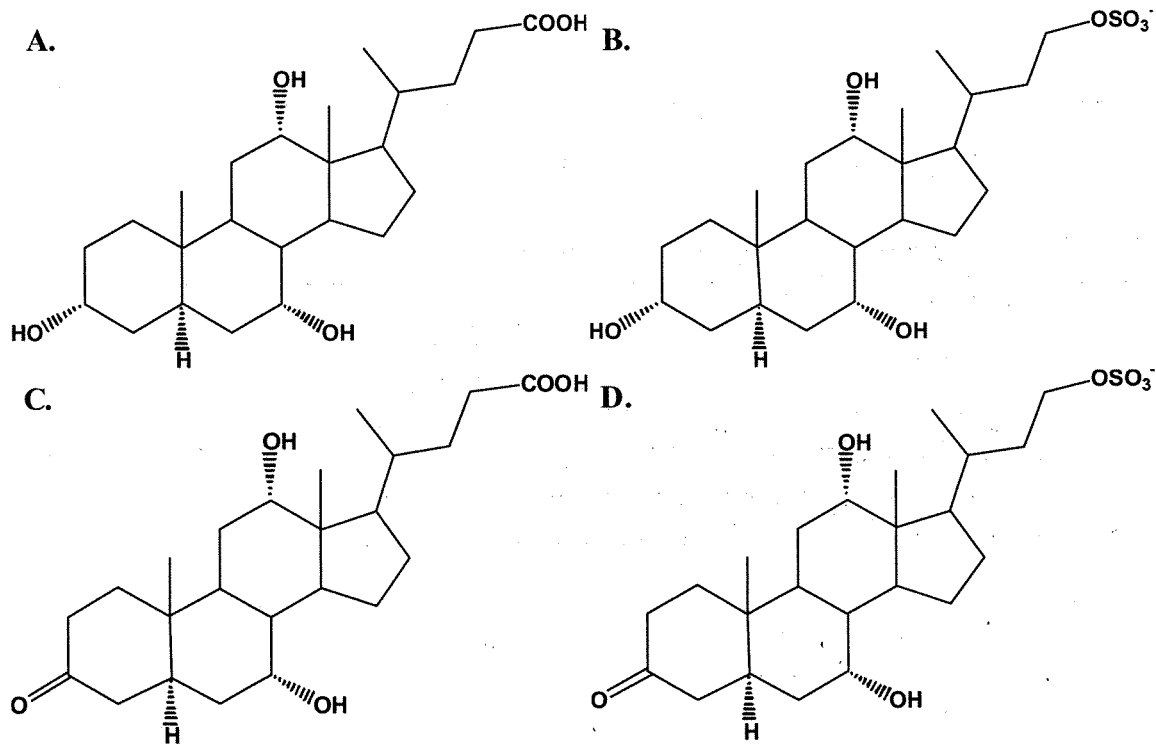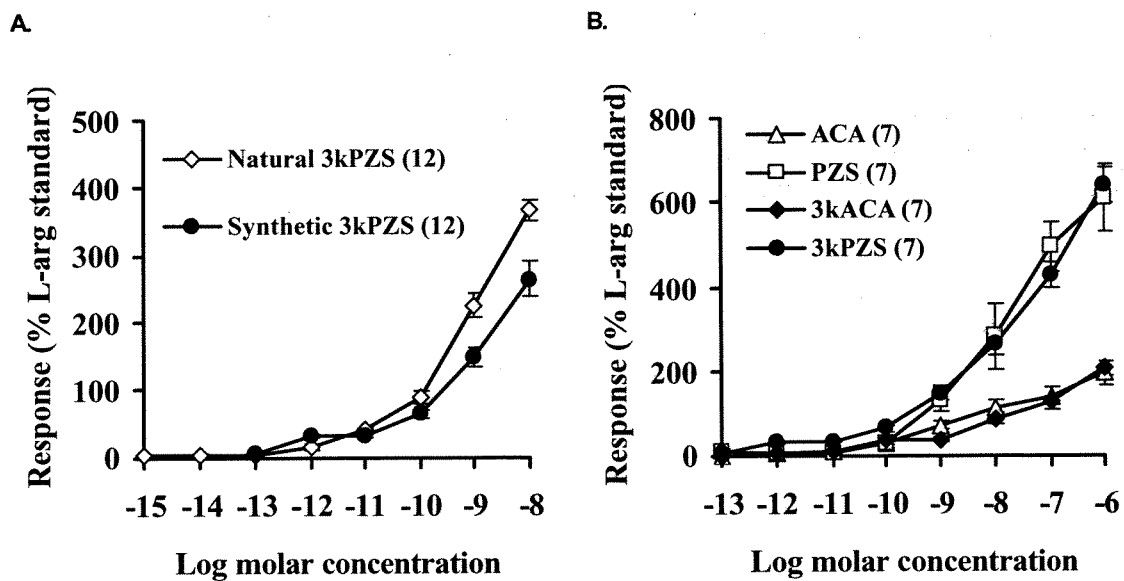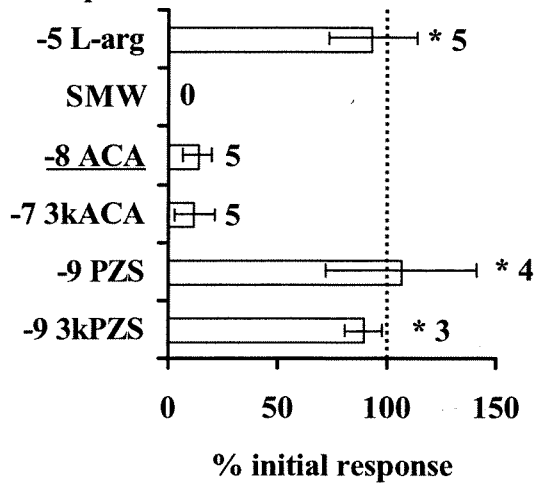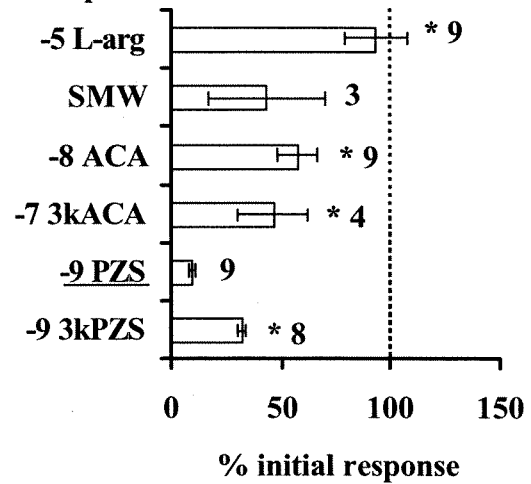
A.

B.

C.

D.

Figure 1.

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A.

B.

Figure 2.

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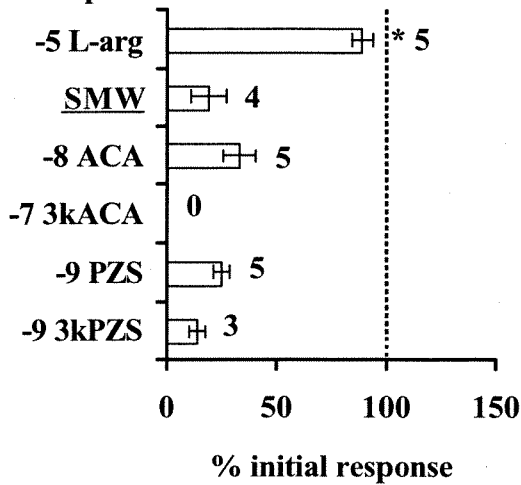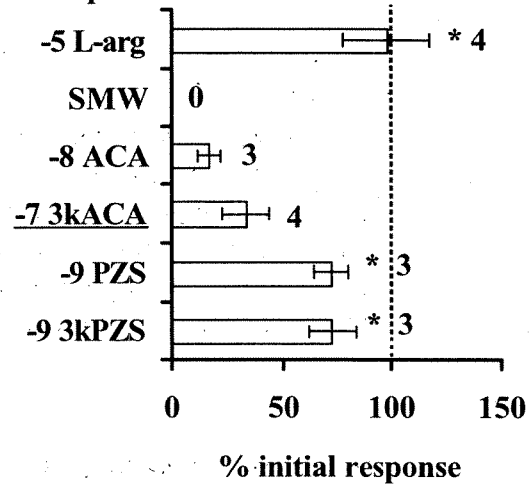
A. Adaptation to ACA

B. Adaptation to PZS

C. Adaptation to SMW
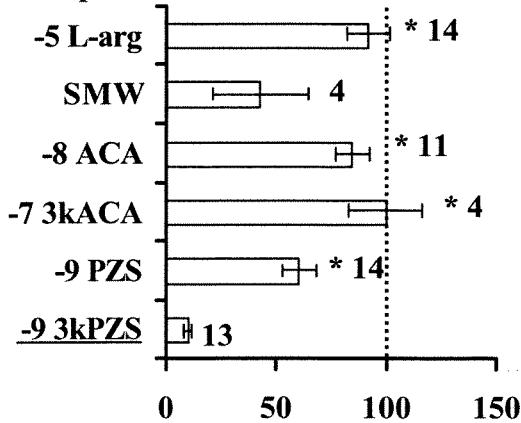
D. Adaptation to 3kACA

E. Adaptation to 3kPZS

Figure 3.

CHAPTER 3

[1]Development and Application of an ELISA for a Sex Pheromone Released by the Male Sea Lamprey (*Petromyzon marinus* L.)

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An Enzyme-linked Immunosorbent Assay (ELISA) has been developed for a conjugated bile acid, $7\alpha,12\alpha,24$-trihydroxy-5α-cholan-3-one 24-sulfate (commonly referred to as 3-keto petromyzonol sulfate [3kPZS]), a pheromone released by reproductively mature male sea lampreys to attract sexually mature females. A polyclonal antiserum against the pheromone was raised by injecting 3keto-petromyzonol 24-hemisuccinate (3kPZ-HS) conjugated to Bovine Serum Albumin into rabbits. The enzyme label was prepared by conjugating 3kPZ-HS to acetylcholinesterase. The standard curve had a working range of 20 pg to 10 ng per well. Intra- and inter assay variations were less than 5% and 12%, respectively. The antiserum had 100% cross-reaction with 3-keto petromyzonol and 3-keto allocholic acid but less than 0.2% cross-reaction with petromyzonol, allocholic acid, cholic acid and taurolithocholic acid sulfate. The assay was applied to water which had been conditioned for 4 h by either larvae, parasitic juveniles, ovulating females, pre-spermiating males or spermiating males. Immunoactive material (average 200 ng/ml, which is equivalent to 500 µg animal/h) was only found in water from the reproductively mature males. It diluted parallel with the standard curve. Assay of water samples collected from 'bisected' male lampreys also established that 99.6% of the immunoactive material emanated from the front end of the fish. This assay has applications in both physiological and ecological aspects of sea lamprey reproduction.

INTRODUCTION

Recently, a bile acid[1], $7\alpha,12\alpha,24$-trihydroxy-5α-cholan-3-one 24-sulfate, or 3keto-petromyzonol sulfate (3kPZS), has been identified as a pheromone released by sexually mature male sea lampreys to attract ovulated females (Li et al., 2002). This provides a useful model for studies of male attractants as most sex pheromones discovered to date are released by females to attract male conspecifics. In contrast to fish pheromones studied to date (Dulka et al., 1987; Sorensen et al., 1988; Sveinsson and Hara, 1995; Yambe et al., 1999) this pheromone is released in relatively large amounts - probably in order to facilitate signaling of male reproductive status over a large active space. This implies that there likely has been selection for high release rate because the pheromone normally is released in a current by males on the spawning grounds. The average yield of powdered 3kPZS, by High Performance Liquid Chromatography (HPLC) fractionation of extracted water conditioned with spermiating males, was c. 250 µg/fish/h. This reported amount, however, did not take into consideration potential losses during purification. Thus the actual release rate has yet to be determined, and is likely to be higher than the reported yield.

Another interesting feature of this male pheromone is its specificity of release. Behavioral studies demonstrated that only conditioned water samples from spermiating males (i.e. those with running milt), and more specifically, only conditioned water samples collected from the anterior portion of the body (the point source most likely being the gills), induced characteristic behavioral responses in sexually mature females (Li et al.,

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[1] Following the lead of Polkinghorne et al. (2001), for ease of discussion and to be consistent with existing literature on biliary compounds, we use the term 'bile acid' to describe 3kPZS even though it is a 'conjugated alcohol' and adult lampreys have no bile duct or gall bladder.

2002). However, the study did not determine whether the lack of attractiveness of conditioned water samples collected from the posterior portion was due to a total lack of released 3kPZS, or a level of 3kPZS that was not high enough to induce observed behavioral responses. Further, the data have hitherto not been clear enough to establish whether males at other developmental stages might also release 3kPZS, but at a rate too low to induce behavioral responses. It also cannot be excluded that the gills are the sole route of 3kPZS release.

Clearly, a simple and robust immunoassay which enables rapid, sensitive and specific measurements of the pheromone in a large number of samples is needed to establish just how much of the pheromone is released by sea lampreys. Although radioimmunoassay (RIA) have been the standard method used for quantification of steroid hormone and bile acid levels in plasma for decades, enzyme-linked immunosorbent assay (ELISA) is becoming the norm (Nash *et al.*, 2000). They have several advantages, not the least of which is the need not to handle and dispose of radioactivity. Many different enzymes have been used as tracers in ELISA, including urease, alkaline phosphatases, horseradish peroxidase and β-galactosidase (Gosling, 1990). However, the commonest, especially for ELISA of steroids appears to be acetylcholinesterase (AChE; Cuisset *et al.*, 1994; Nash *et al.*, 2000; Rodriguez *et al.*, 2000). This is the enzyme which has been used in the present study.

A major challenge in developing an ELISA for 3kPZS was to produce an antibody which would discriminate between 3kPZS and petromyzonol sulfate (PZS). The only difference between these two compounds is that 3kPZS has a 3-keto group while petromyzonol sulfate has a 3-hydroxyl group. PZS was identified over thirty years ago (Haslewood & Tokes 1969) in the bile of larval sea lampreys. Electroolfactographic (EOG) studies have established that it is a potent odorant in adult sea lampreys (Li *et al.*, 1995; Li & Sorensen, 1997), and behavioral studies (Bjerselius *et al.*, 2000) have shown that adult lampreys (of either sex) swim more actively in flowing water which contains a 10^{-10} M mixture of PZS and allocholic acid (ACA; another bile acid found in lamprey larvae bile; Haslewood & Tokes, 1969). Chromatographic and enzyme studies have also shown that both PZS and allocholic acid are released by larval lamprey at rates which are sufficient to produce detectable riverine pheromone plumes (Polkinghorne *et al.*, 2001). The ELISA was applied to water samples which had been conditioned by lampreys and also to water extracts fractionated by HPLC.

MATERIALS AND METHODS

Chemicals and equipment

3kPZS, 3kPZ, petromyzonol (PZ), PZS, ACA and 3-keto allocholic acid (3kACA) were purchased from Toronto Research Chemicals Inc. (2, Brisbane Road, North York, Ontario, Canada M3J 2J8). AChE, acetylthiocholine, cholic acid (CA), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), dimethylformamide (DMF), taurolithocholic acid sulfate, trypsin, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, USA). Freund's complete and incomplete adjuvants for immunization were also obtained from Sigma.

The assay was performed in 96-well polystyrene high-binding microtiter plates

(Costar, product code 3590) from Corning (Acton, MA). The plates were read with a Bio-Rad Benchmark plate reader (Hercules, CA) at 405 nm.

Sep-Paks were purchased from Waters (Milford, MA) and PD-10 desalting columns from Pharmacia (Piscataway, NJ). HPLC was carried out on a Nova-Pak HR C18 column (Waters, 19 x 300mm) using a Waters pump system. The fractionation was monitored using a Photodiode Array detector (Waters) at 200 nm.

Preparation of antibody

In designing a suitable hapten for antibody production, the 'most obvious' method - attaching a carboxymethyloxime group to the 3-keto position (Mount *et al.*, 1988) - was rejected as this was thought likely to produce an antibody which would not discriminate between 3kPZS and PZS. Instead, it was decided to attach a hemisuccinate group to the 24-hydroxyl group of 3-keto petromyzonol (3kPZ). This was conjugated to Bovine Serum Albumin to be injected into rabbits, and to AChE to use as a tracer.

For the preparation of antigen, 3-keto petromyzonol-hemisuccinate (3kPZ-HS) was custom synthesized by Toronto Research Chemicals. To conjugate it to BSA, 21 mg of 3kPZ-HS was dissolved in 1.5 ml of dimethylformamide (DMF) in a 20 ml glass beaker. The beaker was placed in crushed ice within a polystyrene container that was placed on top of a magnetic stirrer. A small magnetic flea was added to the beaker. The ice was prevented from thawing by the occasional addition of small amounts of liquid nitrogen to the container. With constant stirring, 12 μl tri-butylamine and 10 μl isochloroformate were added to the beaker and the reaction allowed to proceed for 40 min. In the meantime, 80 mg BSA was dissolved in 3 ml distilled water, diluted with 3 ml DMF plus 1 drop of 2N sodium hydroxide and chilled on ice. This mixture was added to the beaker and left to stir for a further 3h. After this time, the mixture, which was slightly opaque, was centrifuged for 10 min at 1000 g. The clear supernatant was divided into 2.5 ml aliquots for desalting on PD-10 columns (Nash *et al.*, 2000) using distilled water to elute the protein fraction. The eluates were combined, frozen and freeze-dried.

To produce antisera, 6 mg of the powdered conjugate was dissolved in 1 ml 0.9% saline and mixed with 1 ml Freund's complete adjuvant. One ml antigen-adjuvant mixture was injected into four rabbits. The rabbits were boosted with the conjugate in Freund's incomplete adjuvant two weeks after the first injection. They were bled for the first time at four weeks after the first injection. The serum was collected by centrifugation of the blood at 2700 rpm for 15 min. The supernatant was removed, aliquoted, and stored at –80 °C.

Preparation of enzyme label

The preparation of the enzyme label was based on the procedure described by Nash *et al.* (2000) with slight modifications. Briefly, G4-acetylcholinesterase was generated by treating AChE (1 mg) in 500 μl 0.1 M sodium phosphate buffer, pH 7.0 with 25 μl trypsin solution at 25 μg/ml in the same buffer for 24 h at room temperature. This reaction mixture was loaded on to a PD-10 column and eluted with 3.5 ml of 0.1 M borate buffer, pH 8.5. Next, 3kPZ-HS was activated by dissolving 200 μg in 38 μl of N-hydroxysuccinimide solution (1mg/ml in DMF) and then adding 32 μl of N, N'-dicyclohexylcarbodiimide solution (2 mg/ml in DMF) and leaving it overnight in the dark. Thirty μl of this reaction mixture was reacted with 400 μl G4-AChE stock for 2 h in the dark. This was purified on a PD-10 column by eluting with 3.5 ml of 0.01 M Tris buffer, pH 7.4 containing 0.01 M

MgCl$_2$, 1M NaCl and 0.15 mM NaN$_3$. The eluate was stored at −20 °C in 20 µl aliquots.

Titration of antibody and enzyme label

The optimal dilutions of AChE label and antibody were determined by checkerboard titration (Diamanis and Christopoulos, 1996). The starting dilutions for the titration of the AChE label and the antiserum were 1:40 and 1: 1000, respectively.

Assay procedure

Plates were coated with polyclonal goat anti-rabbit IgG (Sigma; product code R2004) by adding 120 µl of antibody diluted in 0.05 M potassium phosphate buffer, pH 7.4 to each well and incubating overnight at 4 °C. The plates were blocked by addition of 100 µl of 3% BSA in 0.1 M potassium phosphate buffered saline and storage at 4°C for at least 12 h.

After washing the plates three times with wash buffer (0.05 M potassium phosphate buffered saline, pH. 7.4, 0.05% Tween 20), 100 µl of assay buffer (0.1 M potassium phosphate buffered saline, pH. 7.4, 0.1% BSA, 1 mM EDTA, 0.15% sodium azide) was added to each well and serial dilutions of 3kPZS were made in a range of 20 pg to 10 ng/well. Non-specific binding (NSB) and maximum binding (B$_0$) were measured in separate wells. Conditioned water samples, extracts or HPLC fractions were diluted 20 times in assay buffer and 100 µl added to wells in duplicate; 50 µl of the diluted enzyme label (1:2000) was added to all wells and 50 µl of primary antibody (1:500,000) was added to all but NSB wells. Plates were incubated for 2 h at room temperature in a humid chamber. Then, after rinsing three times with wash buffer, 200 µl Ellmans reagent (4.3 mg DTNB, and 4 mg acetyl thiocholine in 20 ml 0.02 M potassium phosphate buffer) was added to each well. The plates were sealed and incubated overnight at room temperature in a humid chamber. Color development was measured at 405 nm.

Assay validation

To test the cross-reactivity of the antibody to structurally related compounds, serial dilutions of ACA, PZ, PZS, CA, 3kACA and 3kPZ were assayed alongside the 3kPZS standard.

Intra-assay variation was determined by assaying eight replicates of 3kPZS standard in the same plate. Inter-assay variation was determined by assaying a sample six times in different plates. In addition, parallelism was determined by diluting, alongside synthetic 3kPZS, water conditioned by a single spermiating male that had been placed in 10 L of lake water for 4 h.

HPLC fractionation of extracts

Up to 12 L of water which had contained spermiating male lampreys was pumped through twelve Sep-Paks (1 L each), which were then washed with distilled water and eluted with 5 ml methanol. The methanol was dried down in a rotary evaporator and the residue redissolved in 280µl solvent B (70% acetonitrile and 0.01% TFA in deionized water) and 720 µl solvent A (0.01% TFA in distilled water) and loaded on to the HPLC column. The column was developed with a gradient of solvent B from 28% to 100% over 50 minutes, at a flow rate of 4 ml/min. One min fractions were collected and diluted in assay buffer.

Separation of 3kPZS and 3kACA

A trial was carried out in which a mixture of synthetic 3kPZS and 3kACA was dissolved in buffers of different pH and then extracted with diethyl ether. This established that synthetic 3kACA, but not 3kPZS, could be extracted into the diethyl ether phase at pH 4. Based on this observation, an extract from 1 L of water that had been conditioned by a spermiating male was redissolved in 100 μl methanol and 900 μl of 0.05 M sodium acetate buffer, pH 4.0. This was extracted four times with 3 ml diethyl ether. The residue was then separated by HPLC as described above and the fractions assayed with the ELISA. Some of the extract which had not been treated with diethyl ether was treated in the same way. After these separations had been performed, standard synthetic 3kACA, 3kPZS and 3kPZ (200 μg each) were run on the column under identical conditions.

To measure the actual amounts of 3kPZS in the conditioned water samples that had been collected from spermiating males, 100 μl of water was mixed with 100 μl of 0.05 M sodium acetate buffer, pH 4.0, and the mixture was extracted 3 times with 700 μl of diethyl ether. 50 μl of the mixture after ether extraction was assayed. The ether extract was dried down, reconstituted in buffer and assayed to establish the amounts of 3kACA-like material present in the water.

Pheromone release at different life history stage

Adult sea lampreys were trapped or collected by hand from tributaries to lakes Huron and Michigan by the staff of the U. S. Fish and Wildlife Service, Marquette Biological Station (MBS), Marquette, Michigan, USA. Parasitic lampreys were obtained from commercial fishermen collecting salmonids. Larval lampreys were collected by the staff of Hammond Bay Biological Station using an electrofishing apparatus. The animals were transported to the main laboratory at the U. S. Geological Survey, Hammond Bay Biological Station, Millersburg, Michigan USA. The adults were sexed and females and males were held in separate flow-through tanks (1000 L) with Lake Huron water (7°C to 20°C). The parasites were not sexed. The same tanks and water were used to hold the parasites. The larval lampreys were held in a tank (100 L) with sand. All the animals were acclimated for at least a week before use. The average body masses of lampreys were 0.63 g (larvae), 125 g (parasites), 240 g (female adults) and 233 g (male adults).

Ten spermiating males, six pre-spermiating males, six parasitic juveniles and six ovulated females were kept separately in 10 L container filled with lake water at 15 to18 °C. Six larval lampreys were kept separately in 250 ml water. After 4 h, 20 ml samples of water were taken and frozen for subsequent direct assay.

Confirming the route of release of the pheromone

Water was collected separately from the head and tail regions of spermiating male sea lampreys. To achieve this, an acrylic plate, with a hole that was slightly larger than the girth of an adult lamprey, was glued into the middle of an acrylic aquarium (thus creating two chambers). The hole was lined with a latex rubber gasket, so that when a lamprey was pushed gently through the hole (to a position just behind the gills), water could not flow from one chamber into the other. A perforated acrylic tube was mounted on one side of the plate - to immobilize the head of the animal. A flexible plastic mesh tube, with an adjustable width, was mounted on the other side. Once the lamprey had been secured, the

head chamber was filled with 10 L of water. The tail chamber was inspected for leaks from the head end then also filled with 10 L of water. Airstones were placed in both chambers and the male was held this way for 1 h. The water was collected at the end of this time and stored at −80 °C until analyzed. This procedure was carried out five times on separate fish

RESULTS

Enzyme label and antibody production

All bleeds from all four rabbits were found to bind the enzyme label. The one with the highest titer (L286) was chosen for further development. Checkerboard titration of the antiserum and enzyme label determined that the optimal combination of dilutions was 1:500,000 for the antiserum and 1:2000 for AChE tracer, respectively.

Standard curve and assay validation

A standard curve was established in a working range of 20 pg/well to10 ng/well. Close parallelism was observed between dilutions of water conditioned by a spermiating male and synthetic standard (Fig. 1). The intra-assay coefficient of variance was 5% and that for inter-assay 12% (for a sample in the middle of the standard curve).

Cross-reaction of other compounds

On a weight for weight basis, the antiserum cross-reacted equally well with 3kPZS, 3kPZ and 3kACA (Fig. 2). The fact that 3kPZ and 3kACA are smaller molecules, however, implies that they do in fact cross-react slightly more strongly than 3kPZS. Cross-reaction with all the other compounds, except PZS (1.6%), was negligible. However, when the PZS was run on HPLC, the cross-reacting material was found in the elution position of 3kPZS (39 to 41 min) and none in the elution position of PZS (43 to 44 min).

Assay of HPLC fractions

When assays were carried out on HPLC fractions of a water extract, the bulk of immunoactivity was found in fractions 39 to 41 (Fig. 3). The total amount of activity in all three fractions was 3.6 mg (which from 20 L water equates to an original concentration of c. 300 ng/ml in the water). Another small peak of immunoactivity was found in fractions 54 to 55.

HPLC analysis of synthetic compounds

The three synthetic compounds which cross-reacted with the antiserum were run on HPLC (Fig. 4A). Their elution positions were confirmed by both UV absorption as: 3kPZS, 40 to 41 min; 3kACA, 54 to 55 min; and 3kPZ, 58 to 59 min.

Separation of 3kPZS and 3kACA

Diethyl ether extraction of water at pH 4 removed the immunoactivity in fractions 54 to 55 (Fig. 4B & C).

Pheromone release at different life history stage

Conditioned water samples from larvae (Larvae), parasites (Para), pre-spermiating

males (PSM) and ovulated females (OF) did not contain pheromone at the limits of detection of the assay, which was 400 pg/ml, while the concentration of the pheromone in the samples from 10 spermiating males was on average 209.5 ng/ml (± 70.6, n=10; Fig. 5). The range was 21.5 ng/ml to 785.2 ng/ml. The proportion of immunoactivity which could be extracted with diethyl ether from these same water samples (after adjusting them to pH 4) varied between 0.2 and 11.5%, with a mean of 3.0% (± 1.1%).

Confirming the route of release of the pheromone

In all five 'bisected' males, >99% of immunoactivity was found in the water bathing the head region (Figure 6). The concentration of the pheromone in the conditioned water samples from the head region ranged between 2.9 and 113.1 ng/ml, with a mean of 49.0 (±15.5) ng/ml. The concentrations in the water bathing the tail region ranged from undetectable to 1.3 ng/ml.

DISCUSSION

An ELISA has been developed for 3kPZS - the male sea lamprey sex pheromone - and successfully applied to the measurement of amounts of pheromone released by captive male lampreys. The standard curve covers a range of 42 fmol to 21 pmol/well and is more sensitive than several previously described RIAs for bile acids with detection limits of 500 fmol to 10 pmol (Davidson et al., 1980; Hashimoto et al., 1990; Matsuoka and Okumura, 1988; Miller et al., 1981), but less sensitive than ELISAs and RIAs for steroids (Nash et al., 2000; Cuisset et al., 1994).

The coupling of 3-keto petromyzonol-24-hemisuccinate to BSA appears to have been successful in producing a relatively specific antiserum. Although it cross-reacts equally well with 3kPZ and 3kACA, the former was not found in the extracts and the latter can be easily removed by solvent extraction. Although the antiserum appeared to cross-react slightly with the migratory pheromone PZS (1.6 %), this was found to be due to contamination. The fact that 3kACA cross-reacts with the antiserum, while ACA does not, highlights the importance of the 3-keto configuration for cross-reactivity.

The assay was validated by checking intra- and inter-assay variation and parallelism of the 3kPZS standard with water which had been conditioned by a spermiating male lamprey. Intra- and inter-assay variances were analyzed according to the procedures of Nash et al. (2000) and were well within the ranges described for other bile acid RIAs (Davidson et al., 1980; Hashimoto et al., 1990; Matsuoka and Okumura, 1988; Miller et al., 1981) and ELISAs (Baqir et al., 1979; Ozaki et al., 1979). Close parallelism of conditioned water was noted with standard 3kPZS - indicating the reliability of the assay in measuring the pheromone in the water samples.

On HPLC, the fractions with a retention time of 39 to 41 minutes were found to contain most of the immunoactivity, with only one other minor peak, in fractions 54 to 55, being found. These two peaks correspond in elution position, and in relative amounts, to the two peaks of EOG activity, and to two out of three of the bands which could be stained by phosphomolybdic acid on Thin Layer Chromatograms, in the original study by Li et al. (2002). The results of the ELISA confirm the findings of Li et al. (2002) that the first (i.e. larger) peak is 3kPZS. They also strongly indicate that the second and minor peak is

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3kACA. The fact that 3kPZS and 3kACA can be easily separated by solvent partition prior to assay means that both compounds can be separately quantified in water samples.

The ELISA method in the present study offers many advantages over the HPLC method described in the earlier study (Li et al., 2002). Firstly, the detection limit of the conventional HPLC methods using an analytical column and U-V absorption falls into the microgram scale, which is too high for studies of individual variations and time course of 3kPZS release. The ELISA, with a detection limit of 20 picogram per well, is far more suitable for these types of studies. Secondly, since the HPLC method requires several extraction and purification steps on water samples prior to loading them onto a column, some pheromone is likely to be lost. However, our ELISA protocol involves only a single and simple solvent partition step. Thirdly, with the HPLC method it takes a long time to prepare and analyze a sample, whereas with the ELISA method a large number of samples can be processed in a relatively short period of time.

The fact that 3kPZS and 3kACA are released together by the adult male lamprey has a remarkable parallel with the larval lamprey, which synthesizes a mixture of PZS and ACA (Haslewood and Tokes, 1969). Since a mixture of PZS and ACA appears to be a more potent pheromonal signal for adult lampreys than PZS by itself (Bjerselius et al., 2000), then possibly a mixture of 3kPZS and 3kACA is a more potent signal for ovulated females. We speculate that 3kACA may be a minor component that regulates or modulates the pheromonal function of 3kPZS. The exact role of 3kACA has yet to be established.

Very little is known about the biosynthesis of sea lamprey bile acids. It is speculated that the pathway that is responsible for the biosynthesis of PZS and ACA is the same as that responsible for the biosynthesis of 3kPZS and 3kACA in spermiating males. The only difference involves the activity of 3α hydroxysteroid dehydrogenase. It is not known yet whether this is normally active (in a reducing mode) in larvae and then becomes switched off in adult males – or whether it is normally inactive (in an oxidizing mode) in larvae and then becomes switched on in adult males.

The concentrations of pheromone in the ten conditioned water samples collected from spermiating male lampreys varied from 21.5 to 785.2 ng/ml (or 53.7 μg to 1.9 mg/fish/h) whereas no activity was found in conditioned water from larval lamprey, parasites, pre-spermiating males and ovulating females. This confirms the original observations of Li et al. (2002) that only mature males produce 3kPZS at an estimated rate of 250 μg/fish/h - based on the weight of dried pheromone obtained from 10 L of conditioned water. Compared to the rough estimation of pheromone release rate from pooled conditioned water, the present ELISA has proved useful for estimating pheromone release rate in the individual mature males.

In an experiment using a bisected aquarium, most of the immunoactivity (>99 %) released by spermiating males was found in the conditioned water samples from the head region, while negligible amounts were found in the conditioned water samples from the tail region. This supports our previous findings that only water from the head region was able to attract ovulated females (Li et al., 2002). It does, however, contradict an earlier study suggesting that urine of sexually mature males attracts mature females (Teeter, 1980). A direct comparison is difficult as Teeter (1980) did not specifically select spermiating males and ovulated females. Our own unpublished data indicates that urine of spermiating males does not contain 3kPZS. It is possible that males do release pheromone via the urine. However, this pheromone is not 3kPZS.

Fish held in the bisected aquaria released similar amounts of pheromone to those held in buckets. These results indicate that constraining a male lamprey in the bisected chamber is not more stressful than holding them in a bucket. The sea lamprey appears to be a good model for studying pheromone biosynthesis under laboratory conditions.

The point source of the pheromone is almost certainly the gills, which in spermiating males contain unique glandular cells (Pickering, 1977). It is not yet known whether these are responsible for de novo synthesis of 3kPZS or whether they act as a 'pumping station' for 3kPZS which has been made in the liver. With the development of an ELISA, we now have a powerful tool for unraveling its mechanism of synthesis and release; and also for determining whether its synthesis is under any sort of hormonal control. One other possible application for the ELISA is to estimate the numbers of male lampreys on a spawning ground - by measuring the amount of 3kPZS in the river water.

In summary, we report an ELISA for 3kPZS which has been validated in a variety of ways. The assay is highly specific - apart from a small amount of cross-reacting material which has been tentatively identified as 3kACA and that elutes after 3kPZS on the HPLC. However, this can be removed by prior extraction of the water samples with an organic solvent. The assay was successfully used not only to show how much pheromone is released by sea lampreys in the laboratory but also to show that it is released only from the head region of spermiating males. The ELISA will undoubtedly provide a useful tool for investigating the physiological, ecological, and behavioral aspects of chemical communication in sea lamprey.

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Figure Legends

FIG. 1.   Dilution curves for synthetic 3kPZS and for water which had been conditioned by a spermiating male lamprey.

FIG. 2.   Cross-reaction of the antiserum with some structurally related compounds. A, 3kPZS (●), 3kPZ(O), PZS(?), PZ(∇); B, 3kPZS (●), 3kACA (∇) ACA(v) and CA( ).

FIG. 3.   HPLC fractionation of an extract of water which had been conditioned by spermiating male lampreys. A, UV absorption at 200 nm; B, immunoactivity in the fractions (measured against synthetic 3kPZS).

FIG. 4.   HPLC fractionation of synthetic and naturally derived bile acids: A, UV absorption of 200 µg of synthetic 3kPZS (peak 1), 3kACA (peak 2) and 3kPZ (peak 3); B, immunoactivity in an extract of water from a spermiating male; C, immunoactivity in the same extract after diethyl ether extraction at pH 4.

FIG. 5.   Amounts of 3kPZS-immunoactive material in water (ng/ml ± SEM) which had been conditioned for 4 h by larvae (Larvae), parasitic juvenile (Para), ovulating females (OF), pre-spermiating males (PSM), and spermiating males (SM). There were ten spermiating males and six of each of the other stages. ND = non-detectable (<400 pg/ml).

FIG. 6.   Concentrations of 3kPZS immunoactivity (ng/ml) in water from either the head or tail regions of spermiated males (n=5). Total volume of water on each side of the bisected tank was 10 L.
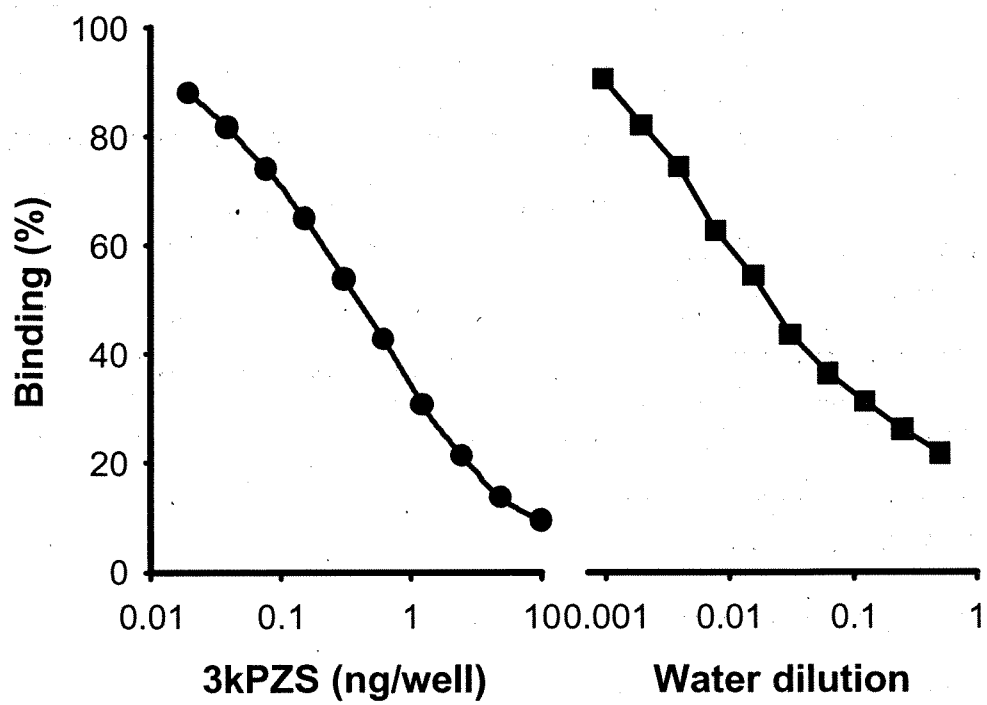
Figure 1

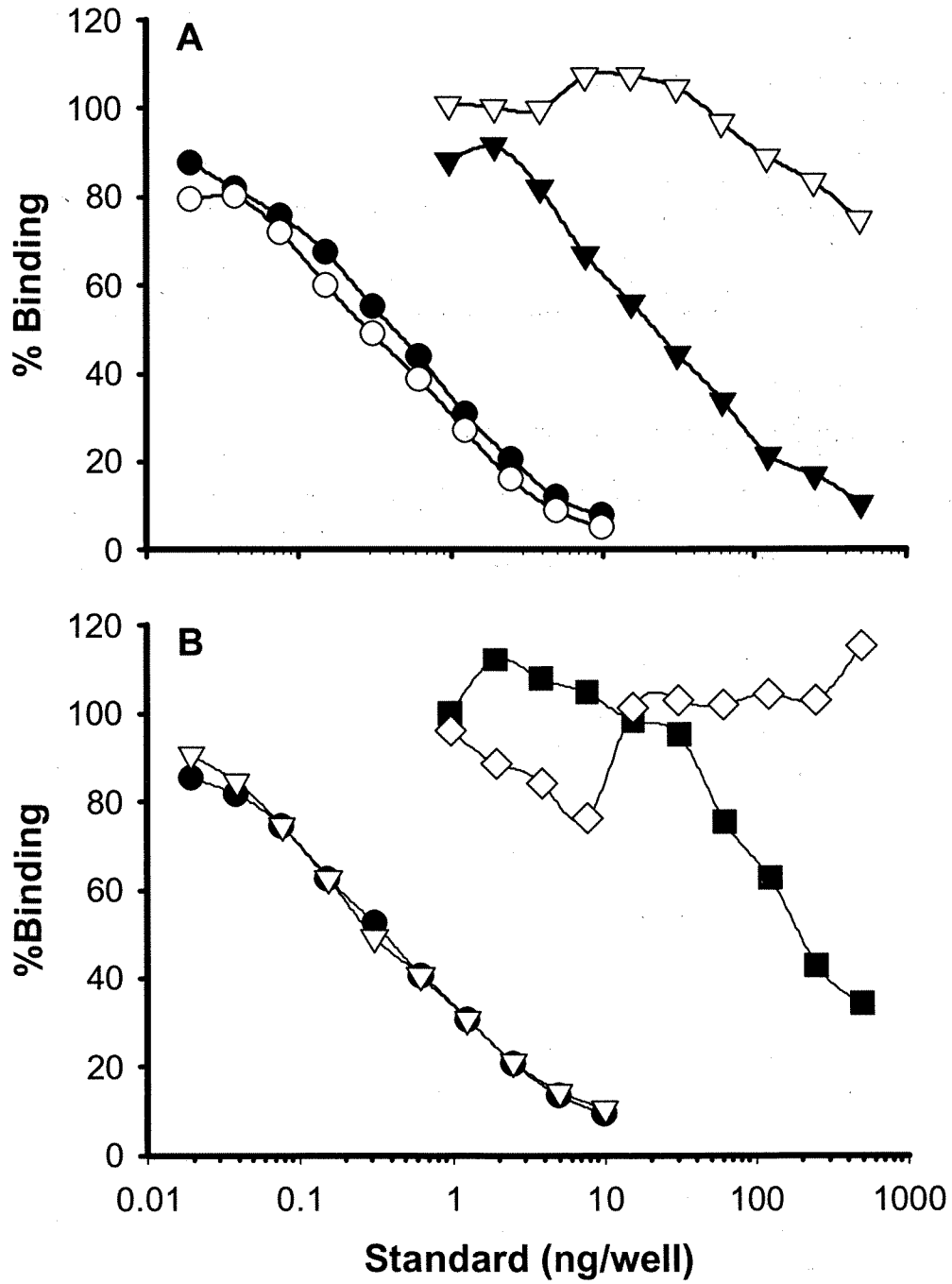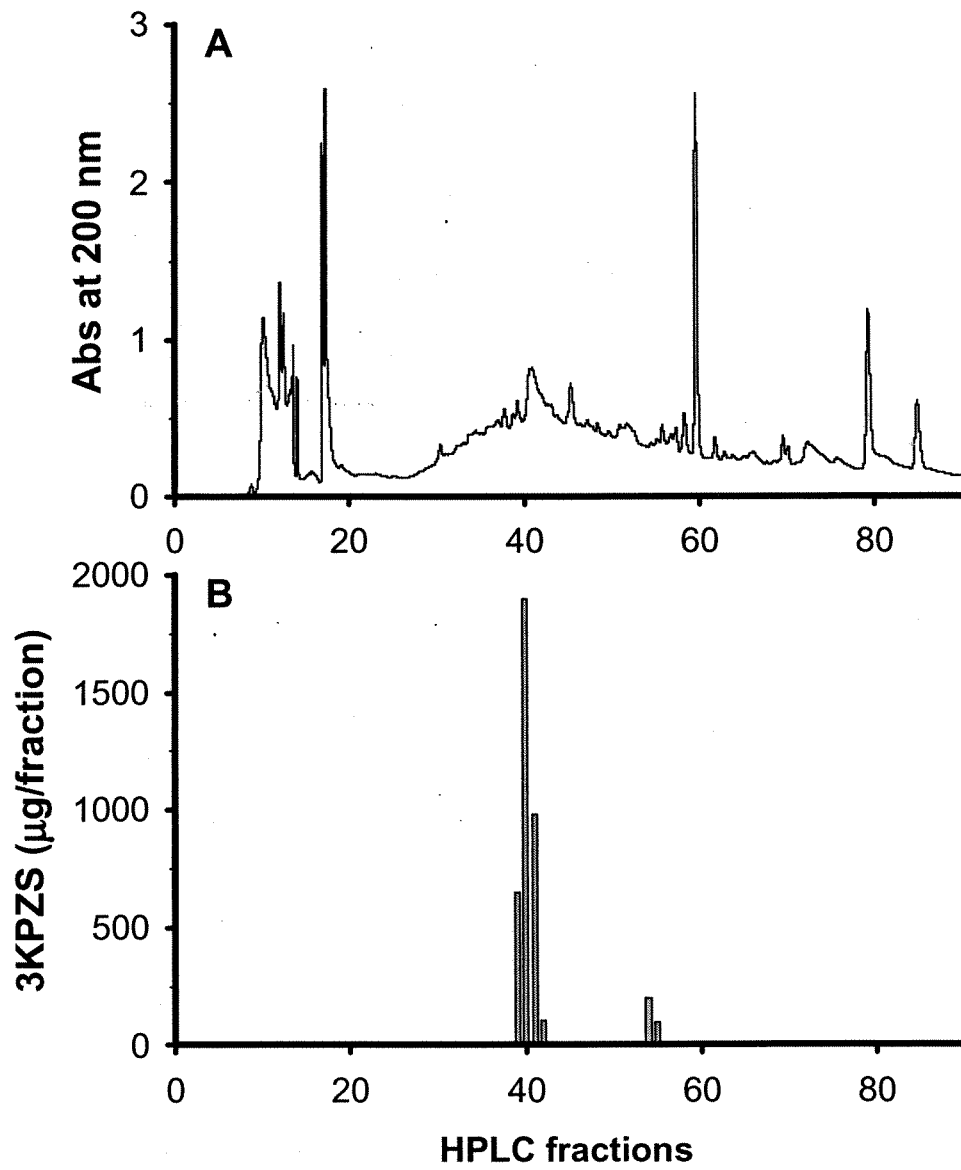Figure axes: Binding (%) on the vertical axis (0 to 100); 3kPZS (ng/well) from 0.01 to 100 on the left panel and Water dilution from 0.001 to 1 on the right panel.
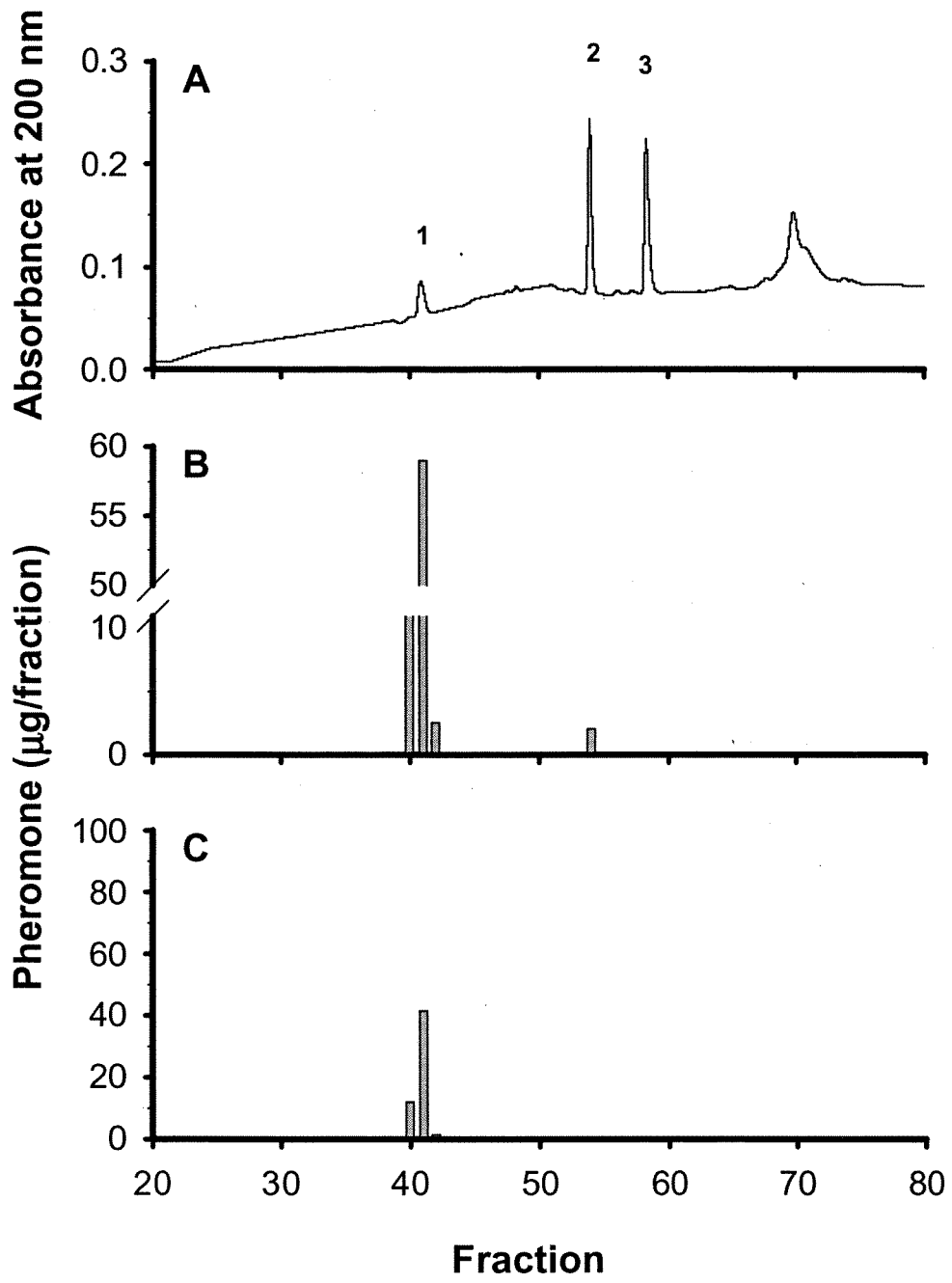
Figure 2

Figure 3

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Figure 4

Figure 5.
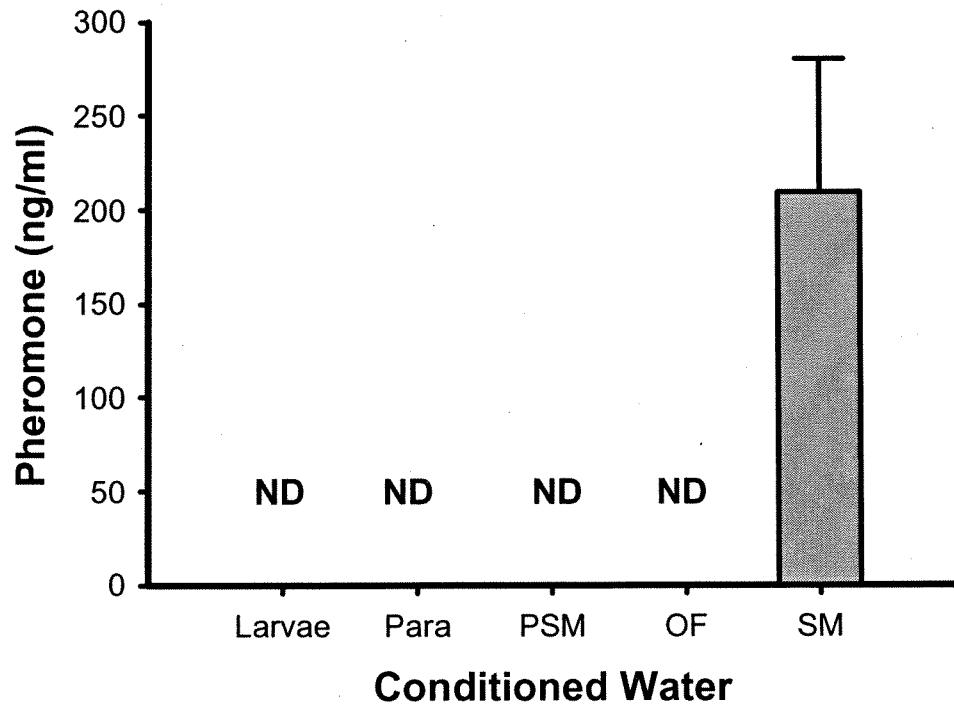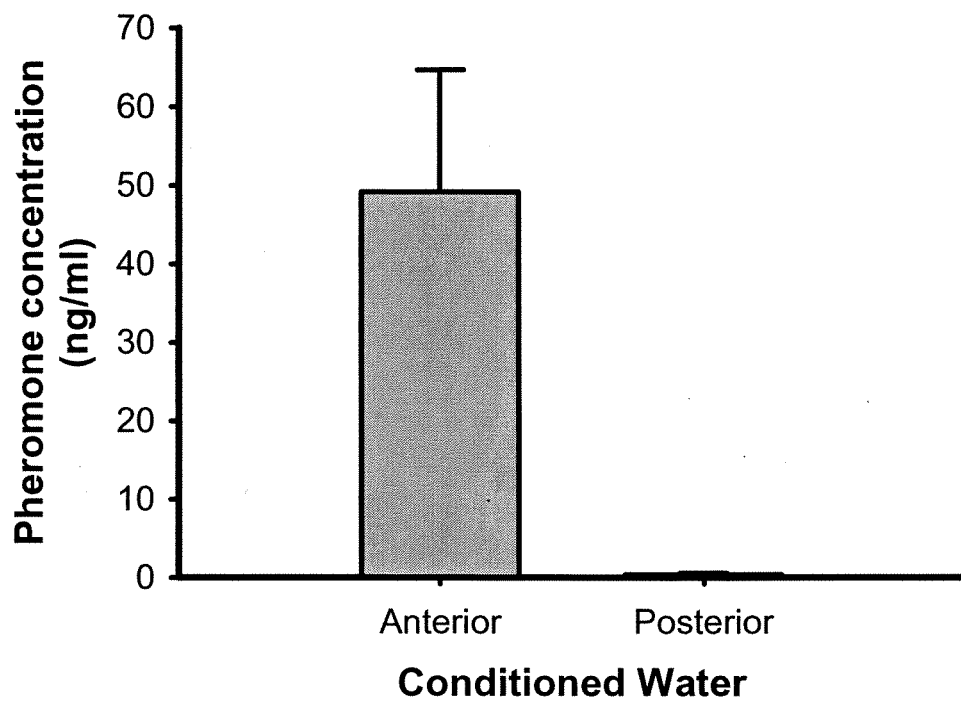
Figure 6.

CHAPTER 4

[3]A Gill Glandular Cell-Mediated Release Mechanism for a Sex Pheromone in the Male Sea Lamprey *Petromyzon marinus* L.[1]

Short title: Gill Release of Sex Pheromone

Keywords: Behavior, Environment, Male sexual function, Pheromones, Spermatogenesis

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ABSTRACT

During the period when they are producing sperm, male sea lampreys (*Petromyzon marinus* L.) release a sex pheromone 7α, 12α, 24-trihydroxy-5α-cholan-3-one-24-sulfate (3 keto-petromyzonol sulfate, 3kPZS) that induces search and preference behaviors in ovulating females. In this study, we conducted a series of experiments to demonstrate that release of this pheromone into water takes place exclusively through the gills. In a behavioral maze, water conditioned with the anterior region of spermiating males induced an increase of search and preference behaviors in ovulating females. Similar behavior was not elicited by water conditioned by the posterior region. The anterior region washings and whole body washings from spermiating males also elicited large and virtually identical electro-olfactogram responses from female sea lampreys, while the posterior washings produced negligible responses. Further, mass spectrometry and immunoassay confirmed that virtually all the 3kPZS released into water was through the gills. Immunocytochemistry revealed some gill epithelial cells and hepatocytes from spermiating males contained dense immunoreactive 3kPZS, but not those from pre-spermiating males. These results demonstrate that 3kPZS is released through the gill epithelia, and suggest that this pheromone or its precursor may be produced in the liver.

INTRODUCTION

Spermiating[4] male sea lampreys *Petromyzon marinus* L. release a bile acid, 7α, 12α, 24-trihydroxy-5α-cholan-3-one-24-sulfate (3 keto-petromyzonol sulfate, 3kPZS), which acts as an attractant for ovulating females [1]. Although this sex pheromone has been identified and its function demonstrated, its mode of synthesis and excretion remains elusive. Bile acids are typically produced in the liver, secreted into the gall bladder, and excreted through the intestine along with feces [2, 3]. Larval sea lampreys appear to have evolved this route as well [4, 5]. However, this same route is not available to adult sea lampreys, which lack gall bladders and bile ducts [6].

In river lamprey *Lampetra fluviatis*, the gills of spawning males contain large glandular cells [7, 8] that have been postulated to "...secrete some substance of sexual significance" [7]. More recently, we showed that water from the anterior region of spermiating males (bathing the gills) contained far more immunoreactive 3kPZS than water from the posterior region [9] and suggested that gills may mediate the release of this pheromone compound [1]. However, there was no proof that this compound emanated from the gills rather than from some other part of the anterior region, and certainly no direct link to the glandular cells. Further, there was no corroborating chemical and biological evidence from any other procedure that 3kPZS was indeed emanated mainly from the anterior region.

The main hypothesis that we set out to prove in the present study is that the male pheromone is released into the water via the glandular cells of the gills. Our expectations are: (1) that the results of ELISA [9], which showed that 3kPZS was released largely from the anterior region of spermiating males, can be confirmed by mass spectrometry, electro-olfactogram and behavior induction in ovulating females; (2) that water emitted by gills (before it touches any other tissues) contain 3kPZS at a level comparable to that estimated from anterior body washings; (3) that the unusual glandular cells in the gills will stain immunocytochemically with antibodies to 3kPZS; and that the gills of spermiating males have many more of these cells than the gills of pre-spermiating males.

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[4] A 'spermiating' lamprey is one from which milt (spermatozoa plus seminal fluid) can be expressed by gentle manual pressure.

MATERIALS AND METHODS

Collection and maintenance of animals

Adult sea lampreys were collected from tributaries to lakes Huron and Michigan by the staff of the U.S. Fish and Wildlife Service, Marquette Biological Station, Marquette, Michigan, USA. The animals were transported to the main laboratory at the U.S. Geological Survey, Hammond Bay Biological Station, Millersburg, Michigan, USA. Males and females were separated and held in flow-through tanks (1000 L) with Lake Huron water at temperatures ranging from 7°C to 20°C. Males and females were checked periodically for spermiation and ovulation according to the criteria and procedures reported [10]. Any spermiating and ovulating individuals were separated into two other tanks respectively.

Collection of washings and extracts

Washings from the anterior and posterior regions of spermiating males were collected using a bisected, acrylic aquarium (Figure 1). A divider with a hole to accommodate a sea lamprey's head was fixed in the middle of the aquarium to make two separate chambers. The hole was lined with a latex gasket which, when the male was in place, prevented water from flowing between the two chambers. A perforated acrylic tube was mounted on one side of the hole to immobilize the anterior region. The posterior region was held in flexible plastic mesh that was tightened according to the size of the male.

During a washing, a spermiating male was anaesthetized with MS-222 and placed headfirst through the gasket/divider, making sure all the gills were located in the forward chamber. The plastic mesh tube around the posterior region was then tightened. The anterior chamber was filled with 10 L of water, the latex gasket inspected for leaks, and then the posterior chamber filled with 10 L of water. Each chamber was aerated and the male was held this way for 1 h. At the end, all the water was collected and either used directly in experiments or extracted as previously reported [1]. For extractions, water was pre-filtered with No. 3 Watman filter paper and then drawn through activated Sep-Pak octadecylsilane cartridges (Waters, Milford, Massachusetts, USA). These were then washed with distilled water, eluted with methanol and stored at -80°C.

To prove that there was no leakage across the latex gasket in the bisected aquarium, two experiments were performed. In the first, males were secured in the aquarium. A dye was then added to one chamber and the other checked visually after 1 h. In the second, females, known not to release 3kPZS [1, 9], were placed in the aquarium and 1 mg of synthetic 3kPZS was introduced into one of the chambers. After 1 h, water samples were taken from both chambers and analyzed for 3kPZS by ELISA [9]. Both experiments were repeated - checking for leakage from either direction.

Behavioral assays on water taken from the anterior and posterior regions of spermiating males

The sex pheromone released by spermiating males induces preference and search behaviors from ovulating females [1, 10]. Therefore we measured these two types of characteristic behavioral responses from ovulating females to chemical stimuli from the anterior and posterior regions of spermiating males using the identical apparatus and protocols developed in the previous studies [1, 10]. Briefly, preference behavior was the amount of time spent in either side of a two-choice maze, while search behavior was the amount of time spent swimming at the head of either side of a two-choice maze. In each test, the behavior of a single female was video recorded before and after the introduction of washings from either the anterior or posterior regions of spermiating males into the odor chamber of the side of the maze chosen randomly by the toss of a coin. The washings were delivered at 75 ml/min using a peristaltic pump. Tests were conducted between 07:00 and 17:00 in water temperatures that ranged from 12 °C to 24 °C. Preference and search

behaviors were scored by naïve observers and the data analyzed with a two-tailed Wilcoxon Signed Ranks Test [1, 10, 11].

Electro-olfactographic (EOG) recordings on water from the anterior and posterior regions of spermiating males

Synthetic and natural 3kPZS induce strong EOG responses from adult female sea lampreys [1], as does water conditioned by spermiating male sea lampreys [10, 12]. Using EOG recording on females, we determined the olfactory potency of washings from the anterior and posterior regions and whole body of spermiating males and established concentration-response relationships, according to established procedures [10, 13]. For each recording, a 10^{-5} M L-arginine standard was pulsed into the olfactory epithelium of a female and the EOG response measured to establish a baseline of electrical activity. Next, blank control water was introduced and the response measured to confirm the absence of odorants from the clean water source used to perfuse the olfactory epithelium (and also to mix the test odorants). Increasing concentrations of test odorants (starting at 10^6 dilution) were then introduced and the responses measured. Measuring the response to the L-arginine standard and blank controls concluded each trial. The epithelium of each female was allowed to recover for at least 3 min between stimuli and each concentration of an odorant was tested at least twice on a female. The magnitude of EOG responses was measured [14] and expressed as a percentage of the L-arginine standard [13].

Mass spectrometry analysis of water and urine from spermiating males

Methanol extracts of washings (1 L) taken from the anterior and posterior regions of spermiating males were dried down under a stream of oxygen-free nitrogen gas at 45°C, reconstituted in chloroform and subjected to fast atom bombardment mass spectrometry (FAB MS, 10KV) in both negative and positive ionization modes.

To determine whether 3kPZS was present in the urine, spermiating males were anesthetized with an MS-222 solution and a catheter was inserted into the urogenital pore [15]. Urine was collected into a 50 ml container that was emptied periodically and stored at -80°C until analysis. The urine was passed through a Sep-Pak and the eluate dried down and subjected to FAB MS as described above.

ELISA of 3kPZS

The procedure for ELISA of 3kPZS has been described [9]. The antibody shows 100% cross reaction with 7α, 12α, 24-trihydroxy-5α-cholan-3-one (3 keto-petromyzonol, 3kPZ) and 7α, 12α-trihydroxy-5α-cholan-3-one-24-oic acid (3 keto-allocholic acid, 3kACA). However, the former was not found in washing extracts from spermiating males [9] and the latter was present at a ratio of only 1:25 (3kACA:3kPZS; [16]). The anterior and posterior washings from spermiating males were diluted 1:25 with assay buffer before ELISA.

ELISA was also carried out on water collected directly from the gills of spermiating males. To do this a male was anesthetized with metomidate hydrochloride (Syndel, Vancouver, British Columbia, Canada), immobilized with gallamine triethiodide (Sigma Chemical Co., St. Louis, Missouri, USA), and placed in a flow-through trough. Water flowed through the mouth and exited the gills at an average rate of 431 ml/min. Water was pipetted directly off the gills and placed into a beaker every three minutes for 0.5 h, making a total of 10 individual samples for each spermiating male. Parts of each sample were stored at -80°C and 100 ml of each sample were extracted using a Sep-Pak and the eluate also stored at -80°C until ELISA analysis.

Hourly release rates of 3kPZS were calculated for the anterior region washings and water collected directly from the gills. For the anterior washings, the 3kPZS level of water was multiplied by the total volume used in the anterior portion of the aquarium (7 L). For water collected directly from the gills, the average 3kPZS level of water for each spermiating male was multiplied by the flow of water across the gills (431 ml/min) and then by 60 min.

Immunocytochemistry for 3kPZS in the gills and liver of male sea lampreys.

Five spermiating and seven pre-spermiating males were killed with an overdose of MS-222. The gills and liver were removed, immersed in either Zamboni's fixative (2% paraformaldehyde and 1.2% saturated picric acid in 0.1 M phosphate buffer (PB) or 4% paraformaldehyde (in PB)) and stored at 4°C. As the male pheromone is a steroidal lipid, whole gill filaments were mounted to minimize the loss of cellular lipids [17]. Each gill pouch was placed under a dissection microscope and single gill filaments were removed. Throughout the immunostaining procedure, the gill filaments were agitated mildly at 4°C. The single gill filaments were rinsed 3 times over 1 h with either PB or with PB plus 0.1% Triton X-100 (PB-TX) for 30 min. The omission of Triton-X from the immunostaining protocol served to assist in keeping the cellular membranes and lipid-containing subcellular structures intact. Non-specific binding was blocked with 5% normal goat serum in PB for 20 min, drained off and exchanged for primary antibody (from four rabbits: codes 184, 185, 286, 285 [9]) diluted with 0.1 M PB or 0.1 M PB-TX (1:1000 to 1:5000) and incubated for 24 h. The tissue was then rinsed three times over 1 h with 0.1M PB, followed by a 3 h incubation in Alexa 488 goat anti-rabbit IgC (1:100; Molecular Probes, Eugene, OR), rinsed and mounted in glycerol or viewed directly with a 40X water immersion lens. Following immuno-staining, some filaments were sectioned with a vibratome (Leica) to allow for viewing of the interplatelet region. The immunostained filament was embedded in 5% agarose (American Bioanalytical, Natick, MA) and sectioned at a thickness of 150 um. All preparations were viewed using a BioRad 1024 Confocal Microscope with the following settings: laser power, 10%; iris, 1.5 mm; gain, 1000X; black level, 0. Fixed liver samples were embedded in 5% agarose, sectioned using a vibratome at a thickness of 200 µm, and stained for 3kPZS by the procedure used for the gill whole mounts (above).

A pre-adsorption control experiment was conducted using a 10-fold molar excess, relative to IgC concentration, of antigen (3kPZS) to antibody dilution. The 3kPZS/antibody mixture was incubated for 24 h at 4°C, with mild agitation, then centrifuged at 100,000 g for 30 min (Sorvall RC M120 GX). The supernatant was used in place of the primary antibody in the previously described immunocytochemical protocol. The pre-adsorption control did not contain any immunostaining. Negative controls were also conducted using pre-immune sera for antisera from rabbits 184 and 185 [16]. Tissues processed with these pre-immune sera were unstained. In addition, a control with the primary antibody omitted was included in each experiment.

Electron microscopic analysis of gill tissues

The gill tissues from spermiating and pre-spermiating male sea lampreys were fixed in buffered 2.5% glutaraldehyde, and then in 1% OsO4. After dehydration, fixed tissues were embedded in Spurtol resin. Ultrathin sections (90 nm) were cut, and then stained with uranyl acetate and lead citrate. The sections were examined in Philips CM10 (Philips) electron microscope.

RESULTS

Dye tests and ELISA measurements of water spiked with 3kPZS revealed that there was no detectable exchange of water between the two chambers of the bisected aquarium (data not shown). The total time spent and the time spent searching in each side of the two-choice maze before odorant introduction showed that ovulating females were not biased to either side ($P>0.10$; data not shown). When water from the anterior region of spermiating males was introduced into the maze, 11 of 14 ovulating females spent more of their total time ($P<0.01$) and six of seven spent more time searching ($P<0.01$) in the side to which the water was introduced. There were no significant increases or decreases in total time spent (seven of 14; $P>0.10$; n=14) or time spent searching (two

of seven; P>0.10; n=7) in the experimental side of the maze when the posterior region water was introduced.

In EOG experiments, the mean response to 10^{-5} M L-arginine was 0.5 mV (SE=0.09 mV; n=6).

The anterior region washings (n=6) of spermiating males were far more potent than those of the posterior region (n=5) at equivalent dilutions (Figure 2), but were equipotent to those from whole spermiating males. The detection threshold for the posterior region washings was approximately 1:100 (v/v) whereas that for the anterior region and whole body chemical stimuli was approximately 1:10,000 (v/v).

Negative FAB MS analyses showed that the most abundant ion in extracts of water from the anterior region of spermiating males was at m/z 471 (n=6; Figure 3A), the same as synthetic 3kPZS [1]. At positive mode, the base peak was at m/z 473. FAB MS detected only trace amounts of this molecule in the extracts of water from the posterior regions (n=6, Figure 3B) and in extracts of spermiating male urine (n=4, data not shown).

The mean concentration of immunoreactive 3kPZS in water collected from the anterior regions of spermiating males was 54 ng/ml (n=13) whereas that from the posterior regions was 0.8 ng/ml (n=13). On average, the total release rate of the anterior region was 379 μg/h (Figure 4). Also, immunoreactive 3kPZS was found in water collected directly off the gills of five spermiating males, however these concentrations showed considerable variation (Figure 4). On average, the release rate of 3kPZS estimated from water collected directly off the gills was 308 μg/h. There was no significant difference between release rates estimated from the anterior region water and gill water (P>0.10, Student t test).

The gill filaments were comprised of gill platelets (lamellae, Figure 5A) covered by low cuboidal platelet cells, with cuboidal to columnar shaped cells in interplatelet regions. In pre-spermiating males, the pheromone immunoreactivity was weak and diffuse on the platelet surface and granular in inter-platelet epithelial cells (Antibody 286; Figure 5A, B, C). In gill tissue from spermiating males, subpopulations of platelet cells were strongly immunoreactive to the pheromone antibodies (Antibody 185; Figure 5D, E). This staining was absent from the nucleus (Figure 5E, 6A). Both platelet and inter-platelet epithelial cells contained supranuclear regions that were intensely immunoreactive to the pheromone antibodies (Antibody 286; Figure 6A-C). These immunopositive cells were cuboidal along the platelet base regions (Figure 6A) and columnar in the inter-platelet regions (Figure 6C). In all samples, pheromone-immunoreactivity was stronger and clearer when Triton-X was omitted from immunostaining procedure than in samples treated with Triton-X (Antibody 185; Figure 6D, E). The fact that the immunoreactivity decreased and blurred following treatment with the lipid solubilizing detergent is consistent with the lipid nature of the pheromone.

Transmission electron microscopy of the gill epithelial cells revealed low microvillar cuboidal platelet cells (Figure 7A) with small electron lucent vesicles, larger electron opaque granules (0.3 mm), round mitochondria, rough and smooth endoplasmic reticulum, and Golgi apparatus. In the basal platelet region and the inter-platelet region the cuboidal cells (Figure 7B) had a dome-shaped apical surface, smooth endoplasmic reticulum, rough endoplasmic reticulum, electron dense vesicles, dense core vesicles and electron lucent vesicles. The supranuclear region of low columnar inter-platelet cells (Figure 7C) contained electron opaque granules and smooth endoplasmic reticulum. The perinuclear cytoplasm of inter-platelet cells (Figure 7D) contained granules of varying sizes and electron density and numerous vesicular and tubular cisternae of smooth endoplasmic reticulum. These ultrastructural characteristics show that these cells are active in lipid metabolism and store secretory products.

In spermiating male liver, the immunostaining (antibody 285) was strong and diffuse in hepatocyte cytoplasm, and strong in widespread cytoplasmic granules (Figure 8A). The liver of pre-spermiating males displayed pheromone-immunoreacitivity (antibody 285) that was weak and diffuse, with scattered intensely stained cytoplasmic granules (Figure 8B). Therefore, the

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pheromone is present in higher levels in the spermiating male liver than in the liver of the pre-spermiating male.

DISCUSSION

The data from the present study clearly support our hypothesis that the male sex pheromone 3kPZS is released through gills, most likely via the glandular cells that appear in the gills of spermiating males. The first sub-hypothesis, that 3kPZS is released almost exclusively from the anterior portion of spermiating males, is unequivocally supported by data from our behavioral assays, EOG recording, ELISA and mass spectrometry analyses. In the two-choice maze, washings collected from the anterior regions of spermiating males induced in ovulating females the preference and search behaviors that are characteristic of those induced by 3kPZS [1] whereas the washings from the posterior region did not. This is corroborated by electrophysiological results that the anterior region water is equipotent to the whole body water in stimulating the female olfactory organ and is about 100 times more potent than the posterior region water. Chemically, both ELISA and mass spectrometry indicate that 3kPZS is present in large amounts in the anterior region water samples but in negligible amounts in the posterior region water and urine samples. These results confirm and expand on the previous ELISA results that the anterior region washings contain large amounts of 3kPZS [9].

The EOG and ELISA data also indicate that the 3kPZS released from the gills accounts for all the 3kPZS released by the whole animal, supporting the second sub-hypothesis. The anterior region washings induce EOG responses at virtually the same magnitude as the whole washings did over a range of dilutions that spans four orders of magnitude. The average release rate from the anterior region, 379 µg/h, is close to the previously reported whole body release rate of c. 500 µg/h [9]. Most importantly, the washings collected directly from the gills contain large amounts of 3kPZS (c. 308 $\mu g \cdot h^{-1}$), which can account for the majority of the 3kPZS release estimated from the washings collected from the anterior region. These results support the gill-release hypothesis set forth in our previous study [1].

Finally, our third sub-hypothesis is clearly supported by immunocytochemical experiments that show the presence of 3kPZS in cells of both the liver and gills. It appears likely that the pheromone, a sulfate and ketone containing bile acid derivative, moves into the water from the platelet and inter-platelet cells of spermiating males. Diffuse immunolabeling on the surface of platelet cells of both stages may be due to the secreted pheromone adhering to the cell surface of these cells. The localization of pheromone-immunoreactive granules in the interplatelet cells of pre-spermiating males suggests that pheromone containing cells in the platelet epithelium first appear in the inter-platelet region during the pre-spermiating phase and then only in the platelet region during spermiation. It is likely that 3kPZS is at least a part of the "…substance of sexual significance" [7].

Our results clearly implicate the involvement, but do not illustrate the explicit role of hepatocytes in biosynthesis of 3kPZS. The granules with strong pheromone-immunoreactivity demonstrate that hepatocytes contain either 3kPZS, 3kACA, or 3kPZ, or all of them, because the primary antibody used for immunocytochemical staining cross-reacts with all three of these compounds. This conforms with the discovery that PZS, a larval bile acid, is produced in the liver [5] and that liver is the exclusive organ for bile acid synthesis [18]. It is possible that 3kPZS is synthesized in the liver, released into circulation, and taken up by the platelet and interplatelet cells. Alternatively, the gill cells may take up a precursor synthesized in the liver and modify it into 3kPZS. Smooth endoplasmic reticulum, a sub cellular site for steroid synthesis is widespread in the platelet and inter-platelet cells. Granules of varying electron density that are prominent in transmission electron micrographs may be the site of the granular localization of pheromone immunoreacitivity of the inter-platelet cells. Large lipid structures with electron lucent centers, previously observed in Type I male glandular cells of river lamprey fixed in osmium tetroxide [8], were absent from sea lamprey.

This difference may be due to the differing fixation protocols, with stabilizing aldehyde pre-fixation being included in the present study, but absent from the earlier study.

In bony fishes, all pheromones identified to date are steroids or prostaglandins - or sulfated or glucuronidated forms of these compounds [19-22]. In the case of free steroids, there is evidence that they, like the lamprey pheromone, are mainly excreted into the water via the gills [23, 24]. However, the mechanism whereby they do so appears to be passive diffusion [23, 25]. There is no evidence for specialized cells such as those that we have demonstrated in lampreys. The release rates of free steroids by bony fishes are also considerably lower than those of 3kPZS by spermiating lampreys [1, 26]. In contrast to free steroids, sulfated steroids in teleosts and elasmobranches do not pass through the gills [23, 25]. Their main site of release appears to be the urinary bladder [24, 27]. Although the urinary bladder has been thought as a source of sex pheromones in the sea lamprey [28], none has yet been firmly identified. The main reason for making these comparisons between vertebrate classes is to underline our belief the mechanism whereby sea lamprey release 3kPZS is an active one, with the specialized cells that appear in the gill epithelium of spermiating males as 'pumps'.

To rely on gills to broadcast 3kPZS possibly represents the evolution of a system mainly to extend the active space of the pheromone signal. Such a system would certainly be advantageous for spermiating male lampreys, that construct nests in sites where water flows at 0.5 to 1.5 m/s [29, 30] and probably have to rely heavily on this pheromone to attract ovulating females from down stream [1, 28].

Bile acids, and their sulfate esters such as 3kPZS, are produced in large quantities by hepatocytes of vertebrates [18]. The transportation of 3kPZS from liver to gills is potentially very efficient because, in the lamprey, hepatic veins carry blood directly to the heart and all blood from the heart passes immediately through the gills [31]. It has been estimated that in a 100 g river lamprey the relative area of the gills is 600 mm^2/g [32]. If the relative surface area in the sea lamprey is similar, its gills would provide an enormous surface area with specialized glandular cells, through which the respiratory activity generates continuous flow to facilitate exchange of the pheromone molecule with the environment.

In conclusion, our experiments demonstrate that the spermiating male sea lamprey sex pheromone, 3kPZS is released through the gills. Although liver and the glandular cells are clearly implicated in the production and release of 3kPZS, their explicit roles need to be examined at the molecular level to elucidate the mechanistic processes whereby this pheromone molecule is produced and released.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Schematic of the bisected aquarium used to collect washings from anterior and posterior regions of spermiating male sea lampreys. (A) indicates the anterior and (B) the posterior chambers of the aquarium. A perforated acrylic tube (C), adjustable mesh tube (D) and latex gasket were used to hold lamprey in place and prevent contamination between the two chambers during washings.

Figure 2. Electro-olfactogram responses of female sea lamprey to washings collected from the anterior, posterior and whole body of spermiating males. Responses are expressed as a percentage of the response to a 10^{-5} M L-arginine standard. Vertical bars represent one standard error.

Figure 3. Representative fast atom bombardment mass spectrometry analyses of extracts from the

anterior (A) and posterior (B) regions of spermiating males.

Figure 4. Mean release rate of 3kPZS estimated from washings collected from anterior and posterior regions as well as directly from the gills of spermiating male sea lampreys. Vertical bars, one standard deviation.

Figure 5. Confocal microscopy of pheromone immunocytchemistry against gill platelets from pre-spermiating (A,B,C) and spermiating (D,E) male lampreys. (A) and (D) are low power images, scale bar shown in (A) is 100 μm. (B), (C) and (E) are high power images; scale bar shown in (B) is 15 μm. All are images of scans in a single Z plane. (B) and (C) show the inter-platelet region sectioned following immunostaining from a single field of view. (B) was collected at 488 nm illumination and the immunostaining is on the platelet surface along the basal region of the platelet. (C) was collected through transmitted light detection of bright field microscopy.

Figure 6. Subcellular localization of pheromone immunostaining. (A) through (C) show the inter-platelet region of a spermiating male gill filament sectioned following immunostaining. (A) and (B) show a single field of view. (A) is viewed at 488 nm, and (B) through bright field microscopy. (C) Immunoreactivity in the supranuclear region of epithelial cells in the inter-platelet epithelium. (D) and (E) show pheromone immunoreactivty in the platelet and inter-platelet regions following lipid solubilization with 0.1% Triton-X100.

Figure 7. Transmission electron microscopy of gill platelets from a spermiating male. (A) A platelet cell. (B) A cuboidal cell. (C) The supranuclear cytoplasm of an inter-platelet cell. (D) The perinuclear cytoplasm of an inter-platelet cell.

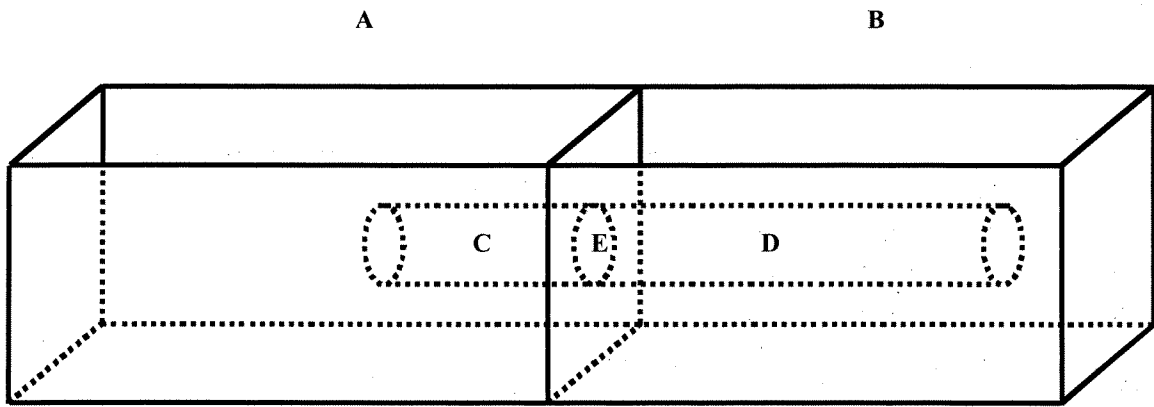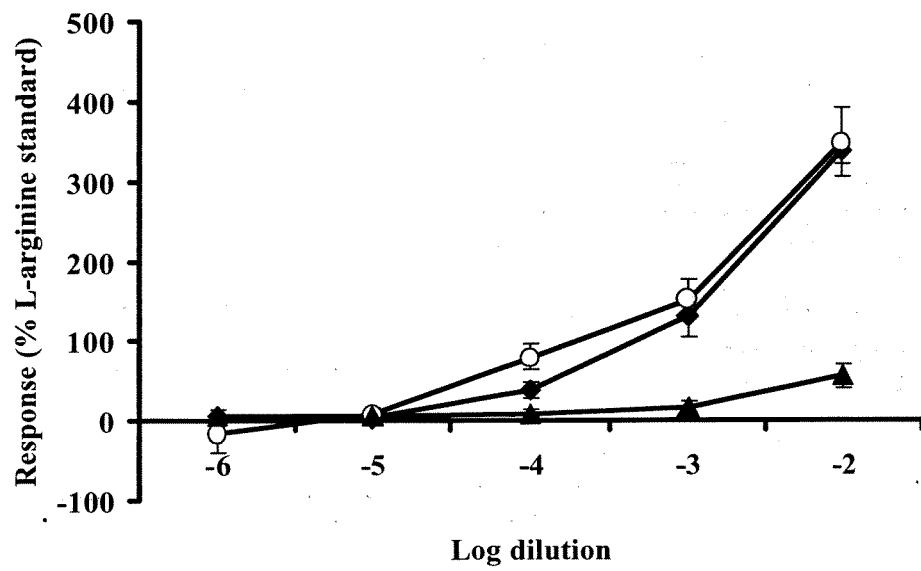Figure 8. Pheromone immunostaining in the liver of spermiating (A) and pre-spermiating (B) male sea lampreys. Scale bar, shown in (A) is 50 μm.
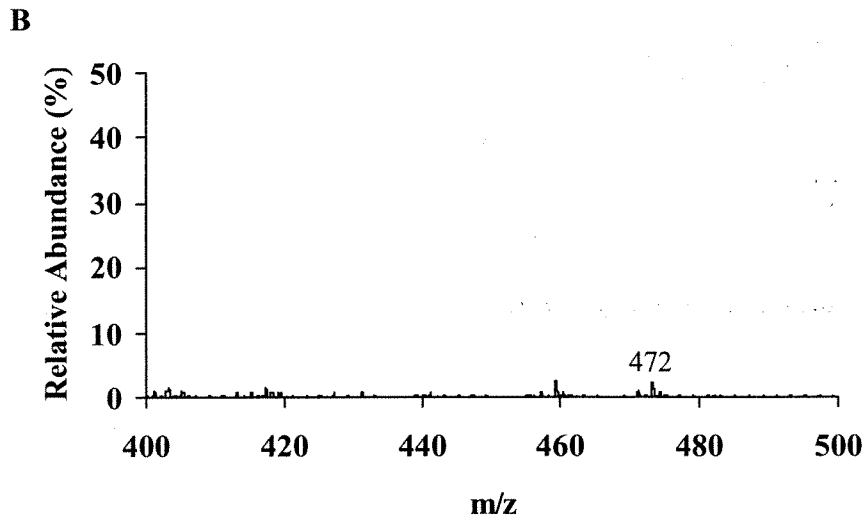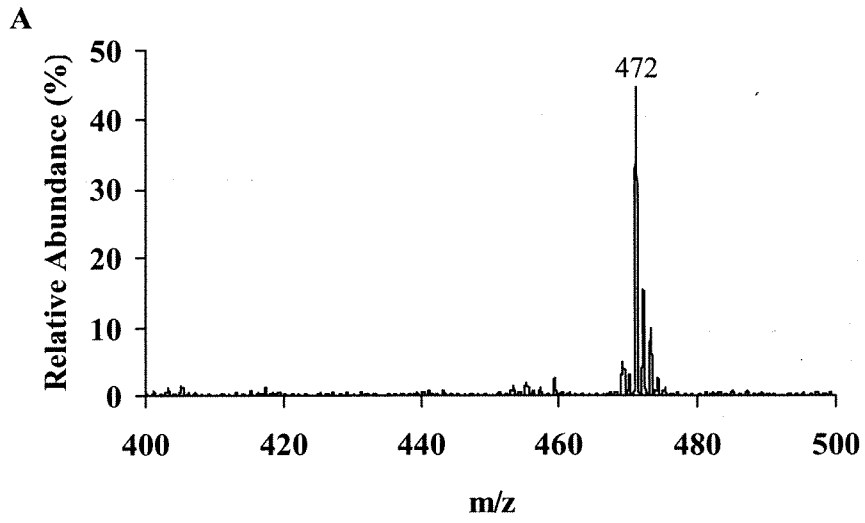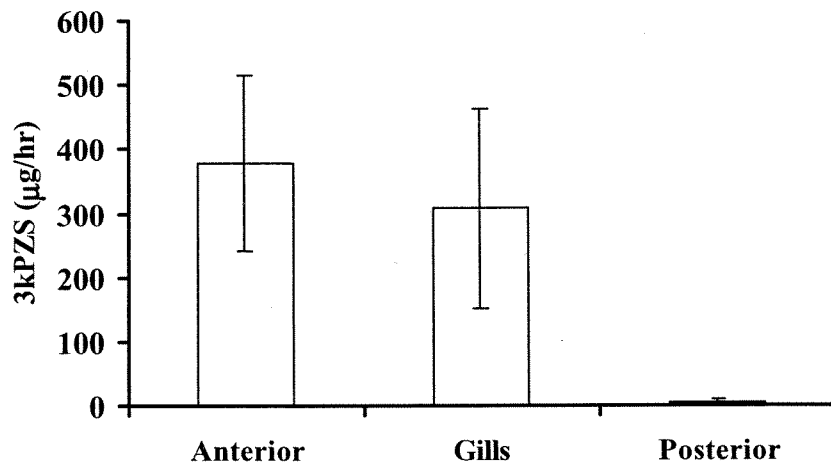
Figure 1.

Figure 2.

Figure 3.
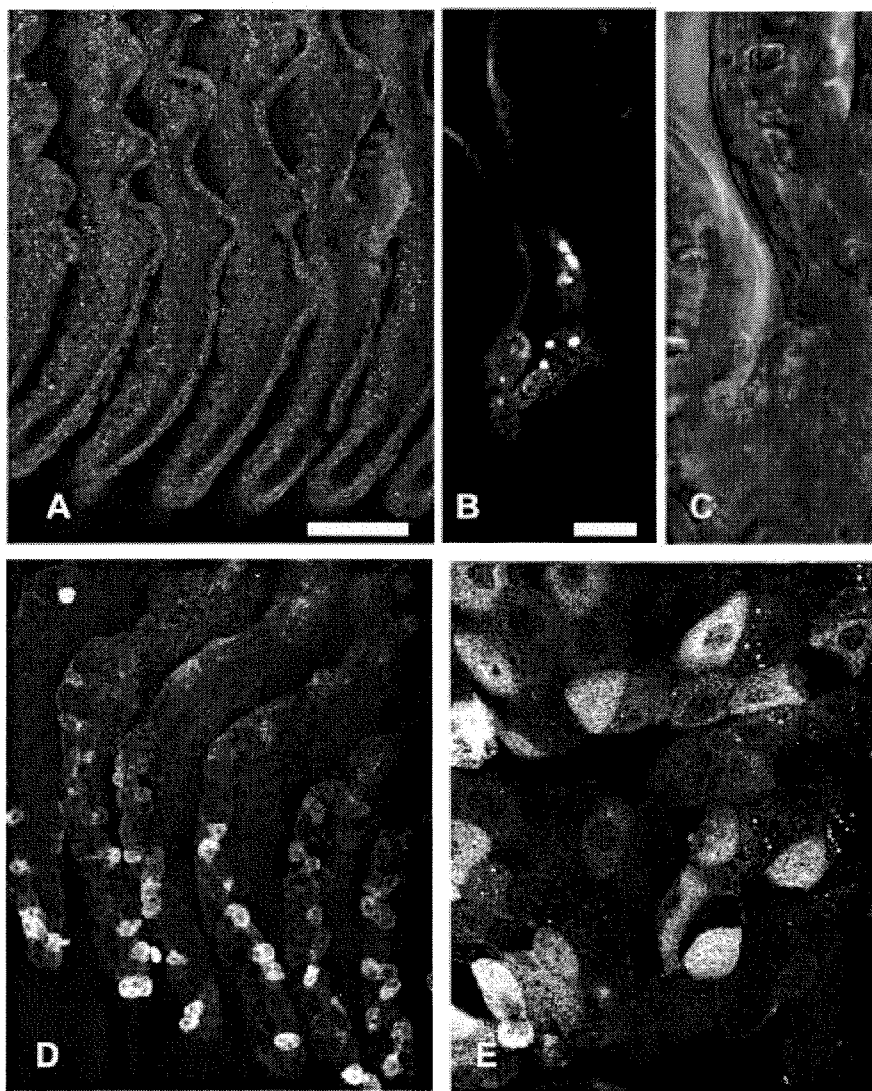
Figure 4

Figure 5.
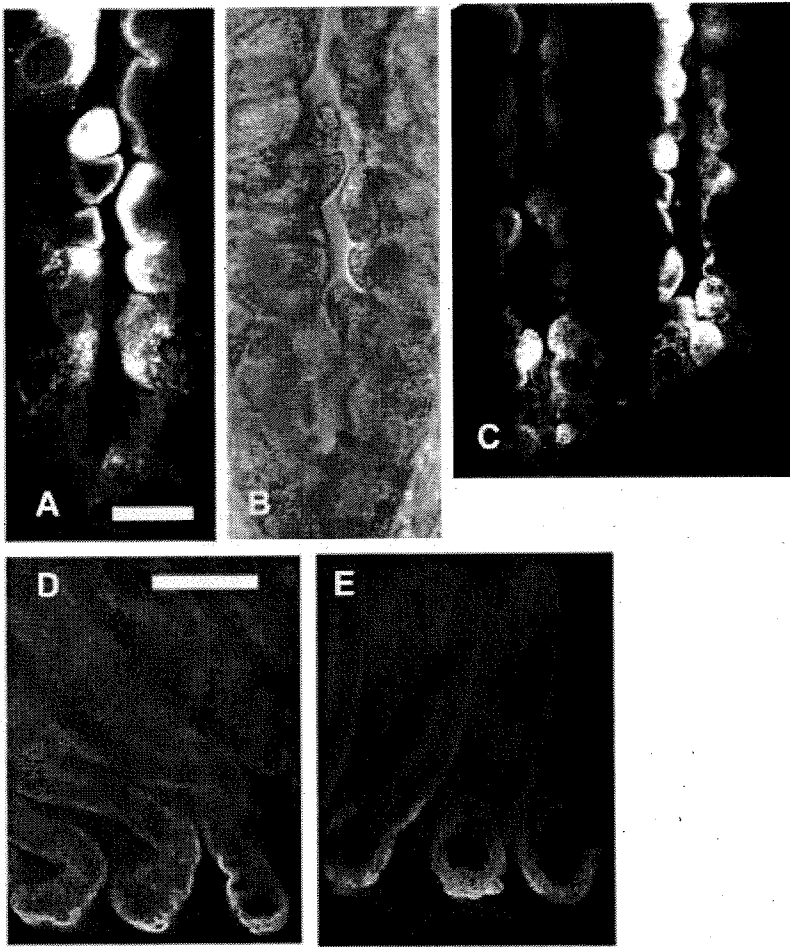
Figure 6.
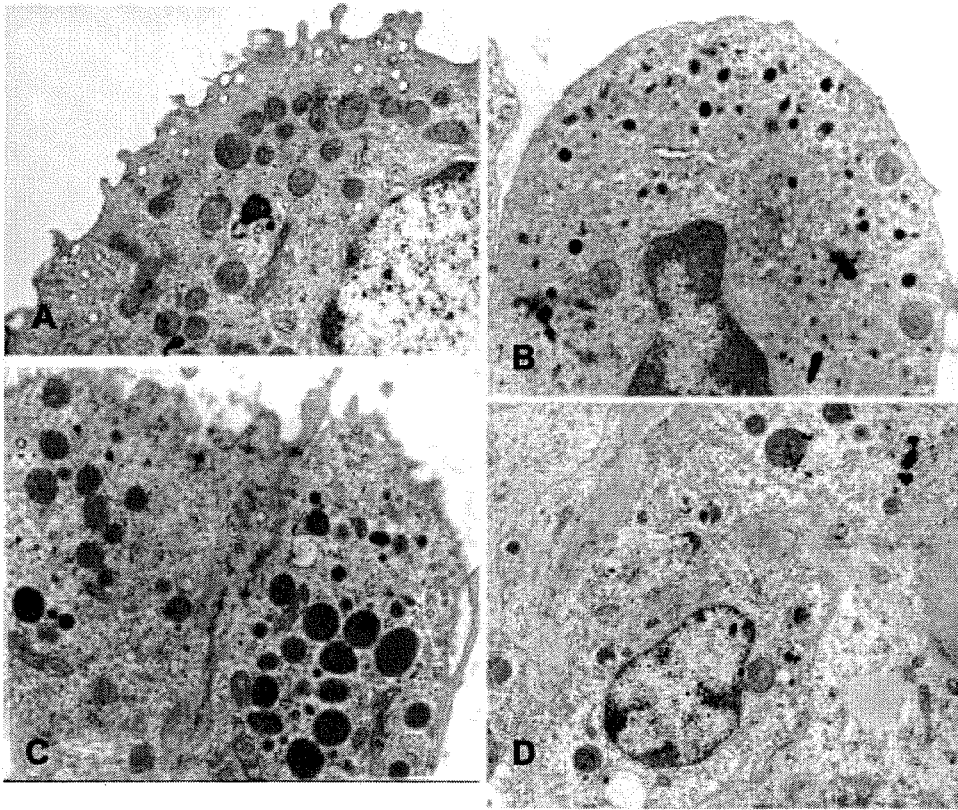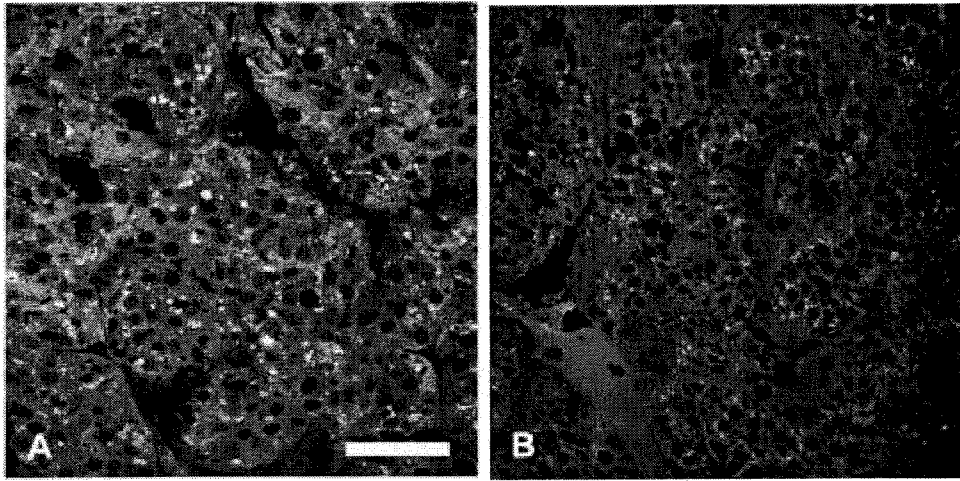
Figure 7.

Figure 8.

[5]Pheromones of the male sea lamprey, *Petromyzon marinus* L: structural studies on a new compound, 3-keto allocholic acid, and 3-keto petromyzonol sulfate.

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Abstract

This study reports the results of chemical and chromatographic studies which establish the presence of 3-keto allocholic acid (3kACA) in water extracts from spermiating male sea lamprey, *Petromyzon marinus*. This is the second compound to be isolated and identified from these extracts. The first was 3-keto petromyzonol sulfate (3kPZS), which was shown to act as strong pheromonal attractant for ovulated females. Some new characterization data on 3kPZS (utilizing an only recently available synthetic preparation of the compound) is also included. The possibility that a mixture of 3kACA and 3kPZS might be a more potent pheromonal attractant than either compound alone is discussed.

Keywords: lamprey; bile; pheromone; sterol; 3keto-allocholic acid; 3keto-petromyzonol sulfate

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[5] This manuscript has been accepted for publication by *Steroids*.

1. Introduction

The sea lamprey is a jawless fish with a complex life history that consists of larval, parasitic and adult stages. Larval sea lampreys inhabit streams for up to 17 years [1]. After a radical metamorphosis, they enter a parasitic stage and migrate to lakes or ocean to predate on fish [2]. When approaching sexual maturation, adults migrate back to streams to reproduce [3].

It has been demonstrated that sea lampreys use bile acids or alcohols, or both, as pheromones at two stages of their life history. When they are larvae, they synthesize [4] and release into the water [5, 6] $3\alpha,7\alpha,12\alpha,24$-tetrahydroxy-$5\alpha$-cholan-24-sulfate (petromyzonol sulfate; PZS) and $3\alpha,7\alpha,12\alpha$-trihydroxy-5α-cholan-24-oic acid (allocholic acid; ACA), neither of which have been detected in adults [5, 6]. It has been proposed that PZS and ACA are pheromones which serve to attract adults back to suitable spawning streams based on the facts that the olfactory organs of adults are exquisitely sensitive to PZS and ACA [5, 7], that larvae release the two compounds at a rate that is sufficient to produce detectable riverine pheromone plumes [6] and that a mixture of the two compounds elicits positive rheotaxis in migratory adults which are placed in a two-choice chamber [8].

Once the adults are on the spawning grounds, sexually mature spermiating males[6] have been shown to release $7\alpha,12\alpha,24$-trihydroxy-5α-cholan-3-one-24-sulfate (3-keto petromyzonol sulfate; 3kPZS) into the water [9, 10]. This conjugated bile alcohol is strongly attractive to ovulated females[7], is released from the gill region of the males and only differs from the larval pheromone, PZS, by the possession of a 3-keto, as opposed to a 3-hydroxyl group. The evidence suggests that males use this pheromone to attract gravid females to their nest sites.

During the course of purification and identification of 3kPZS [9], it was noted that spermiating, but not non-spermiating males, released at least two other compounds into the water. It was also noted that, after HPLC of water extracts from spermiating males, there were two fractions that had a stimulatory effect on the olfactory epithelium of adult lampreys. The fraction with the higher potency corresponded to 3kPZS, and that with the lower potency to one of the unidentified compounds. In the course of development of an ELISA for 3kPZS [10] it was discovered that both fractions were able to displace enzyme-labeled 3kPZS from the antibody. Based on this evidence – and also the similar immunological and chromatographic properties of synthetic compound – it was postulated that the second fraction was $7\alpha,12\alpha$-dihydroxy-5α-cholan-3-one-24-oic acid (3-keto allocholic acid; 3kACA). Since this identification could only be considered as 'tentative', we set out to chemically characterize (and hence 'definitively' identify)

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[6] 'Spermiating' males are those from which milt can be expressed by gentle manual pressure

[7] 'Ovulated' females are those from which eggs can be expressed by gentle manual pressure

the compound. The results are presented below. Also presented are some previously unpublished data which show that the chromatographic behavior of natural 3kPZS, before and after treatment with sulfatase and 3α-Hydroxysteroid Dehydrogenase (3α-HSD) is identical to that of synthetic 3kPZS.

2. Materials and Methods

2.1 Chemicals

Synthetic 7α,12α,24-trihydroxy-5α-cholan-3-one (3-keto petromyzonol; 3kPZ), PZS, 5α-cholane-3α,7α,12α,24-tetrol (petromyzonol; PZ), PZS, 3kPZS, ACA and 3kACA were purchased from Toronto Research Chemicals (North York, ON, Canada) and dissolved at a concentration of 1 mg/ml in ethanol. The chemical structures of these synthetic bile acids are shown in Fig.1. Snail juice sulfatase, 3α-HSD, β-nicotinamide adenine dinucleotide (NAD), and 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) were purchased from Sigma (St. Louis, USA). HPLC grade methanol, acetonitrile (ACN) and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). Deuterated dimethyl sulfoxide (DMSO) was obtained from Cambridge Isotope Laboratories (Andover, MA).

2.2 Animals

Adult sea lamprey were trapped or collected by hand from tributaries to lakes Huron and Michigan by the staff of the U. S. Fish and Wildlife Service, Marquette Biological Station (MBS), Marquette, Michigan, USA. The animals were transported to the main laboratory at the U. S. Geological Survey, Lake Huron Biological Station, Millersburg, Michigan USA. These adults were held in flow-through tanks (1000 l) with Lake Huron water (7°C to 20°C).

2.3 Extraction of pheromones from water

A spermiating male lamprey was held for 4 h in a tank with 10 l lake water. The lamprey was then removed and the water passed through Whatman Grade 3 filter paper and then through Sep-Pak C18 cartridges (Waters, Milford, MA, USA; 1 l of water per cartridge). After being washed with 5 ml deionized water, the cartridges were purged with 5 ml methanol. The methanol eluates were pooled and dried down using a rotary evaporator. 300 l of lamprey holding water from 30 sea lampreys was processed to obtain c. 2 mg of natural compound.

2.4 HPLC fractionation and analysis of compounds

Extract equivalent to 20 l water was reconstituted in 720 μl of 0.01% TFA (v/v) and 280 μl of 70% ACN/0.01% TFA and loaded onto a reverse phase preparative HPLC column (Nova-Pak, 39 x 300 mm, Waters). The column was developed with: 20% ACN in 0.01% TFA for 10 minutes; a linear gradient from 20% to 70% ACN in 0.01% TFA for the next 50 min; 70% ACN in 0.01% TFA for the final 20 min. The flow rate was 4 ml/min and ultra-violet (U-V) absorption was monitored at 200 nm. Fractions were collected every 1 min. In experiments that

were designed to compare the behavior of natural and synthetic compounds, an analytical reverse phase column (3.9 x 300 mm, Waters) was used at a flow rate of 0.5 ml/min.

2.5 TLC analysis of compounds

Thin layer chromatography (TLC) was performed on silica plates (LK6DF; Whatman). The solvent was a mixture of chloroform/ethanol/acetic acid (200:50:1 v/v/v). Samples and standards were run for 45 min. The plates were then dried, sprayed with 5% phosphomolybdic acid in ethanol (w/v) [11] and heated at 100 °C for *c.* 5 min.

2.6 Hydrolysis of the sulfate group of 3kPZS

Natural and synthetic 3kPZS (100 μg each) were dissolved in 500 μl 0.5 M sodium acetate buffer, pH 5.0 and incubated overnight at 37 °C with 1000 units of snail juice sulfatase [12]. The reaction mixture was loaded onto a C18 Sep-Pak and eluted with 5 ml methanol. The methanol eluates were dried down and subjected to TLC and HPLC analyses.

2.7 Conversions with 3a-Hydroxysteroid Dehydrogenase

Enzymatic conversion of 3kPZS and ACA was performed by the method of Macdonald *et al.* [13] and Schwartz *et al.* [14] with a slight modification. Natural 3kPZS, synthetic 3kPZS and synthetic 3kPZ (100 μg each) were incubated with 1 unit of 3α-HSD and 2 mg NAD in 0.1 M Tris-HCl buffer, pH 7.2 for 2 hours. A control experiment was carried out by incubation of the compounds in 0.1 M Tris-HCL buffer, pH 7.2 without enzyme or NAD. The reactants were concentrated with a Sep-Pak cartridge, eluted with 5 ml methanol and dried down. Enzymatic conversion of ACA to 3kACA was carried out by the method of Macdonald *et al.* [13]. ACA (2 mg) was incubated for 2 h in 0.05 M CAPS buffer, pH 10.8 containing 20 mg NADH and 5 units 3α-HSD at 37°C. The reactants were concentrated with a Sep-Pak cartridge, eluted with 5 ml methanol, dried down, redissolved in 100 μl methanol and subjected to TLC and HPLC.

2.8 Mass Spectrometry and Nuclear Magnetic Resonance

Mass spectra were obtained using a JEOL HX-110 double-focusing Fast Atom Bombardment (FAB) mass spectrometer (JEOL, Peabody, MA, USA) which could be operated in either the positive or negative ion mode. Ions were produced by bombardment with a beam of Xe atoms (6 keV). The accelerating voltage was 10 kV and the resolution was set at 3000. Samples were prepared for mass spectrometry by drying down the HPLC fractions and re-dissolving them in methanol. High resolution mass spectrometry was performed by peak matching with a resolution of 10,000. FABMS was done at the NIH MS facility at MSU.

Three mg each of ACA and 3kACA and ca. 2 mg of natural 3kACA were dissolved in 700 μl of deuterated DMSO and subjected to Nuclear Magnetic Resonance (VXR-S 500MHz NMR Spectrometer; Varian Inc., Palo Alto, CA). ^{1}H, ^{13}C, COSY, HMBC and HMQC NMR experiments were performed on all samples at 25 °C with a deuterium lock.

3. Results

3.1 HPLC purification of putative 3kACA

Several batches of the second HPLC peak (shown as eluting at 58 min in the paper by Li *et al.* [9]) were pooled and rerun on the same preparative HPLC column (Fig. 2 A). Fractions eluting around 57 to 58 min, where there was a noticeable UV absorption peak at c. 200 nm, were dried down. The total amount of the compound obtained was c. 2 mg.

3.2 Mass spectrometry of natural and synthetic3kACA

Mass spectrometry showed that both natural and synthetic 3kACA had the same mass of 406 Dalton (Fig. 3 B & C). High resolution mass spectrometry analysis confirmed the molecular mass of the natural compound to the −2.5 ppm level (data not shown).

3.3 Chromatographic behavior of natural and synthetic 3kACA

ACA was treated with 3α-HSD and NAD. On TLC (Fig. 3), the main product was isopolar with natural 3kACA. Some of the ACA was not converted.

On HPLC (Fig. 4), the main peak of absorption of natural 3kACA ran in the same position as the main peak of absorption of synthetic 3kACA. The ability of 3kACA to adsorb UV at 200 nm is conferred by the double bond between the third carbon and the oxygen atom (i.e. the 3-keto group). Thus ACA, which has a 3α-hydroxyl group, does not show up on HPLC.

3.4 NMR analysis of synthetic and natural 3kACA

One- and two- dimensional ^{1}H-NMR analysis of natural and synthetic 3kACA further confirmed the chemical identity of the natural compound. The chemical shifts which are characteristic of other bile acids [16,17] were clearly visible in the one-dimensional ^{1}H-NMR spectra of natural 3kACA, synthetic 3kACA and synthetic ACA (Fig. 5). These included: the C-18 (0.592 ppm), C-19 (0.904 ppm), and C-21 (0.894 ppm) methyl groups; the 7α-hydroxyl (3.59 ppm) and 12α-hydroxyl (3.76 ppm) groups in 3kACA and the extra 3α-hydroxyl (3.82 ppm) group in ACA. The C-19 methyl proton peak of ACA at 0.68 ppm has shifted to overlap with the C-21 methyl group at 0.90 ppm in 3kACA. This was demonstrated by peak integration, which revealed double the number of protons (6) in the peak at 0.90 ppm in comparison to those (3) in the peak at 0.59 ppm in both natural and synthetic 3kACA. The 'hidden' C-19 methyl group in one-dimensional ^{1}H-NMR was clearly visible in the 2-D HMQC NMR spectrum (data not shown). In addition, 2D-HMQC analyses confirmed that both natural and synthetic 3kACA displayed exactly the same chemical shift for major functional groups (data not shown). Combined application of several NMR techniques were made to assign all the carbon and proton shifts for synthetic 3kACA (Table 1). The majority of carbon resonances were between 10 to 80 ppm except for C-24 (carboxyl) and C-3 (carbonyl), which had shifts of 174 ppm and 210 ppm, respectively.

3.5 Elution position of natural and synthetic 3kPZS on HPLC

Both natural and synthetic 3kPZS (100 µg each) were run separately or combined (50 µg of each) on the analytical HPLC column. In all cases, the main peak of UV adsorption (200 nm) appeared in the same fractions (Fig. 6 A). Both compounds were also isopolar on TLC (data not shown).

3.6 Hydrolysis of the sulfate group of 3kPZS

Sulfatase treatment of natural and synthetic 3kPZS (either separately or as a mixture) resulted in the appearance of a new UV-absorbing peak which had the same elution position as 3kPZ (Fig. 6 B). In all cases, hydrolysis was incomplete as there was a peak which remained in the position of the standard.

3.7 3α-HSD conversion of 3kPZS to PZS

Treatment of synthetic and natural 3kPZS with 3α-HSD and NAD at pH 7.2 converted all of the synthetic compound and most[8] of the natural compound into compounds which had the same polarity as PZS (Fig. 7). Unexpectedly, some hydrolysis of the sulfate group appeared to have occurred in the natural compound as there was also a faint spot in the elution position of 3kPZ and a prominent spot in the elution position of PZ. Synthetic PZ and PZS were, as expected, unaffected by treatment with 3α-HSD under the same conditions, while synthetic 3kPZ was mostly converted to a compound with the same polarity as PZ.

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[8] Phosphomolybdic acid-stained spots on TLC are not quanitifiable

4. Discussion

4.1 Identification of 3kACA

The original study in which 3kPZS was identified as the major pheromone of spermiating male lampreys [9] showed that at least two other compounds were released concurrently into the water and that one of them appeared to have electro-olfactographic (EOG) activity. Following the development of an ELISA for 3kPZS, this particular compound was found to cross-react in the assay and also to elute in the same position as synthetic 3kACA on HPLC [10]. The present study, through the use of mass spectrometry, NMR, HPLC, TLC and enzymatic modification, confirms this identification and the chemical structure of the compound in fraction 58 was designated as in Fig.1 D. The amount of compound which was used in this study was only about 2 mg and this was extracted from c. 300 l of water. The amount of 3kPZS which could be extracted from the same volume of water was in excess of 50 mg. This is in agreement with the ELISA results - which indicated that the ratio of 3kPZS to 3kACA was c. 20:1 [10]. The strongest evidence for the identify of 3kACA was provided by NMR analysis which indicated the presence of all the major functional groups which have been found in previous NMR studies on bile acids and steroids [9,15-17]. The resonance of one carbon was found to be 210 ppm, suggesting the existence of a keto group. The chemical shift of the C-19 methyl group of 3kACA (by +0.23) compared to that of ACA, indicating the keto group is formed at C-3 (that has previously been shown to cause a chemical shift of the C-19 methyl group by +0.242 ppm; [18]). In addition, 7α and 12α OH groups were also identified from NMR spectra in both natural and synthetic 3kACA.

The concurrence of 3kACA and 3kPZS in water extracts from spermiating males has a strong parallelism with the concurrence of ACA and PZS in water extracts from larval lampreys [6] although the ratio of PZS to ACA (3:1) in larvae was lower than that of 3kPZS to 3kACA in spermiating males [10]. In larvae, there is strong evidence that a mixture of PZS and ACA has a much stronger pheromonal effect than either compound alone [8]. Whether this is the case for a mixture of 3kACA and 3kPZS in spermiating males remains to be established. However, it does seem likely. In insects, it is well known that pheromonal signals most often consist of multicomponent mixtures and that at least two principal pheromone components seem to be necessary for eliciting attraction responses [19]. For example, *Heliothis virescens* produce (Z)-11-hexadecenal and (Z)-9-tetradecenal in the ratio of 16:1 and species *Helicoverpa zea* produce the same compounds in the ratio of 16:0.1 [20,21]. However, in some species, ratios between 10:1 − 100: 1 have been found to elicit responses [22].

4.2 Further identification studies on 3kPZS

In our previous study on 3kPZS [9], we only had a small supply of synthetic 3kPZS that we had been able to produce by enzymatic oxidation of the 3α-hydroxyl group of PZS. This restricted the number of comparisons that we were able to make between the natural and synthetic 3kPZS. We have now been able to complete these studies. We have confirmed that both synthetic and natural 3kPZS have the same chromatographic properties and behave in the same way

when treated with 3α-HSD and sulfatase. The only difference that we noted was that a proportion of the natural compound appeared to have broken down to 3kPZ (which was then converted to PZ by the 3α-HSD). The reason for this has not been fully established. However, the natural compound that was used in this experiment had been stored at 1 mg ml^{-1} in ethanol at -20 °C for over one year, whereas the synthetic compound had been made up freshly. The possibility is that the sulfate group is unstable in long-term storage.

4.3 Relationship between adult and larval bile acids and alcohols

It seems likely that the pathway of biosynthesis that is responsible for producing PZS and ACA in the larvae is the same as that responsible for producing 3kPZS and 3kACA in spermiating males. As mentioned previously, there is only one difference between the two sets of compounds; and this difference only needs the intervention of one enzyme - 3α-HSD. This is a widespread enzyme in steroid-producing tissues [23]. Because bile acids and alcohols are derived from cholesterol - and cholesterol already has a 3α-hydroxyl group - it is more likely that PZS and ACA are precursors of 3kPZS and 3kACA than vice versa. Although the oxidation of 3α-hydroxyl bile acids to 3-keto bile acids does not appear to be common in vertebrates, a low rate of production of 3-keto bile acids has been demonstrated in the liver of the guinea pig and hamster [24].

Because adult lampreys often enter streams with abundant larvae to reproduce, larval and spermiating male bile acids and alcohols will both be present in stream water during the spawning season. In order to recognize and distinguish between the two sets of compounds, ovulated female lampreys must have evolved at least two sets of olfactory receptors. The characteristics of these receptors are currently under investigation. However, Li et al. [7] have already shown that the olfactory epithelia of sea lamprey have separate and independent olfactory receptors for different types of bile acids. Thus it would not be unexpected that they can readily distinguish between PZS and 3kPZS (and between ACA and 3kACA) despite the small difference between the compounds.

In summary, a new bile acid, 3kACA, has been definitively identified in water washings of spermiating male lamprey. Since the HPLC fractions in which this compound elutes also have EOG activity, it is possible that it enhances or in some other way modifies the pheromonal activity of 3kPZS. Also presented in this paper is some previously unpublished evidence that the 3-keto group of both natural and synthetic 3kPZS can be converted to a 3α-hydroxyl group by 3α-HSD; and that the sulfate group can be removed by enzymatic hydrolysis.

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Table 1. ^{13}C and ^{1}H resonance assignments for 3keto allocholic acid.

Number	Type	Carbon	Proton
1	CH2	22.67	1.64, 1.67
2	CH2	43.96	1.75, 2.25
3	C	210.62	-
4	CH2	28.83	1.50, 1.50
5	CH	41.35	1.88
6	CH2	36.81	1.27, 1.41
7	CH	65.39	3.59
8	CH	46.04	1.75
9	CH	38.85	1.92
10	C	34.90	-
11	CH2	27.09	1.16, 1.75
12	CH	70.82	3.76
13	C	45.76	-
14	CH	38.16	1.60
15	CH2	30.78	2.07, 2.25
16	CH2	30.71	1.22, 1.64
17	CH	39.49	1.29
18	CH3	9.96	0.89
19	CH3	12.28	0.60
20	CH	34.95	1.28
21	CH3	16.85	0.88
22	CH2	37.86	1.82, 1.87
23	CH2	37.67	2.08, 2.37
24	C	174.83	-

Figure 1. Chemical structures of bile acids from sea lamprey. Petromyzonol sulfate (A), 3 keto petromyzonol sulfate (B), allocholic acid (C), and 3 keto allocholic acid (D).

Figure 2. Purification of putative 3keto allocholic acid on a preparative HPLC column (A) and mass spectrometry analyses of natural compound in fractions 58 from the HPLC (B) and synthetic 3 keto allocholic acid (C). Both compounds were subjected to FAB-MS analysis (negative mode) which indicated a mass of 406 Da.

Figure 3. TLC separation of allocholic acid (lane 1), allocholic acid which had been treated with 3α-HSD and NAD (lane 2) and compound in fraction 58 of HPLC (lane 3).

Figure 4. HPLC separation (on an analytical column) of a mixture of c. 50 μg of the material in HPLC fractions 58 and 50 μg of 3α-HSD-treated allocholic acid (A) and a mixture of c. 50 μg of the material in HPLC fractions 58 and 50 μg of synthetic 3kACA (B). In both cases, there is only one peak of absorbance at 200 nm and in exactly the same elution position.

Figure 5. One dimensional ^{1}H-NMR analysis of synthetic allocholic acid (A), synthetic 3keto allocholic acid (B) and HPLC fraction 58 (C). The positions of all but a few trace peaks in B and C are identical between 0.5 ppm to 4.5 ppm. The peak ascribable to the 3α-hydroxyl group is missing in B and C. The numbers refer to the peaks representing the C-18, C-19 and C-21 methyl groups which are found in all 3α-hydroxylated bile acids. In the case of there being a 3-keto group, the C-19 peak shifts to overlie the C-21 peak. This can be seen in B and C.

Figure 6. HPLC separation (on an analytical column) of synthetic and natural 3 keto petromyzonol sulfate before treatment with snail juice sulfatase (A) and after (B). A; A mixture of 50 μg natural and 50 μg synthetic 3 keto petromyzonol sulfate was loaded . The sharp UV-absorbing peak at 34/35 min represents 3 keto petromyzonol sulfate. B; A mixture of synthetic and natural 3 keto petromyzonol sulfate after sulfatase treatment was loaded. Note that both compounds behave identically. Also, in all cases, hydrolysis was incomplete and the peak at 34/35 min represents undigested 3 keto petromyzonol sulfate and that at 49/50 min 3 keto petromyzonol.

Figure 7. TLC separation, after treatment with 3α-HSD and NADH, of natural 3 keto petromyzonol sulfate (lane 1), synthetic 3 keto petromyzonol sulfate (lane 3), 3 keto petromyzonol (Lane 5), petromyzonol sulfate (Lane 6) and petromyzonol (Lane 7). Untreated 3 keto petromyzonol sulfate and 3 keto petromyzonol were run in lanes 2 and 4, respectively. Note that petromyzonol sulfate and petromyzonol were unaffected by the enzyme treatment as C-3 already possesses a hydroxyl group.
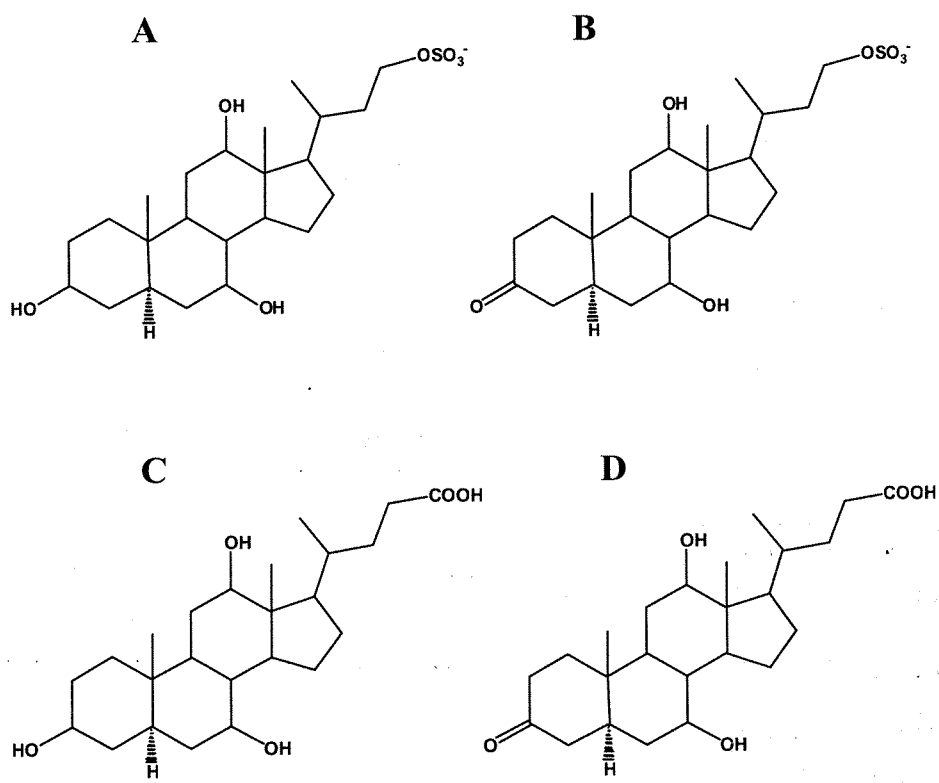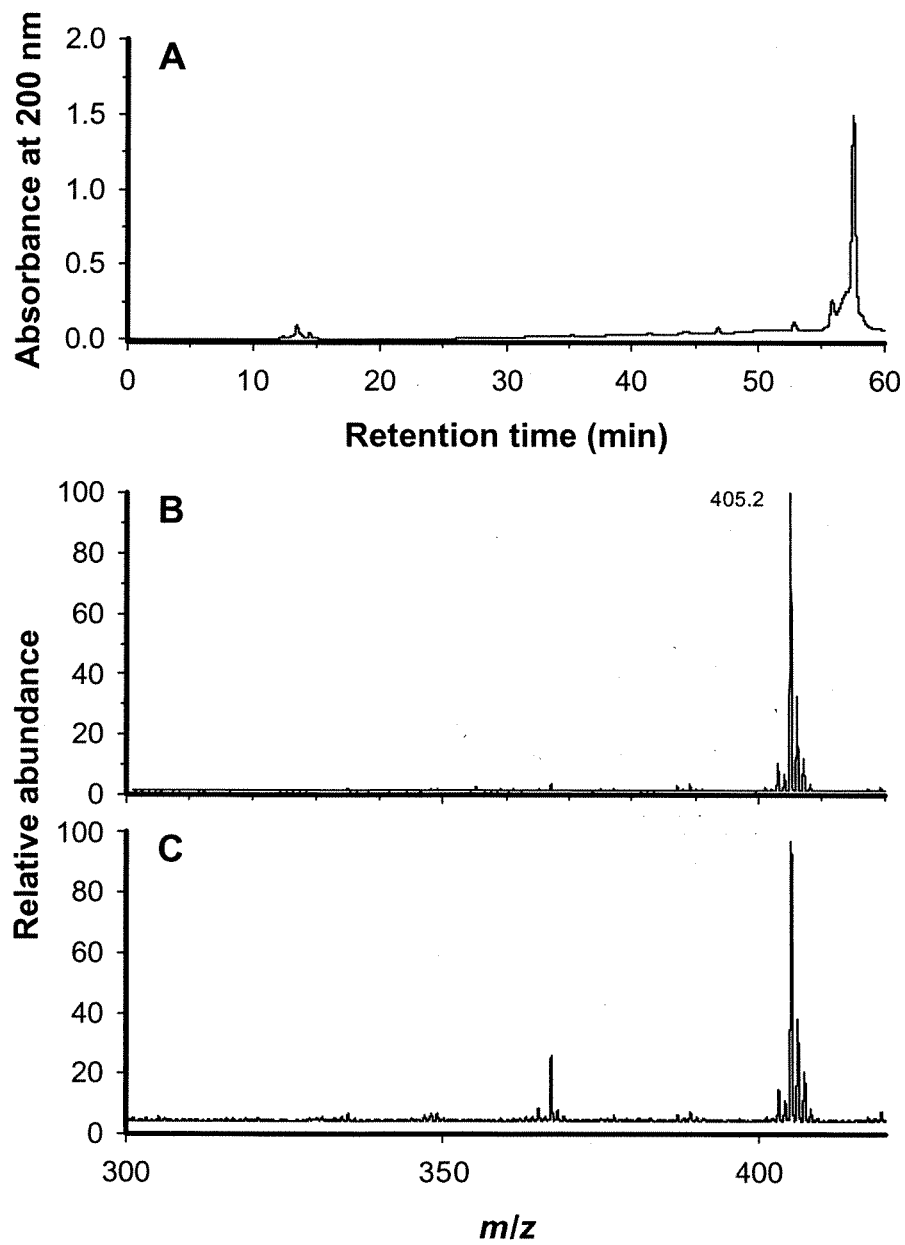
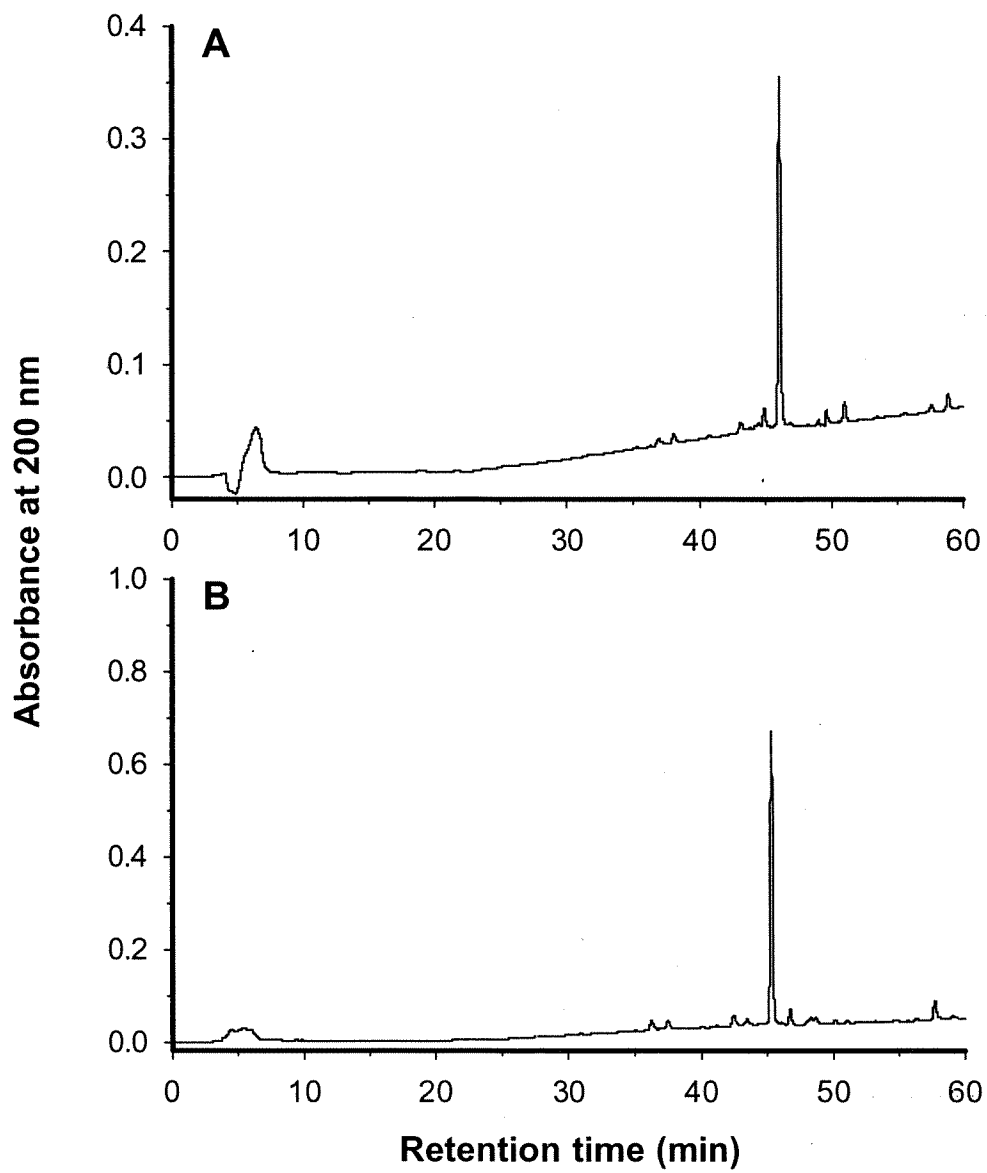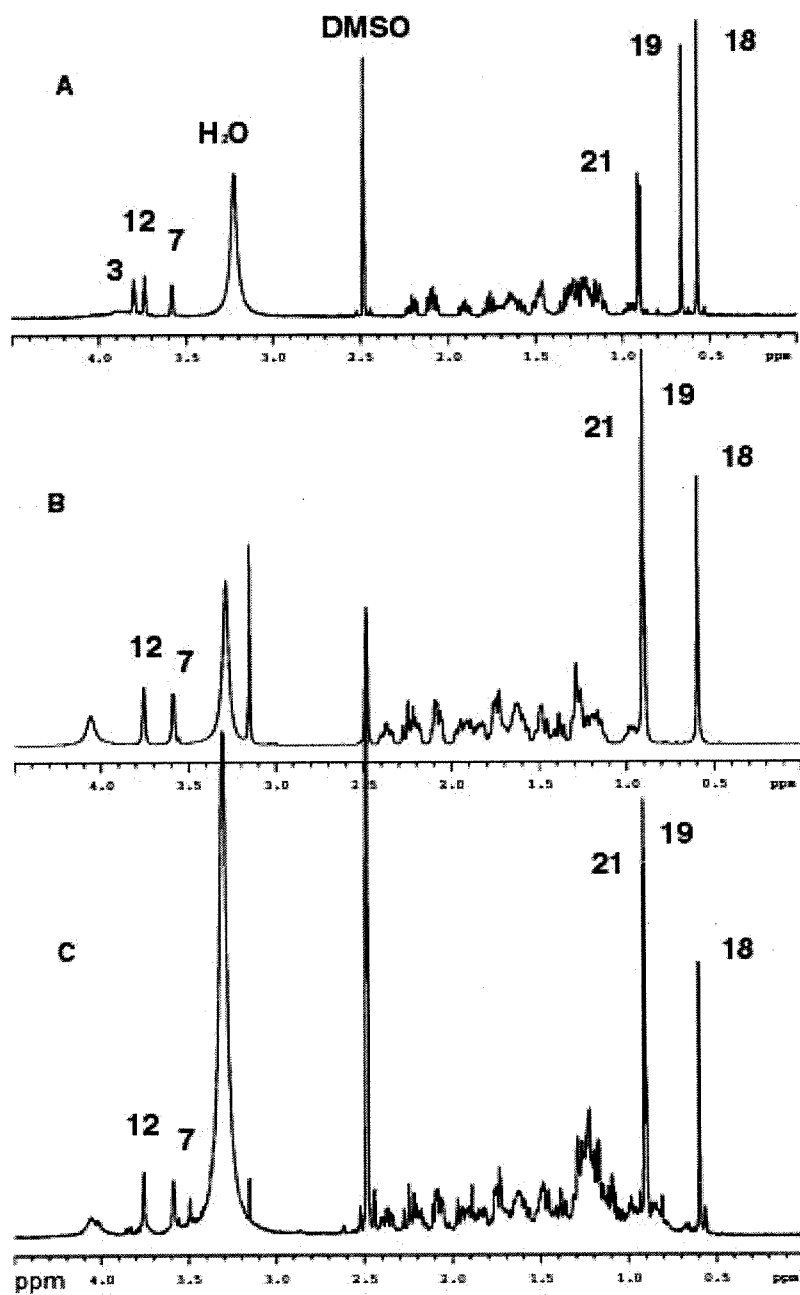A

B

C

D

Figure 1

Figure 2
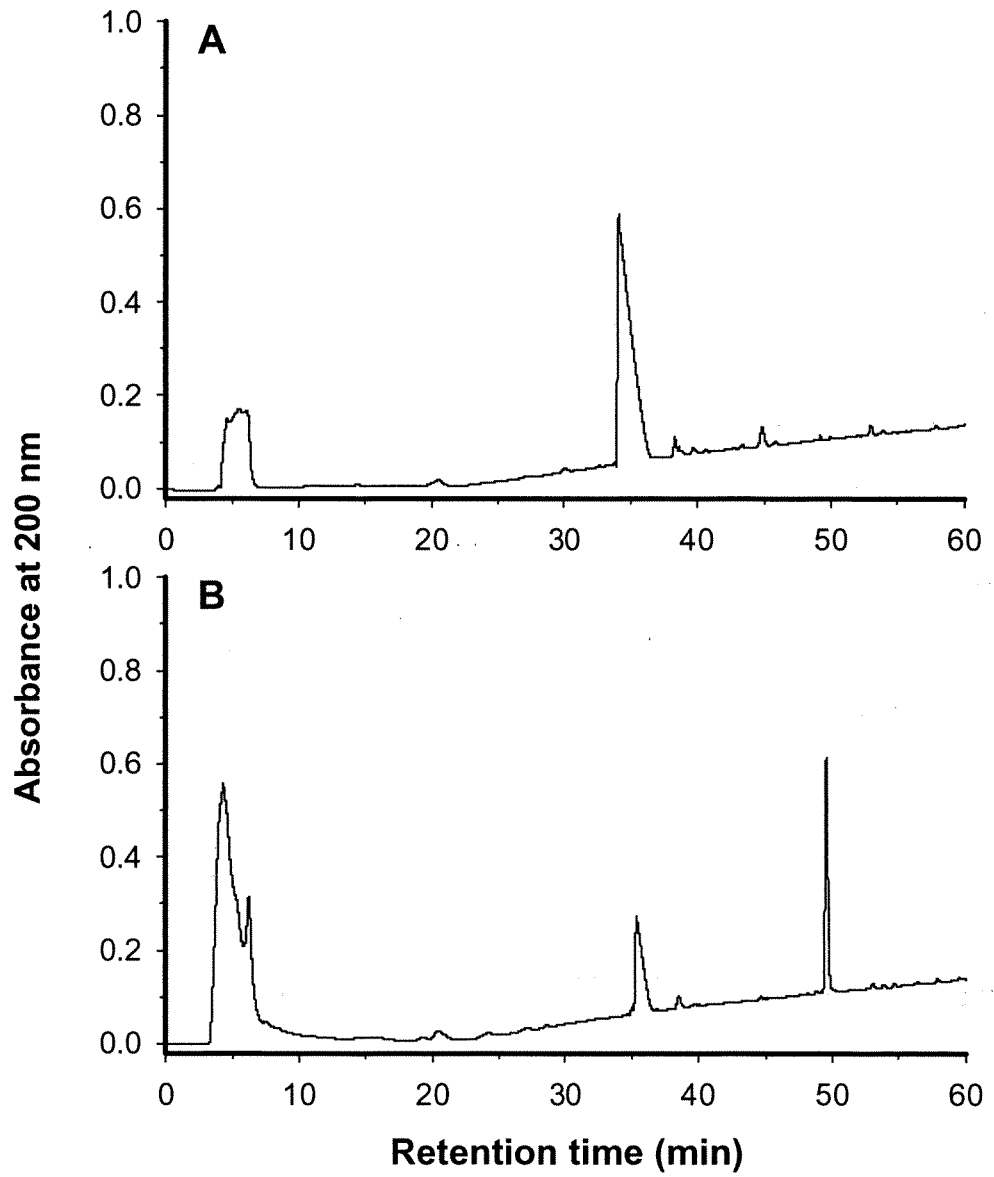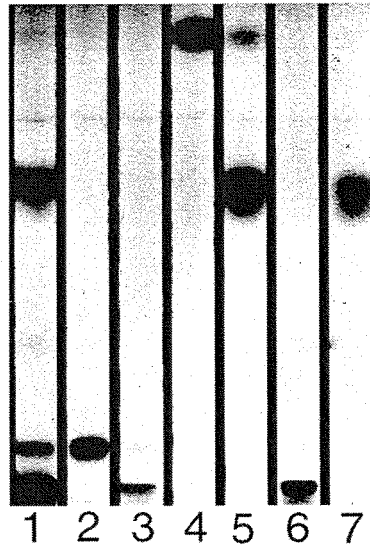
Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

APPENDIX II

[9]**Chemosterilization of male sea lamprey *Petromyzon marinus* does not affect sex pheromone release**

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Abstract

Release of males sterilized by injection with bisazir is an important experimental technique in management of sea lamprey (*Petromyzon marinus*), an invasive, nuisance species in the Laurentian Great Lakes. Sea lampreys are semelparous and sterilization can theoretically eliminate a male's reproductive capacity and, if the ability to obtain mates is not affected, waste the sex products of females spawning with him. It has been demonstrated that spermiating males release a sex pheromone that attracts ovulating females. We demonstrated that sterilized, spermiating males also released the pheromone and attracted ovulating females. In a two-choice maze, ovulating females increased searching behavior and spent more time in the side of the maze containing chemical stimuli from sterilized, spermiating males. This attraction response was also observed in spawning stream experiments. Also, electro-olfactograms showed that female olfactory organs were equally sensitive to chemical stimuli from sterilized and non-sterilized, spermiating males. Finally, fast atom bombardment mass spectrometry showed that extracts from water conditioned with sterilized and non-sterilized, spermiating males contained the same pheromonal molecule at similar levels. We concluded that injection of bisazir did not affect the efficacy of sex pheromone in sterilized males.

Keywords: Sea lamprey, *Petromyzon*, pheromone, bisazir, sterile male release

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Introduction

The sea lamprey (*Petromyzon marinus*) is a non-indigenous nuisance species in the Laurentian Great Lakes and a potentially lethal parasite feeding on the blood of larger fishes such as lake trout (*Salvelinus namaycush*) and lake whitefish (*Coregonus clupeaformis*). Its establishment in the upper Great Lakes during the 1920s through 1940s was a significant factor in the collapse of commercial fisheries and brought about dramatic biological and ecological changes in the fish communities (e.g., Smith 1971; Coble et al. 1990). Despite an intensive program started in the 1950s to control their populations (Smith and Tibbles 1980), the sea lamprey remains a factor in the mortality of larger fishes in the Great Lakes (e.g., Spangler et al. 1980; Kitchell 1990).

An important new method being experimentally implemented in sea lamprey management is the sterile male release technique (e.g., Bergstedt et al. 2003; Schleen et al. 2003; Twohey et al. 2003). In this technique, the chemosterilant bisazir (P,P-bis(1-azirindinyl)-N-methylphosphinothioic amide) is injected intra-peritoneally into adult males (Hanson and Manion 1978). Sperm from males injected with bisazir can still fertilize eggs, but the resulting embryos die before reaching the larval stage (Hanson and Manion 1980). Laboratory and field studies with bisazir treatment demonstrated that sterilized males mate successfully with females (Hanson and Manion 1978) and when released into spawning streams reduce the abundance of sea lamprey offspring (Bergstedt et al. 2003). In the current application of the sterile male release technique the observed proportion of sterilized males in male spawning populations was typically near expected values calculated by mark and recapture estimates, but the overall percent egg viability was consistently higher than the theoretical effect of the number of sterilized males released (Bergstedt et al. 2003). One factor potentially contributing to the difference between the actual and theoretical egg viability could be the effect of bisazir on pheromone production of male sea lampreys. Non-sterilized, spermiating male sea lamprey release a potent sex pheromone, $7\alpha,12\alpha,24$-trihydroxy 5α-cholan-3-one 24-sulfate, which induces preference and locomotor activities of ovulating females (Li et al. 2002). This pheromone functions to attract females that are ready to spawn. Clearly, if bisazir affected the release of this pheromone, the ability of sterilized males to obtain mates would be reduced. The present study explores this possibility.

As invasive fish species increasingly threaten the health of our aquatic ecosystems, the sterile male release technique is one of the few potential control methods with no identifiable environmental effects. It has the potential to become an important technique in management of these nuisance species, for which bisazir could be used as a sterilant. Where studied, most fish species are shown to rely heavily on the olfactory sense to mediate reproduction (e.g., Solomon 1977; Liley 1982; Stacey et al. 1994). Although each species would have to be tested independently, initial determination of whether sterilization of sea lampreys with bisazir affects sex pheromone biosynthesis and release would bear strongly on its potential for broader use.

The overall goal of this study was to determine if bisazir-sterilized, spermiating male sea lampreys release the same sex pheromone at similar levels found in non-sterilized, spermiating males and if they elicit the same behavioral response from adult sea lampreys. We applied a multidisciplinary approach to characterize whole animal and electrophysiological responses to, as well as chemistry of, chemical stimuli produced by sterilized males. The following specific questions were posed: 1) In a two-choice maze, do ovulating females show preference and searching behaviors when exposed to water conditioned with bisazir-sterilized, spermiating males? 2) In a spawning stream, are female sea lampreys attracted to water conditioned with

bisazir-sterilized, spermiating males? 3) Do female olfactory organs show the same electrophysiological response to water conditioned with bisazir-sterilized and non-sterilized, spermiating males? 4) Do extracts of water conditioned with bisazir-sterilized and non-sterilized, spermiating males contain the same pheromone molecule? Our results showed that bisazir-sterilized, spermiating male sea lampreys released the same sex pheromone at similar levels found in non-sterilized, spermiating males.

Materials and methods

Collection and maintenance of animals

Adult sea lampreys were collected from tributaries to lakes Huron and Michigan by the staff of the U. S. Fish and Wildlife Service, Marquette Biological Station, Marquette, Michigan, USA. The animals were transported to the Sterilization Facility and main laboratory at the U. S. Geological Survey, Hammond Bay Biological Station, Millersburg, Michigan, USA. Males were identified by their raised dorsal ridge and females by their enlarged, soft abdomen and absence of dorsal ridge (Vladykov 1949). Males averaged 470 mm and 233 g while females averaged 465 mm and 240 g. The sexes were separated and held in flow-through tanks (1000 L) with Lake Huron water (7 °C to 20 °C).

Sterilization of male sea lamprey

Males were sterilized in the sterilization facility by robotic injection with a dose of 100 $mg\cdot kg^{-1}$ body weight of bisazir (Hanson and Manion 1978; 1980; Twohey et al. 2003a). Quality assurance monitoring showed that males used during this study received no less than the correct dose of bisazir (Twohey et al. 2003a). Sterilized males were held in the sterilization facility for at least 48 hours after injection before being used in any procedure.

Identification of maturity

Each sex was further assigned one of two maturity classifications. For males, gentle pressure was applied to the abdomen to induce emissions into a petri dish. White emissions indicated spermiation. Depending on treatment status, males that did not emit sperm were classified as non-sterilized, pre-spermiating males or as sterilized, pre-spermiating males. Those that did emit sperm were classified as non-sterilized, spermiating males or as sterilized, spermiating males. To determine whether white emissions correctly indicated spermiation, emissions from ten males classified as spermiating and ten classified as non-spermiating were checked under a microscope for sperm. All 20 males were classified correctly. For females, gentle pressure was applied to the abdomen to induce egg emission. Females that did not release eggs were classified as pre-ovulating females and those that did release eggs were classified as ovulating females.

Induction of spermiation and ovulation

Sterilized and non-sterilized males were allowed to spermiate naturally or were induced to spermiate by holding them at 18 °C and then injecting them intra-peritoneally with [D-Ala^6]-luteinizing hormone-releasing hormone (pGlu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH$_2$, Sigma Chemical Co., St. Louis, Missouri USA) at a dose of 100 $\mu g\cdot kg^{-1}$ body weight (Li 1994). This injection was repeated three and five days later. Males were checked each day until spermiation occurred. Females were treated similarly to induce ovulation.

Chemical stimuli used in experiments

Either male sea lampreys or water conditioned with male sea lampreys were used as the chemical stimuli in experiments. Conditioned water was collected by holding individual males of classification for 4 h in polyethylene buckets filled with 10 L of water. These buckets were aerated and kept in a water bath of 18 °C. The conditioned water was either used immediately for behavioral assays, frozen immediately at -80 °C for subsequent electro-olfactographic analyses, or extracted using C18 solid phase extraction cartridges and the elute frozen at -80 °C for chemical analysis.

Experiment 1: In a two-choice maze, do ovulating females show preference and searching behaviors when exposed to water conditioned with bisazir-sterilized, spermiating males?

Preference and searching tests were used to assess the attraction behaviors of adult sea lampreys of each sex and maturity class to chemical stimuli produced by sterilized males of both maturity classes. Preference behaviors were measured as the amount of time spent in each side of the maze, while searching behaviors were measured as the amount of time spent swimming at the head of each side of the maze and were categorized as described by Li et al. (2002). These tests were carried out in the same two-choice maze, under similar conditions and following the same protocol of Li et al. (2002). Briefly, in each test, the behavior of a single test subject was video recorded before and after the introduction of chemical stimuli from either sterilized spermiating or pre-spermiating males into the odor chamber of the side of the maze chosen randomly by coin toss. The chemical stimuli were either five sterilized males per test (placed directly into the odor chamber) or water conditioned with sterilized spermiating males (delivered into the odor chamber at 75 mL·min^{-1} using a peristaltic pump). Tests were conducted between 07:00 and 17:00 in water temperatures that ranged from 12 °C to 24 °C.

The times spent in each side of the maze and the time spent swimming at the head of each arm of the maze by the test animal in the treatment (stimulus) and control (no stimulus) side before and after chemical stimulus introduction were summed from the videotapes by a reviewer unaware of which side contained the stimulus. The data were used to calculate an index of preference or searching (I), described by Li et al. (2002) for each individual test subject: $I = (At/(At+Bt)) - (Ac/(Ac+Bc))$, where Bt and At are either the time spent or time spent searching in the treatment side of the maze before and after chemical stimulus introduction and Bc and Ac are the time spent or time spent searching in the control side of the maze before and after chemical stimulus introduction. These indices compare test subject behavior in each side of the maze before and after chemical stimulus introduction. A positive I value indicates a preference or increase in preference or searching behavior, while a negative value indicates an avoidance or decrease in the behaviors.

The I values were analyzed with a two-tailed Wilcoxon Signed Ranks Test (Rao 1998) to determine if there was differences in preference and searching behavior in each combination of test subject and chemical stimulus source class. Sample sizes varied due to availability of test subjects (Tables 1 and 2). Tests in which proportions and I values could not be generated were not used in analyses. These situations comprised approximately 20% of all tests and occurred when test subjects did not explore both sides of the maze in the initial recording period or did not explore either side of the maze in the experimental recording period, causing zero index values dictated by the Wilcoxon test to be deleted.

Experiment 2: In a spawning stream, are female sea lampreys attracted to water conditioned with bisazir-sterilized, spermiating males?

To assess whether sterilized, spermiating male chemical stimuli attract females in their spawning habitat, we monitored ovulating and pre-ovulating female responses to sterilized male chemical stimuli under similar conditions and in the same 65-meter section of the Ocqueoc River used by Li et al. (2002). Tests were conducted between 07:00 and 17:00, in water temperatures ranging from 12 °C to 24 °C, using the protocol of Li et al. (2002). A day before testing, a female sea lamprey (ovulating or pre-ovulating) was fitted externally with a radio tag (Model 393, Advanced Telemetry System, Isanti, Minnesota, USA) according to Kelso and Gardner (2000). The tagged female and the five sterilized, spermiating and five sterilized, pre-spermiating males were transported to the study site the following morning. The sterilized, spermiating and sterilized, pre-spermiating males were randomly assigned to two upstream cages. The tagged female was placed in a downstream acclimation cage, exposed to the two chemical stimuli sources for 2 h and then released.

In each test, the subject's location was observed visually or determined with a directional radio antenna and receiver (Lotek Engineering Inc., Newmarket, Ontario, Canada) and recorded on a map grid of the site every 5 min. If test subjects failed to move from the release site within 1 h, they were removed. If test subjects did move from the release site, they were observed until 1) they reached one cage or the other and stayed there for an hour; 2) they swam past the cages; or 3) 4 h elapsed from the start of the test. A contingency table was used to tally the behavior of ovulating (N = 15) and pre-ovulating female (N = 10) test subjects. Responses were categorized as swimming to the sterilized, spermiating males, swimming to the sterilized, pre-spermiating males, or not choosing (staying at an intermediate position within the stream section or not leaving the acclimation cage). A Fisher's Exact Test was used to compare the choice of females (proc freq; 1998 SAS Institute Inc., Cary, North Carolina, USA).

Experiment 3: Do female olfactory organs show the same electrophysiological response to water conditioned with bisazir-sterilized and non-sterilized, spermiating males?

The olfactory potency of an L-arginine standard and chemical stimuli from sterilized, spermiating males, non-sterilized, spermiating males and pre-spermiating males were examined with electro-olfactogram recordings of female sea lampreys. Electro-olfactogram recordings measure summated generator potentials of the olfactory neurons (Ottoson 1956; Getchell 1974). The recordings were conducted according to the procedures established by Li et al. (1995). Briefly, a female was anaesthetized with metomidate hydrochloride (3 $mg \cdot kg^{-1}$ body weight) (Syndel, Vancouver, Canada), immobilized with gallamine triethiodide (150 $mg \cdot kg^{-1}$ body weight) (Sigma Chemical Co., St. Louis, Missouri, USA), and placed in a water-filled trough. The head of the female remained above the water and the gills were supplied with aerated Lake Huron water. The olfactory lamellae were then exposed and perfused with water from a source that could deliver either clean water or the same clean water containing chemical stimuli on demand.

Differential electrical responses between the skin surface and the sensory epithelia in response to each chemical stimuli were recorded using two Ag/AgCl electrodes (type EH-1S, World Precision Instruments, Sarasota, Florida, USA) filled with 3 M potassium chloride and bridged with 8% gelatin/0.9% saline-filled glass capillary tubes. Two experiments were conducted using this setup.

In the first experiment, we determined dose-response relationships (Li et al. 1995) for water conditioned with sterilized, spermiating (N = 8), non-sterilized, spermiating (N = 8) and non-sterilized, pre-spermiating (N = 4) males as test stimuli. In a given trial, a 10^{-5} M L-arginine standard was pulsed into the olfactory epithelium of a female for 5 seconds and the electro-olfactogram response measured to establish a baseline of electrical activity.

Next, blank control water was introduced and the response measured to confirm the absence of response from the clean water supply used both to perfuse the olfactory organ and to dilute the chemical stimuli for testing. Increasing concentrations of test stimuli starting at 10^6 times dilution were then introduced and the responses measured. Measuring the response to the L-arginine standard and blank controls again at the end of the dilution series concluded each trial. The epithelium of each female was allowed to recover at least 3 min between stimuli and each of the test stimuli concentrations were assayed at least twice on a female. Female electro-olfactogram response magnitudes were measured in mV and expressed as a percentage of the L-arginine standard.

In the second experiment, we used cross-adaptation as described by Li and Sorensen (1997) to determine the olfactory sensitivity of females to water conditioned with sterilized (N = 5) and non-sterilized (N = 5), spermiating males. In a given trial, baseline olfactory responses of females to blank control water, a L-arginine standard, and the test stimuli (water conditioned with sterilized and non-sterilized, spermiating males) were recorded using electro-olfactograms. The test stimuli were used at concentrations that elicited equipotent olfactory responses at about 200% of the L-arginine standard. During adaptation, the olfactory epithelium of the female was continually perfused with the adapting stimulus (either sterilized or non-sterilized, spermiating male test stimuli) for 5 min after which a 5 second pulse of the same stimulus at the same concentration being used for the adaptation was introduced to the nose to establish that the sensory epithelium was completely adapted. Next, a 5 second pulse of the L-arginine standard was introduced to confirm that the olfactory epithelium of the female was still active. A 5 second pulse of the alternate test stimulus (not being used as the adapting stimulus) was then introduced to see if any responses were generated. Switching the epithelial adapting stimulus back to clean water completed the trial. The epithelium of the female was allowed to recover for 30 min and then it was tested again using the alternate test stimulus as the adapting stimulus. The effects of adaptation on female responses to male stimuli were analyzed with a two-way analysis of variance (ANOVA). Once the main effect of adaptation has been established, effects of each adapting stimuli on olfactory responsiveness to other stimuli were determined by t-tests with equal variance (proc glm, 1998 SAS Institute Inc., Cary, North Carolina, USA).

Experiment 4: Do extracts of water conditioned with bisazir-sterilized and non-sterilized, spermiating males contain the same pheromone molecule?

Water conditioned with sterilized (N = 3) and non-sterilized (N = 3), spermiating males were separately pre-filtered with No. 3 Whatman filter paper, passed through methanol-activated C-18 solid phase extraction cartridges (Waters, Milford, Massachusetts, USA) and eluted with 100% methanol. All six extracts were assayed for relative abundances of molecules by subjecting them to fast atom bombardment mass spectrometry (10 KV) analyses at both negative and positive ionization modes.

Results

The total time spent and time spent searching by test subjects in the two sides of the maze before chemical stimulus introduction did not differ significantly (P > 0.10; data not shown), indicating that adults were not biased to either side. Ovulating females, however,

showed strong preference responses to sterilized, spermiating male chemical stimuli (Table 1). Eleven out of twelve ovulating females spent more time in the side of the maze containing five sterilized, spermiating males (P < 0.01, N = 12), and nine of ten ovulating females spent more time in the side containing water conditioned with sterilized, spermiating males (P < 0.02, N = 10). In contrast, none of the three other classes of test subjects showed a preference response to sterilized, spermiating male chemical stimuli (P > 0.10, see Table 1 for sample sizes), and ovulating females did not show a preference response to sterilized, pre-spermiating male chemical stimuli (P > 0.10, N = 10; data not shown).

Consistent with the preference results, there was an increase in searching behavior by ovulating females exposed to chemical stimuli from sterilized, spermiating males (Table 2): all eight ovulating females spent significantly more time searching in the side of the maze containing five sterilized, spermiating males (P < 0.01, N = 8) and seven of eight ovulating females spent significantly more time searching in the side containing water conditioned with sterilized, spermiating males (P < 0.01, N = 8). In contrast, none of the three other classes of test subjects showed significant increases or decreases in searching behavior (P > 0.10, see Table 2 for sample sizes), and ovulating females did not show significant increases or decreases in searching behavior (P > 0.10, N = 8; data not shown) in response to sterilized, pre-spermiating male chemical stimuli.

Table 2 here   The chemical stimuli released by sterilized, spermiating males influenced the behavior of ovulating females, but not of pre-ovulating females, within the spawning stream section. Females were not biased toward either side of the stream. Among the 15 ovulating females tested, ten swam to and then stayed at the cage containing sterilized, spermiating males and five did not choose a stimulus source. Of the ten that did choose, the mean time to swim the 65 m to the sterilized, spermiating males was 29 min. No ovulating females swam to the cage containing sterilized, pre-spermiating males. Among the ten pre-ovulating females tested, three swam to the cage containing sterilized, spermiating males, two swam to the cage containing sterilized, pre-spermiating males, and five did not choose a stimulus source. The distributions of choices differed significantly between ovulating and pre-ovulating females (P = 0.024). Although the behavior was not quantified, we observed ovulating females swimming repetitively around and against the cage containing sterilized, spermiating males for 1 h. The three pre-ovulating females that swam to the sterilized, spermiating male cage and the two that swam to the sterilized, pre-spermiating male cage did not display this behavior, but paused briefly at the cages and continued swimming upstream.

In electro-olfactogram experiments the chemical stimuli released by sterilized (N = 8) and non-sterilized (N = 8), spermiating males induced similar responses from the olfactory epithelium of adult females, but chemical stimuli from non-sterilized, pre-spermiating males (N = 4) did not at equivalent concentrations (Figure 1). Sterilized and non-sterilized, spermiating male conditioned waters both had detection thresholds of approximately 10^5 times dilution. The detection threshold for non-sterilized, pre-spermiating male chemical stimuli was approximately 10^2 times dilution, one thousand times more concentrated than the dilution required for detection of sterilized and non-sterilized, spermiating male chemical stimuli.

Figure 1 here   Cross–adaptation experiments demonstrated that female olfactory responses were affected by continuous exposure to spermiating male chemical stimuli (ANOVA; P < 0.01).

Although the responses to L-arginine standard did not significantly change before and during adaptation (t-test; $P > 0.05$), there were substantial differences among responses to sterilized and non-sterilized, spermiating male chemical stimuli before and during adaptation (t-tests; $P < 0.01$). Both sterilized and non-sterilized, spermiating male chemical stimuli suppressed olfactory responsiveness to themselves and each other when used as adapting stimuli (Figure 2 a). For example, when sterilized spermiating male chemical stimuli (Figure 2 b; $N = 5$) were used as the adapting stimuli, the initial responses were 2.17 ± 0.23 mV for sterilized, spermiating male chemical stimuli and 1.52 ± 0.39 mV for non-sterilized, spermiating male chemical stimuli, while during adaptation, the responses of sterilized, spermiating male chemical stimuli to itself was 0.08 ± 0.02 mV and to non-sterilized, spermiating male chemical stimuli was 0.18 ± 0.13 mV. When non-sterilized, spermiating male chemical stimuli were used as the adapting stimulus (Figure 2 c; $N = 5$), the same pattern of olfactory suppression occurred for each chemical stimuli.

Negative and positive fast atom bombardment mass spectrometry showed that the base peak (the most abundant molecule) of crude extracts of all three samples of water conditioned with sterilized, spermiating males had a molecular weight of 472 Dalton, the same as extracts from the three samples of water conditioned with non-sterilized, spermiating males (Figure 3 a and b). The isotopic patterns of sterilized and non-sterilized, spermiating males are virtually identical and no other major peaks are present in either spectrum.

Discussion

The combined results of all four experiments show that bisazir-sterilized male sea lampreys during spermiation are capable of attracting ovulating females, and this attraction is mediated through the release of a sex pheromone in an amount similar to that released by non-sterilized males during spermiation. In the first experiment, when placed in a two-choice maze, ovulating females both preferred and spent more time searching in the treatment side of the maze containing chemical stimuli from sterilized, spermiating males. These results are nearly identical to those of Li et al. (2002) where 22 of 22 and eight of eight ovulating females preferred and spent more time searching in the side of the maze conditioned with non-sterilized, spermiating males and 12 of 15 and seven of seven ovulating females preferred and spent more time searching in the side conditioned with non-sterilized, spermiating male washings. Searching behavior could be the mechanism underlying the attraction response that leads female sea lampreys to male partners in a spawning stream. Sea lamprey often nest in rapids and chemical stimuli from nesting animals are flushed downstream (Applegate 1950). Individuals responding to chemical stimuli by searching and swimming against the current stand a better chance of reaching the nesting individuals upstream (Li et al. 2002). Further, ovulating, but not pre-ovulating females were attracted to sterilized, spermiating males in their natural spawning habitat, further demonstrating that only ovulating females that are ready to spawn are attracted to spermiating males. It is notable that in a previous study conducted in the same stream section under similar conditions, nine out of 13 ovulating females chose the side conditioned with five non-sterilized, spermiating males (Li et al. 2002). Whereas in this study ten out of 15 ovulating females chose the side conditioned with five sterilized,

spermiating males.

The virtually identical levels of behavioral responses observed in the first experiment may be attributable to the virtually identical levels of sex pheromone production and release between sterilized and non-sterilized, spermiating males, as demonstrated in experiments 2 and 3. In the second experiment, electro-olfactogram results showed that sterilized and non-sterilized, spermiating male chemical stimuli produced similar dose-response relationships in females, demonstrating that the chemical stimuli from both types of animals have the same potency. The olfactory potency of non-sterilized, spermiating male chemical stimuli was first observed by Bjerselius et al. (1996). Our results confirm the potency and show that sterilized males can be just as potent. Also, electro-olfactogram cross-adaptation experiments showed that chemical stimuli from sterilized and non-sterilized, spermiating males suppressed olfactory responsiveness to each other and to themselves after olfactory adaptation to one had occurred, demonstrating that water conditioned with sterilized and non-sterilized, spermiating males are of the same quality in terms of their olfactory stimulatory effectiveness.

Our fast atom bombardment mass spectrometry analysis further showed that the same pheromonal molecule is responsible for the behavioral and electrophysiological responses elicited by chemical stimuli from both sterilized and non-sterilized, spermiating males. The most abundant molecule contained in extracts of water conditioned with sterilized and non-sterilized, spermiating males has the same molecular weight and isotopic patterns that are characteristic of $7\alpha,12\alpha,24$-trihydroxy 5α-cholan-3-one 24-sulfate, the molecule demonstrated to induce behaviors identical to those induced by non-sterilized, spermiating males (Li et al. 2002) and by sterilized, spermiating males. All four of our experiments demonstrate the biosynthesis and release of a sex pheromone by sterilized, spermiating males. The close similarity of our results to that of Li et al. (2002) also demonstrate that the mechanisms regulating pheromone production and release were intact in males sterilized by bisazir treatment.

For a sterile male release technique to be effective, it is essential that the sterilization process does not negatively affect the mating competitiveness of sterilized individuals (Knipling 1964). Previous field tests demonstrated that sterile male sea lampreys displayed nest-building behaviors and obtained mates (Hanson and Manion 1978). Our study expanded on these findings and further demonstrated that the male sex pheromone signaling system is not affected by bisazir treatment. Bisazir may induce lethal mutations in gametes (Hanson and Manion 1980) that cause premature death in heterozygotes if dominant, and in homozygotes if recessive. Spawning behaviors of sea lampreys, on the other hand, are under direct regulation by the central nervous system, which in turn is influenced by the hypothalamus-pituitary gland-gonad axis (Sower 1990). Since the release of sex pheromone coincides with spermiation (Li et al. 2002), a process also regulated by the hypothalamus-pituitary gland-gonad axis (Sower 1990), it is possible that sex pheromone release is also regulated by this axis. It is not likely that the dosage of bizasir injected for sterilization will affect this system, and thus, reproductive behavior, pheromone release, and ultimately mating efficacy. Further, it appears that the slightly higher than expected levels of egg viability from the sterile male release technique (Bergstedt et al. 2003) is not due to the effect of bisazir treatment on pheromone synthesis and release.

Recently, the potential up-regulation of pheromone biosynthesis and release was proposed for the sterile male release technique program of sea lamprey management (Li et al. 2003). The concept is that sterilized males would be induced to synthesize and release the sex pheromone at higher concentrations, for longer periods of time, or both. A similar concept, where cues (similar to natural pheromonal compounds) are synthesized to be more potent, have been developed for the oriental fruit fly and applied in their control (Lanier 1990). If a technique to up-regulate the sex pheromone in sea lampreys can be developed, it would potentially improve the efficacy of the current sterile male release technique, which is mainly limited by the number of males that can be collected for sterilization and release (Twohey et al. in 2003b).

The present study supports the premise of up-regulating pheromone release by demonstrating that pheromone release in sterilized males appears to be intact, thus suggesting that a technique that alters the pheromone production in a non-sterilized male is likely to be as effective in a sterilized male. However, the utility of this concept requires further study. First, a technique that can alter the pheromone production and release has yet to be developed. Second, it has not been experimentally proven that sterilized males releasing higher levels of pheromone, or for longer periods of time would actually recruit more mates. We are currently pursuing both issues.

Numerous invasive fish species are currently affecting fish communities in the Great Lakes (Michigan Sea Grant 1994), large lake ecosystems worldwide (Hall and Mills 2000), and other aquatic ecosystems across North America (Courtenay et al. 1986). In the Great Lakes, the Eurasian ruffe (*Gymnocephalus cernuus*) is invading areas currently occupied by native yellow perch (*Perca flavescens*) (Fullerton et al. 2000), and the round goby (*Neogobius melanostomus*) may be displacing native sculpins (*Cottus sp.*) and preying on native lake trout eggs (Chotkowski and Marsden 1999). However, resistance to widespread use of non-selective pesticides severely limits efforts to control these or any invasive fish species. Also, alternatively, non-chemical control methods have been developed for other pests, but most are not easily transferred to fish.

It is evident that teleost fish use gonadal steroids, prostaglandins and bile acids as sex pheromones (e.g., Stacey et al. 1994; Vermeirssen and Scott 2001; Zhang et al. 2001). Since in the sea lamprey, as discussed above, function of the gonadotropin releasing hormone-gonadotropin-sex steroid axis appears not to have been affected by bisazir treatment, which mainly acts on gametes, it is possible that production of pheromonal sex steroids also will not be affected by this sterilant. Pheromonal cues have been demonstrated in both the Eurasian ruffe and round goby (Murphy et al. 2001). Up-regulation of pheromone production could increase the potential of the sterile male release technique for control of these and other invasive fish species that use sex pheromones.

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Table 1. Chemical stimuli from sterilized, spermiating male sea lampreys (*Petromyzon marinus*) induced preference responses from ovulating females only. N: sample size. Preference is the number of test subjects that spent proportionately more time after stimulus introduction in treatment (scented) side. P-values were determined using a Wilcoxon Signed Ranks Test (2-tailed) using indices of preference. NS: not significant ($P > 0.10$). Preference ratio is the mean ratio (standard deviation) of the time spent in seconds in the treatment/control sides after stimulus introduction.

*indicates ovulating female tests using water conditioned with sterilized,

Test subject	N	Preference	P	Preference ratio
Ovulating female	12	11	< 0.01	7.0 (5.5)
*Ovulating female	10	9	< 0.02	2.0 (1.0)
Pre-ovulating female	10	6	NS	1.7 (1.0)
Non-sterilized spermiating male	11	7	NS	1.4 (1.8)
Non-sterilized pre-spermiating male	10	6	NS	2.2 (1.6)

spermiating males as the stimulus source.

Table 2. Chemical stimuli from sterilized, spermiating male sea lampreys (Petromyzon marinus) increased searching responses from

ovulating females only. N: sample size. Searching is the number of test subjects that spent proportionately more time swimming after stimulus introduction in treatment (scented) side. P-values were determined using a Wilcoxon Signed Ranks Test (2-tailed) using indices of preference. NS: not significant (P > 0.10). Searching ratio is the mean ratio (standard deviation) of the time in seconds spent swimming in the treatment/control sides after stimulus introduction. *indicates ovulating female tests using water conditioned with

Test subject	N	Searching	P	Searching ratio
Ovulating female	8	8	< 0.01	3.9 (1.5)
*Ovulating female	8	7	< 0.01	3.3 (4.0)
Pre-ovulating female	7	3	NS	1.3 (1.3)
Non-sterilized spermiating male	10	5	NS	0.8 (1.2)
Non-sterilized pre-spermiating male	8	5	NS	2.4 (2.7)

sterilized, spermiating males as the stimulus source.

Figure Legends

Figure 1. (a) Representative electro-olfactogram (EOG) responses of female sea lampreys (*Petromyzon marinus*) to chemical stimuli from sterilized, spermiating males (SSM, diamonds), non-sterilized, spermiating males (SM, open circles), and non-sterilized, pre-spermiating males (PSM, filled circles). *Std* designates the response to a 10^{-5} M L-arginine standard and *Con* the response to a blank water control. Numbers along the x-axis indicate the logarithmic value of the dilution from the original conditioned water collected by holding one male in 10 l for 4 h. (b) Female sea lamprey EOG dose-response relationships to SSM (N = 8), SM (N = 8), and PSM (N = 4) chemical stimuli. Responses are measured as a percentage of the response to a 10^{-5} M L-arginine standard. Vertical bars represent one standard error.

Figure 2. (a) Representative electro-olfactogram (EOG) responses of female sea lampreys (*Petromyzon marinus*) to chemical stimuli from sterilized spermiating male (SSM) and non-sterilized spermiating male (SM) before (white bars) and during (shaded bars)

adaptation to a SSM chemical stimuli. *Std* designates the response to a 10^{-5} M L-arginine standard and *Con* the response to a blank water control. Female EOG responses to a 10^{-5} M L-arginine standard, SSM and SM chemical stimuli both before and during adaptation to SSM (b) and SSM (c) chemical stimuli were measured. Vertical bars represent one standard deviation.

Figure 3. Negative and positive fast atom bombardment mass spectrometry (10 KV) spectrum of (a) extracts of water conditioned with non-sterilized spermiating male sea lampreys (*Petromyzon marinus*) and (b) extracts of water conditioned with sterilized spermiating males.

Figure 1.

Figure 2.

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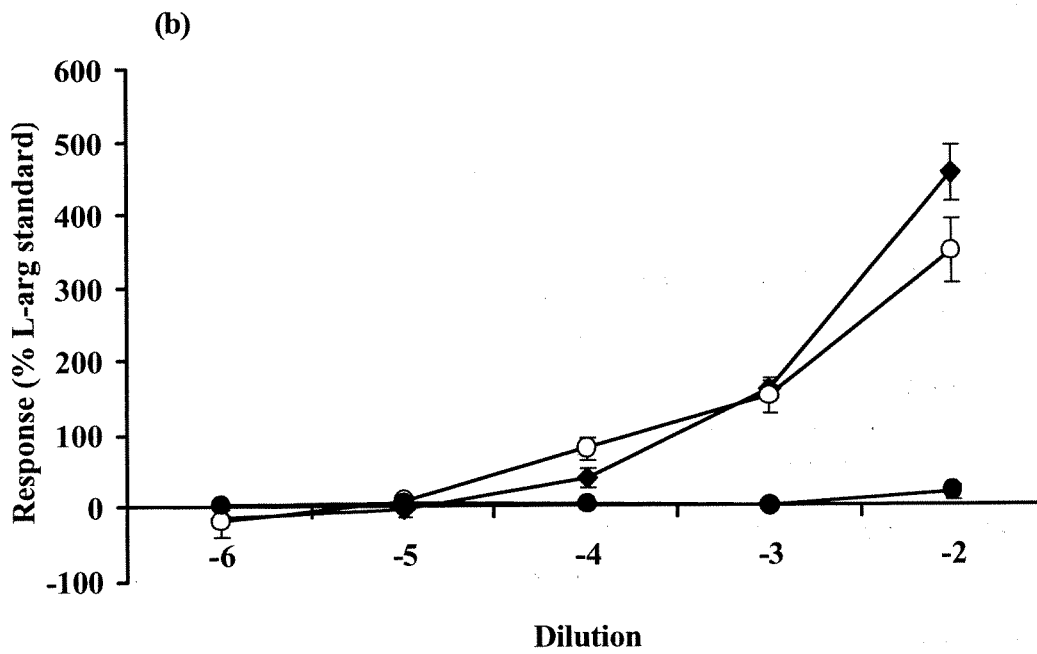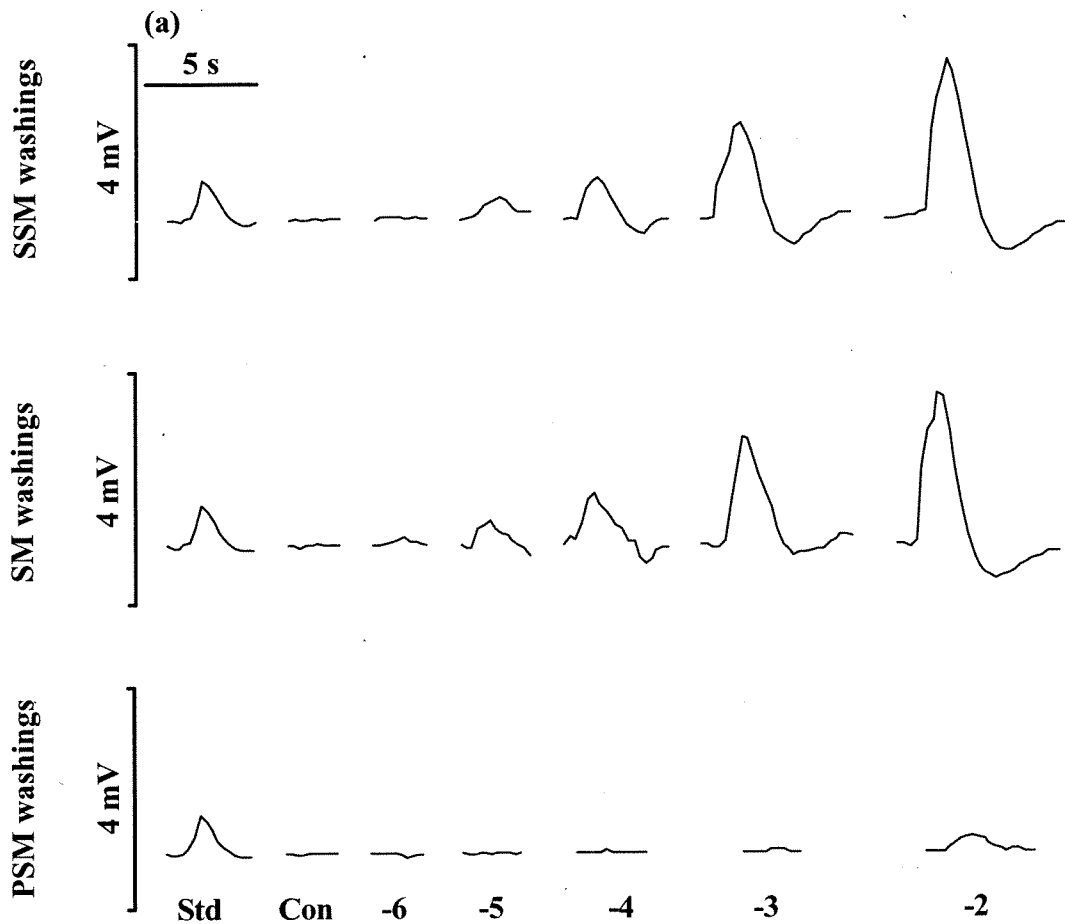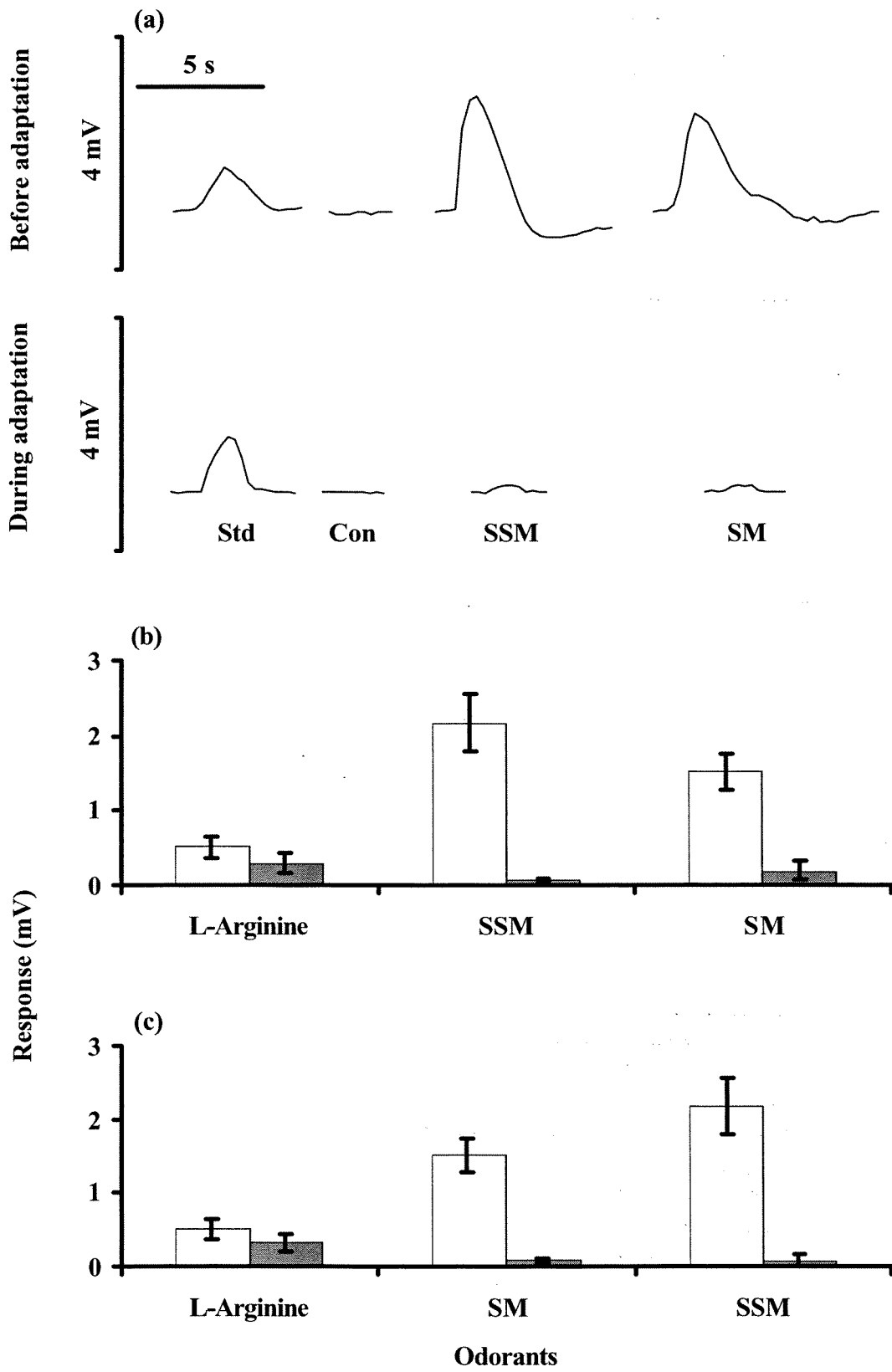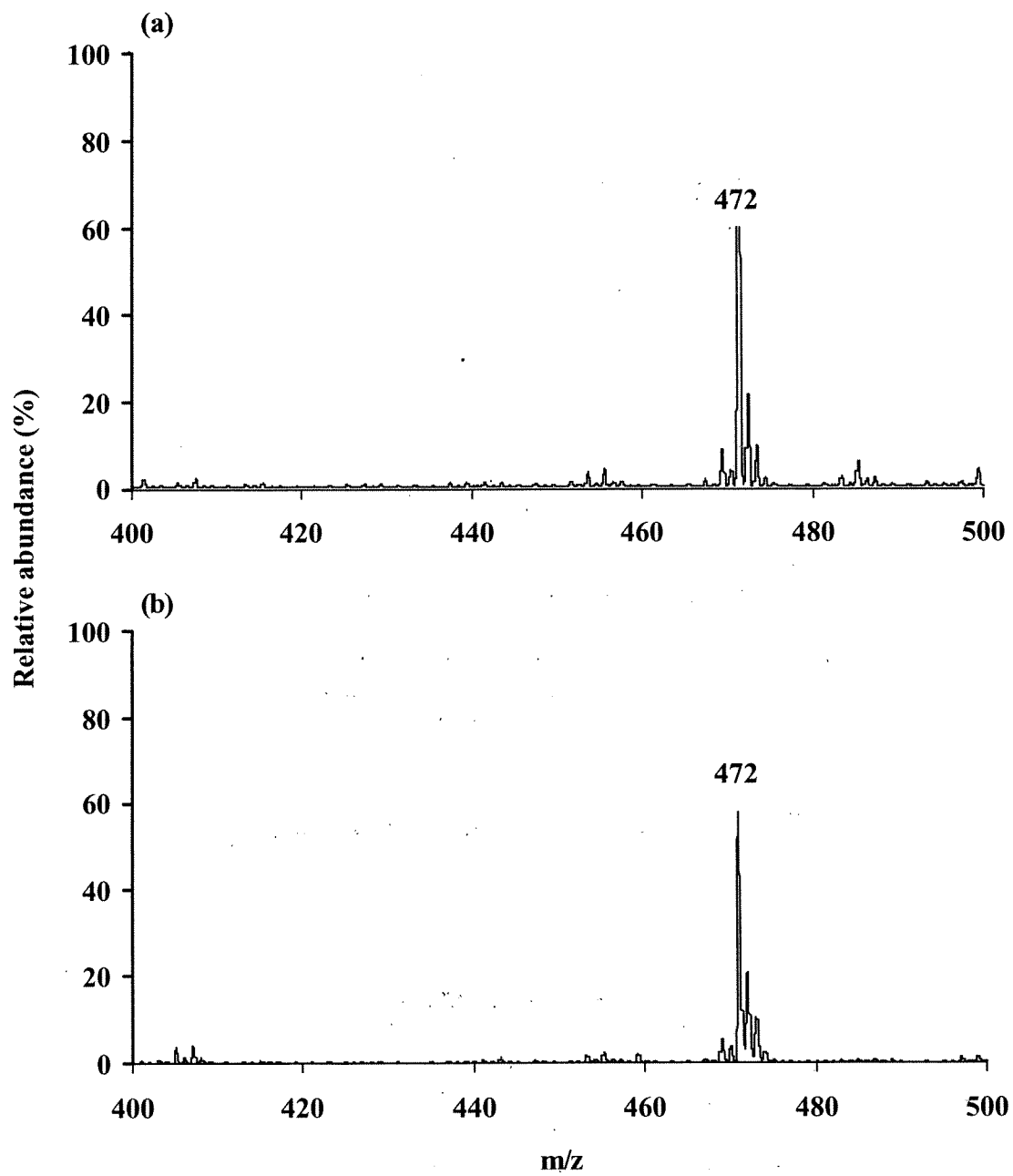
Figure 3.

[10]15α-Hydroxytestosterone produced *in vitro* and *in vivo* in the sea lamprey, *Petromyzon marinus*

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ABSTRACT

Prior research has shown that the testes of lampreys are able to synthesize 15-hydroxylated steroid hormones *in vitro*. Here we show that testes of the sea lamprey *Petromyzon marinus* L. are able to convert tritiated testosterone into tritiated 15α-hydroxytestosterone (15α-T) in high yield. The identity of the tritiated 15α-T has been confirmed by: co-elution with standard 15α-T on high performance liquid chromatography (HPLC); co-elution on thin layer chromatography (TLC); co-elution of acetylated tritiated and standard 15α-T on TLC; and strong binding to an antiserum developed against 15α-T. The strong reaction between the tritiated 15a-T and the antiserum has been used to develop a radioimmunoassay (RIA). The RIA operates over the range of 500 pg to 2 pg per tube; and can be applied directly to plasma samples. This assay has been used to demonstrate that 15α-T is present in blood plasma of the sea lamprey. The concentrations of 15α-T in captive lamprey were found to be as follows (pg/ml; mean $\pm$ sem, n): parasitic stage (reproductively immature), < 20 pg/ ml, n=7; pre-ovulatory females, 156 $\pm$ 30 pg/ ml, n=8; ovulated females, 62 $\pm$ 9, n=5; pre-spermiating males, 275 $\pm$ 19, n=8; spermiating males, 216 $\pm$ 48, n=8. When spermiating male plasma was fractionated on HPLC, immunoreactivity was found exclusively in the expected elution position of 15α-T. The biological significance of this steroid has yet to be established.

Key words: sea lamprey, Petromyzon, endocrinology, steroid, androgen

I. Introduction

The sea lamprey, *Petromyzon marinus* L., is a useful model species in studies of comparative and evolutionary endocrinology. As a member of superclass Agnatha, the lamprey is one of the earliest evolving vertebrates still alive today. It has an anadromous life cycle, with filter-feeding ammocoetes living in stream bottoms, parasitic phase immature lamprey feeding in large bodies of water, and reproductive adults migrating to tributary streams to spawn (Hardisty and Potter, 1971). Additionally, information concerning basic lamprey biology may be useful in a conservation context due to the effort spent controlling the invasive sea lamprey population in the Laurentian Great Lakes and conserving dwindling populations in Europe (Maitland, 1980).

Previous research on lampreys has established that, similar to teleosts and higher vertebrates, the hypothalamus-pituitary-gonadal axis, which includes two forms of GnRH, regulates reproduction (for reviews see Sower, 1998; Sower and Kawauchi, 2001). However, the identity of the gonadal steroids in lampreys and their role in reproduction is still not clear, especially with regard to the existence of androgens. There have been several studies in which measurements have been made of plasma concentrations of testosterone in the sea lamprey (Sower *et al.*,

1985a, 1985b; Linville *et al.*, 1987; Katz *et al.*, 1982; Weisbart *et al.*, 1980). In most cases, the amounts of testosterone were either undetectable or very low (<1 ng/ml) – the exception being the paper by Weisbart et al. (1980) in which a single value of 4.2 ng/ml was reported. This was the only study that did not use radioimmunoassay (RIA) to measure the steroid. Despite the low concentrations, Linville *et al.* (1987) found significant differences in plasma testosterone concentrations between male and female lampreys and an association between testosterone concentrations and stage of maturation. However, this finding was in contrast to that of Sower *et al.* (1985a), which found no differences between the sexes and no relationship to the stage of reproduction. Sower *et al.* (1985b) also showed that testosterone concentrations did not change in response to GnRH injection. The story with other species of lamprey is very similar. Testosterone (or at least a substance or substances cross-reacting with testosterone antibodies) is present, but at low concentrations that bear no relationship to stage of maturation, gender or treatment (Fukayama and Takahashi, 1985; Kime and Larsen, 1987; Rinchard *et al.*, 2000). The only exception is the brook lamprey, *Lampetra planeri* Bloch, in the blood of which Seiler *et al.* (1985) recorded concentrations of testosterone of up to 15 ng/ml.

Attempts have also been made to demonstrate the *in vivo* (Weisbart et al., 1977) and *in vitro* biosynthesis of testosterone by the gonads of immature (Weisbart and Youson, 1975) and mature sea lamprey (Weisbart *et al.*, 1978). In both cases, no testosterone was found. In the latter study, it was also reported that testicular 3β-hydroxysteroid dehydrogenase activity (that is required for the production of 4-ene steroids such as testosterone) was relatively low in comparison to other vertebrates.

The most likely reason for the apparent absence of testosterone in lamprey comes from a study by Kime and Rafter (1981). These authors incubated the testes of the river lamprey, *Lampetra fluviatilis* L., *in vitro* with either radio-labeled testosterone or radio-labeled progesterone. In both cases, the steroids were rapidly transformed into, respectively, 15β-hydroxytestosterone and 15α-hydroxyprogesterone. When Kime and Callard (1982) incubated testes of the sea lamprey with radio-labeled androstenedione they identified both 15α-hydroxyandrostenedione and 15α-hydroxytestosterone (15α-T). These findings prompted the authors to predict that the functional steroids in lampreys (whether androgens, estrogens or progestagens) differed substantially from those of most other vertebrates in possessing a 15-hydroxyl group. To date this has never been tested. The aim of the present study was to establish whether or not 15α-T is present in the blood plasma of reproductively mature sea lamprey.

Our first objective was to establish whether the incubation of sea lamprey testes with tritiated testosterone would yield 15α-T. Our second objective was to develop an immunoassay – whether by enzyme-linked immunoassay (ELISA) or RIA – in order to determine whether 15a-T is present in sea lamprey plasma. Our third objective was to determine whether there were any differences between the sexes and between stages of maturity in plasma concentrations of 15α-T.

2. Material and Methods

All chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Sea lamprey were collected in streams by U.S. Fish and Wildlife Serve employees, and transported to either Michigan State University (East Lansing, MI) for incubation experiments or Hammond Bay Biological Station (Millersburg, MI) for plasma sampling where they were held at 10° C. Blood was obtained from the caudal vein using heparinized syringes, held at 4° C for 20 min, and centrifuged at 2500 rpm for 20 min. The plasma was removed and stored at -80° C.

2.1 Synthesis of 15-hydroxylated standards

15α-T was prepared by a fifteen-step synthesis beginning with DHEA. The 15α-hydroxy group was introduced using a modification of the procedure described by Hosoda et al. (1976). A 15-hydroxyandrostane derivative was obtained and, after change of protection, was transformed into the testosterone series using the method described by Raggio and Watt (1976). The final product had a melting point and optical rotation that was identical to 15α-T obtained through microbial transformation (Tamm et al., 1963). The fully resolved NMR spectrum corresponded to established data (Kirk et al., 1990). 15ß-T was prepared as in Cerny et al. (1996).

2.2 In vitro biosynthesis of tritiated 15α-T

To confirm the biosynthesis of 15α-T, testicular tissue from two freshly killed non-spermiating adult male lampreys was finely diced with a scalpel. Replicate 500 mg portions of diced tissue from each lamprey were placed in 50 ml polypropylene tubes containing 10 ml L15 medium (at 6° C) and 25 µCi [1,2,6,7-^{3}H]-testosterone. The tube was then shaken for 4 h at 17° C. At the end of this period, the medium was removed from each tube, filtered and passed through an activated Sep-Pak C18 extraction cartridge (Waters, Milford, MA). Each cartridge was washed with 5 ml distilled water and eluted with 5 ml methanol. The solutions were stored at –20° C.

In order to characterize the products of the reaction, 50 µl of methanol extract was mixed with 10 µg each of T, 15α-T and 15β-T (dissolved in 20 µl ethanol), dried down under a stream of nitrogen at 45° C, redissolved in 500 µl acetonitrile/water/TFA (28/72/0.01, v/v/v) and loaded onto a reverse-phase HPLC column (Waters, Milford, MA; 5 µm octadecylsilane; 4.6 mm x 250 mm; fitted with a guard module). Two solvents were used to deliver a gradient to the column. Solvent A was 0.01% TFA in distilled water and solvent B was 70% acetonitrile and 0.01% TFA in distilled water. The pattern of development was as follows: 0 → 10 min, 28% B; 10 → 60 min, 28% → 100% B; 60 → 80 min, 100% B. The eluate was monitored for UV absorption with a photodiode array detector (Waters). Fractions were collected every 1 min between 20 and 60 min into scintillation vials. After addition of 3 ml ScintSafe Econo 1 scintillation fluid (Fisher Scientific, Pittsburgh, PA) the vials were counted on an LS 6500 Scintillation Counter (Beckman Coulter).

Further characterization of radioactive 15α-T was carried out by thin layer chromatography (TLC). A 1.5 ml aliquot of methanol extract from one of the incubations was mixed with 20 µg 15a-T and testosterone, dried down and fractionated on HPLC as described above. Part of the fraction (30 µl containing 50,000 dpm) corresponding to the elution position of 15a-T was placed in a glass tube containing 10 µg 15a-T. The solvents were removed, replaced by 100 µl pyridine and 100 µl acetic anhydride, and left overnight at room temperature. A further 30 µl of the same fraction was mixed with 10 µg of 15α-T and 15ß-T in a separate glass tube. The solvents in both tubes were evaporated and replaced with 40 µl ethyl acetate. These were loaded onto separate lanes of a TLC plate, which was developed with chloroform/ethanol (50/2; v/v). The positions of the standards were noted by placing the plate under a UV source. The lanes were then divided into 4 mm fractions, scraped off, placed in scintillation vials, mixed with 3 ml scintillation fluid and counted.

Further confirmation of the identity of radioactive 15a-T was carried out by testing its ability to bind to different dilutions of antiserum developed to 15α-T-3-CMO (as described below), 15ß-T-3-CMO and 15α-P-3-CMO (and to a control serum). This required a further 1.5 ml of the methanol extract to be fractionated on HPLC without being mixed with any standard. The antibody dilutions were made up in 100 µl assay buffer in glass tubes (as described below) and the radioactive 15a-T was added in a further 100 µl buffer at the rate of 5,000 dpm per tube. After overnight incubation at 4° C, 500 µl of ice-cold charcoal solution (50 mM sodium phosphate pH 7.4, 0.1% gelatin, 1.0% dextran-coated charcoal) was added to each tube. The tubes were kept in ice for 20 min, and then centrifuged at 1000 g for 12 min. The supernatants were poured into 8 ml scintillation vials, mixed with 6 ml scintillation fluid and counted.

2.3 Preparation of 15a-T radiolabel for use in radioimmunoassay

Approximately 1 g of minced testicular tissue was added to 12 ml L-15 medium containing 150 µCi tritiated testosterone and 7.3 mg NAD. After incubation at 10° C for 4 h, the medium was filtered and loaded onto an extraction cartridge as described above. The 15a-T label was preferentially eluted by passing through the cartridge 10 ml of 23% acetonitrile and 0.01% TFA in distilled water (v/v/v). This was then diluted with 20 ml distilled water and passed through a fresh extraction cartridge, washed with 5ml distilled water and eluted with 5 ml ethanol. The extract was stored at -20° C.

2.4 Development of antiserum

In order to develop an antiserum, a mixture of 15 mg 15α-T, 17 mg carboxymethyloxime hydrochloride, and 25 mg sodium acetate was dissolved in 1.5 ml methanol and left overnight at 4° C. The methanol was then dried down under a stream of nitrogen at 45° C and redissolved in 400 µl methanol, followed by 1 ml of water which had been adjusted to pH 2.0 with acetic acid. The product from the reaction, 15α-T-CMO, was extracted from this solution by shaking it twice with 4 ml ethyl acetate. The ethyl acetate was evaporated and the extract redissolved with a small amount of methanol and then precipitated by addition of diethyl ether. The powder was dried under vacuum.

Five mg 15α-T-CMO was dissolved in 1.5 ml of dimethylformamide (DMF) in a 20 ml glass scintillation vial. The vial was placed in crushed ice within a polystyrene container that was placed on top of a magnetic stirrer. A small magnetic flea was added to the beaker. The ice was prevented from thawing by the occasional addition of small amounts of liquid nitrogen to the container. With constant stirring, 3.5 μl tri-butylamine and 2.5 μl isochloroformate were added to the vial and the reaction was allowed to proceed for 40 min. In the meantime, 20 mg BSA was dissolved in 1 ml distilled water and then diluted with 1 ml DMF and 1 drop of 2 N sodium hydroxide. This mixture, after being chilled on ice, was added to the vial and left to stir for a further 3 h. After this time, the mixture (which was slightly opaque) was centrifuged for 10 min at 1000 g. The clear supernatant was made up to 2.5 ml with distilled water and then desalted on a PD-10 column (Nash *et al.,* 2000), using 3.5 ml distilled water to elute the protein fraction. The eluate was frozen and freeze-dried.

Approximately 2 mg 15α-T-CMO-BSA was dissolved in 1 ml saline and 1 ml Freund's Complete Adjuvant and each injected intradermally into two rabbits. Booster injections using the same amount of powder, but suspended in Freund's Incomplete Adjuvant, were given at four, six, and eight weeks following the first injection. Blood (20 ml) was obtained eight weeks after the first injection and allowed to clot before being centrifuged at 2500 rpm for 15 min. The serum was removed and frozen in 0.5 ml aliquots at -80° C.

2.5 Radioimmunoassay (RIA) procedure

RIAs were conducted in glass culture tubes (10 mm x 75 mm, Fisher Scientific, Pittsburgh, PA) according to Scott *et al.* (1980). The assay buffer consisted of 50 mM sodium phosphate pH 7.4, 0.2% BSA, 137 mM NaCl, 0.40 mM EDTA, 0.77 mM sodium azide. Nine standards were made up in duplicate over the range 500 to 1.95 pg/ 100μl/tube. The tubes containing unknowns also had a volume of 100 μl. Binding reagent was made up by adding radiolabel and antiserum to 20 ml of assay buffer in amounts such that, when 100 μl was dispensed to all tubes, each tube contained 7,000 dpm and, in the absence of any standard, 50% of the radiolabel was bound to the antiserum. Blank tubes, to which no antibody was added, and tubes necessary to determine the total and maximum DPM counts were also included in the assay. All tubes were incubated overnight at 4 °C, and then placed on ice and separated with charcoal as described above.

The specificity of the antiserum was tested by making, in 100 μl buffer, six five-fold serial dilutions of 15a-T over a range of 10,000 to 0.8 pg/tube; of 15ß-T and T over a range of 10,000 to 4 pg/ tube; and other several other steroids over a range of 10,000 to 500 pg/tube. A further 100 μl of buffer containing 7,000 dpm radiolabel and the 15α-T antiserum at a dilution of 1:100,000 (v/v) was added to each tube. After overnight incubation at 4° C they were separated with dextran-coated charcoal as described above.

Some cross-reaction was found with 15β-T (see Results). An experiment was thus performed to determine whether this was due to: genuine cross-reaction; possible contamination of the standard with 15α-T; or a mixture of both.

Small amounts of standard 15a-T (200 ng) and 15ß-T (2 µg) were loaded on to separate lanes of a TLC plate (type LK6DF; Whatman International). The plate was then developed with chloroform/ethanol (50/3; v/v) and the positions of the steroids detected by placing the plates under a UV light source. Both lanes were divided into 5 mm sections that were scraped into glass tubes. Assay buffer (1 ml) was added to each tube, held overnight at 4 °C, vortexed, and centrifuged briefly. Replicate 100 µl aliquots from each tube were assayed for 15a-T.

Preliminary experiments with several plasma extraction procedures (involving diethyl ether, ethyl acetate or dichloromethane) indicated that there appeared to be little or no interference with the assay if plasma was added directly to the tubes. An experiment was conducted to determine whether plasma proteins that bind 15α-T are present. Plasma from male lampreys was diluted 1:2, 1:4, 1:8, and 1:16 in both assay buffer and assay buffer without EDTA (100µl/tube). Radiolabel was added to all tubes (100 µl containing 7,000 dpm). The tubes were then incubated and separated as described above for the RIA. In a second experiment, 2 ml male plasma (pooled from several males) was mixed with 20 ng 15a-T. The plasma was then diluted four times with the same plasma to yield a range of dilutions of 10 to 1.25 ng/ ml. Each of these dilutions was then assayed using aliquots of 25, 50 and 100 µl aliquots. The two lower aliquots were made up to 100 µl with assay buffer.

Inter-assay variation was determined by measuring the amount of 15a-T in the same plasma sample (c. 2 ng/ml) in six separate assays. Intra-assay variation was determined by measuring 15a-T in the same sample six times in the same assay.

2.6 Concentrations of 15α-T in lamprey plasma

Plasma was collected as described above from seven parasitic lamprey, five ovulating females and eight each of pre-spermiating males, pre-ovulatory females and spermiating males. Parasitic phase lampreys were held for at least one week without feeding. For each sample, 50 µl of plasma was assayed in duplicate.

To confirm that cross-reacting material in plasma had the same chromatographic properties as 15α-T, 5 ml pooled male sea lamprey plasma was passed through an extraction cartridge. The steroids were eluted with 7 ml methanol and then dried with a rotary evaporator (RE 200, Yamato, Orangesburg, NJ). The residue was subjected to HPLC separation as described above. All fractions were assayed for 15α-T using 20 µl/ fraction.

3. Results

Incubation of lamprey testes with tritiated testosterone resulted in two major peaks on HPLC (Fig. 1). The first and largest of these peaks corresponded to the elution position of 15α-T and the second to the elution position of testosterone. The conversion rates of ^{3}H-testosterone to 15α-T in the two testes were 85.6 % and 64.5 %.

Further confirmation of the identity of the radioactive 15α-T was obtained by running some of the HPLC fraction on TLC. The bulk of the radioactivity eluted in the same position as standard 15α-T (Fig. 2). Very little was associated with 15β-T. Furthermore, when the radioactive peak was mixed with standard 15α-T and acetylated, both radioactivity and UV absorption co-migrated.

Incubation of 1 g lamprey testis with 150 μCi tritiated T yielded approximately 75 μCi 15α-T. The dilution of antiserum that was required to bind only 50% of this radiolabel (at 7000 dpm/ 200 μl) was 1:100,000 (v/v). The radioactive 15α-T bound strongly to the 15α-T antiserum, slightly to the 15β-T antiserum and not at all to the 15α-P antiserum or control serum (Fig. 3).

There was negligible cross-reaction between the 15α-T antiserum and most of the tested steroids (Fig. 4). However, there was substantial, but non-parallel, cross-reaction of the 15β-T (36% towards the top of the standard curve and 6.4% towards the bottom). By running the 15β-T on TLC, it was established that a large part of the cross-reaction was due to probable contamination of the 15ß-T standard with 15α-T (Fig. 5). Some cross-reaction was still associated, however, with the elution position of 15ß-T.

Intra-assay variation for 15α-T was c. 5% over the middle of the standard curve. Inter-assay variation was c. 14%.

Male lamprey plasma did not bind to tritiated 15a-T even at a dilution of 1:2 (v/v). Plasma dilutions yielded values near expected when assayed in 25 μl or 50 μl volumes (Fig. 6). Assay of plasma in 100 μl volumes yielded values slightly lower than expected.

No 15a-T (< 20 pg/ml) was detected in plasma obtained from parasites. Significant differences existed in plasma 15α-T concentrations among other groups (ANOVA, $F_{3, 25}$ = 6.53, P = 0.002, Fig. 7). In pairwise comparisons (with a Bonferroni correction) ovulating females were found to have significantly lower concentrations than pre-spermiating males (P = 0.002) and spermiating males (P = 0.028). After HPLC fractionation of male plasma, only one fraction contained cross-reactive material and that was in the expected elution position of 15a-T (Fig. 8).

4. Discussion

This study provides conclusive evidence that the testis of the sea lamprey is able to produce 15α-T both *in vitro* from exogenous testosterone and *in vivo*. *In vitro* production using testosterone as a precursor has so far only been demonstrated in the river lamprey (Kime and Rafter, 1981; Golla *et al.*, 2000). Kime and Callard (1982) found 15α-T as one of the products of incubation of sea lamprey testes with radioactive androstenedione. Kime and Rafter (1981) obtained high yields of 15β-T from testes of the river lamprey. However, in both the present study and that by Kime and Callard (1982) this isomer was not formed – suggesting that the sea lamprey lacks a 15β-hydroxylase.

The 15α-hydroxylating activity in the sea lamprey testis is so strong and specific that it has enabled us to use lamprey testicular tissue to produce tritiated 15α-T suitable for use in RIA. This proved to be beneficial as we had originally

set out to develop an ELISA for 15α-T. However, we experienced persistent problems with replication, specificity and generation of 'false positives' with the prototype ELISA. These problems were ameliorated when we developed the RIA.

The amounts of the steroid that we have found in plasma are no higher than those of testosterone that have previously been measured in this species. However, as opposed to the situation with testosterone (with the exception of one study – see Introduction) there are very clear differences in 15α-T concentrations between the sexes and stages of maturation. Also, we have HPLC evidence that what we are measuring is in fact 15α-T and not some other cross-reacting compound(s). None of the testosterone RIAs in any of the previous studies were similarly validated. We have also established (unpublished studies) that 15α-T concentrations of between 2 and 4 ng/ml are found in the plasma of individual GnRH-injected males.

One possibility for the relatively low amounts found in the present study may be due to the fact that the fish had been held in tanks for some weeks before they were sampled. It is known that captivity causes a substantial reduction in sex steroid concentrations in teleost flatfish (Vermeirssen et al., 1998). However, this possibility remains to be established in the lamprey. Another possible reason for low amounts of 15α-T is that it is cleared very rapidly from the plasma. The fact that there is no steroid binding activity in the plasma would certainly be a factor that favors its rapid excretion (see discussion by Weisbart et al., 1980).

The RIA for 15α-T has the ability to measure 15α-T over a wide range, and has low cross-reactivity with all hormones tested, with the exception of 15β-T. However, TLC fractionation of 15β-T with subsequent quantification of the amount of 15α-T in the fractions via RIA revealed that the 15β-T standard used in these analyses was heavily contaminated and that the actual detection rate of 15β-T is very low (0.87%). Additionally, since 15β-T does not appear to be synthesized by the testes of sea lamprey, cross-reactivity is not a concern. The use of plasma, rather than a plasma extract, in the assay did not appear to be a problem.

Although we have demonstrated that 15α-T is produced by lamprey gonads and is present in the blood, we still cannot state whether it has a function. For this, we would need to demonstrate the presence of receptors in one or more putative target organs – and then to demonstrate that injection of 15α-T causes a specific biochemical, physiological or behavioral change. Larsen (1974) has already shown that treatment of river lamprey with exogenous testosterone causes the development of male secondary characters in the river lamprey. This does not exclude the possibility that the testosterone exerted its activity through conversion to 15α-T by endogenous enzyme (see discussion by Kime and Larsen, 1987).

An attempt has already been made to identify DNA sequences that are common to vertebrate androgen receptors by searching sea lamprey liver mRNA with PCR primers (Thornton, 2001). The results of this study were negative, suggesting that androgen receptors evolved after the divergence between cyclostomes and gnathostomes, and that lamprey therefore may not utilize androgens at all and instead use estrogens as the main steroids in both males and

females. This hypothesis is supported by the fact that 17β-estradiol is present in the blood of male sea lampreys at up to 3 ng/ml (e.g. Sower *et al.*, 1985a). However, since the search for lamprey androgen receptors was performed using PCR primers for known (gnathostome) androgen receptors, the presence of unique receptors with unique DNA sequences for '15α-hydroxylated androgens' cannot be excluded.

If 15α-T is a functional hormone, there are several possible reasons why the lamprey may utilize it rather than testosterone. As a parasite feeding on other vertebrates, the sea lamprey would possibly need a mechanism to distinguish endogenous from exogenous ingested hormones. Using a unique set of hormones, such as 15-hydroxylated steroids, would allow lampreys an easy way to distinguish between the two. It is also possible that the 15-hydroxylated steroids are not an evolved response to parasitism, but are simply a primitive form of steroid hormone.

Studies of the endocrine systems of other parasites would be useful in distinguishing between the above two hypotheses. Unfortunately, there has been very little research performed on the steroids of other parasites, although one study did find that ticks inactivate ingested ecdysteroids by conjugating them with fatty acid chains (Connat *et al.*, 1986). Most studies have instead focused on the effect of parasites on host steroids. However, based on studies of this type, it seems likely that host hormones affect parasites feeding upon them. Lawrence (1991) found that various host steroids were found to promote growth and/or reproductive activities in a variety of invertebrate parasites. In certain cases, such as that of the coccidian, the parasite's endocrine system becomes strictly correlated with the host's in order to synchronize reproductive activities (Porchet-Hennere and Dugimon, 1992). In situations such as that of the lamprey where the parasite and host are very similar, having a system to distinguish endogenous from exogenous hormones would perhaps be essential in order for the lamprey to prevent its own body from responding to the host's hormones especially since the sea lamprey feeds upon several species with differing reproductive seasons. Further research on sea lamprey endocrinology would help illustrate how parasites in general cope with host hormones to prevent endocrine disruption.

In contrast, examining the endocrine systems of animals related to lamprey may help to distinguish if 15-hydroxylated steroids are an incidental event in evolution. Hagfish are the only other group of agnathans still alive today, though hagfish are scavengers and not parasites, so they do not need a mechanism to cope with exogenous hormones. However, it has been demonstrated that the hagfish produce some unusual steroids, including 5, 6, and 7-hydroxylated hormones (Kime *et al.*, 1980; Kime and Hews, 1980). Based on hagfish endocrinology, it appears possible that unusual steroids are due to incidental or ancestral events in evolution.

Lampreys are not the only animals that produce steroids with a 15-hydroxyl group, although steroids of this type are rare, and the reasons for the 15-hydroxyl group are rarely explored. Estrogens with a 15-hydroxyl group have been found *in vivo* in the peripheral plasma of the laying turkey (Brown *et al.*, 1979) and *in vitro* in bovine adrenal glands (Levy *et al.*, 1965). Bullfrog liver slices were shown

to produce 15α- and 15β-hydroxydeoxycorticosterone *in vitro* (Schneider, 1965). Additionally, 15-hydroxylated steroids have been isolated from human pregnancy urine (Giannopoulos and Solomon, 1967,1970; Giannopoulos *et al.*, 1970). Hepatic microsomes from mice have been shown to metabolize testosterone by hydroxylation, some of which occur at the 15α or 15β positions (Ford *et al.*, 1975). A final instance of 15-hydroxylation has a definitive reason; it was determined that in rat liver microsomes cytochrome p450 hydroxylates steroids at several different points including the 15-C, which may make the steroids more water-soluble in order to aid in excretion (Hrycay *et al.*, 1976; Gustafsson, 1970).

Further research is needed to elucidate the function of 15α-T and determine the reason for the unique structure of the hormone. It is clear that knowledge of sea lamprey hormones can provide useful information for comparative endocrinology and the evolution of steroid hormones.

Acknowledgments

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Fig. 1. ^{3}H counts (DPM) in fractions following HPLC analysis of 20 μl of media from incubation of testicular tissue with ^{3}H testosterone. Arrows show where 15a-T (15a-hydroxytestosterone), 15ß-T (15ß-hydroxytestosterone), and T (testosterone) standards elute.

Fig. 2. ^{3}H counts (DPM) from TLC fractionation of 30 μl of incubation media after purification with HPLC. Black bars represent the purified product of incubation, and grey bars represent the acetylated product. Arrows show the elution points of 15a-T (15a-hydroxytestosterone) standard, acetylated 15a-T, and 15ß-T (15ß-hydroxytestosterone) standard.

Fig. 3. Ability of antibodies raised against different steroids to bind purified 15a-T produced *in vitro*. 15a-T is 15a-hydroxytestosterone, 15a-P is 15a-hydroxyprogesterone, and 15ß-T is 15ß-hydroxytestosterone.

Fig. 4. Ability of various common steroids and bile acids to displace radio-labeled 15a-T (produced *in vitro*) from the antibody raised against 15a-T. 15a-T is 15a-hydroxytestosterone, 15ß-T is 15ß-hydroxytestosterone, T is testosterone, 11KT is 11keto-testosterone, Ad is androstenedione, E2 is estradiol, CA is cholic acid, 15a-P is 15a-hydroxyprogesterone, 3kPZS is 3keto-petromyzonol sulfate, 3kACA is 3keto-allochoic acid, PZS is petromyzonol sulfate, and ACA is allochoic acid.

Fig. 5. Assay for 15a-T (15a-hydroxytestosterone) of TLC fractions from 2 μg of 15ß-T (15ß-hydroxytestosterone) standard (filled bars) and 200 ng of 15a-T standard (open bars). Arrows indicate the elution points of 15a-T and 15ß-T. There is only a small positive result in the fraction containing 15ß-T, indicating that actual cross-reactivity is small. However, there are positive results in other fractions in the 15ß-T lane, indicating that the standard is contaminated.

Fig. 6. Expected amounts of 15a-T plotted against observed amounts in the recovery experiment. Known amounts of 15a-T were added to plasma, which was assayed in different quantities and dilutions.

Fig. 7. Differences in 15α-T concentrations among sexes and stages of maturity. P is parasitic phase lamprey (n=7), OF is ovulated females (n=5), POF is pre-ovulatory females (n=8), PSM is pre-spermiating males (n=8), and SM is spermiated males (n=8). Dotted lines are means, solid center lines are medians, boxes cover the 25-75% range, whiskers show the 10% and 90% percentiles, and dots are values either below 10% or above 90% percentile.

Fig. 8. Amount of 15a-T found in HPLC fractions from 5 ml pooled male sea lamprey plasma based on RIA of 20 μL of each fraction. An arrow shows where 15a-T elutes.
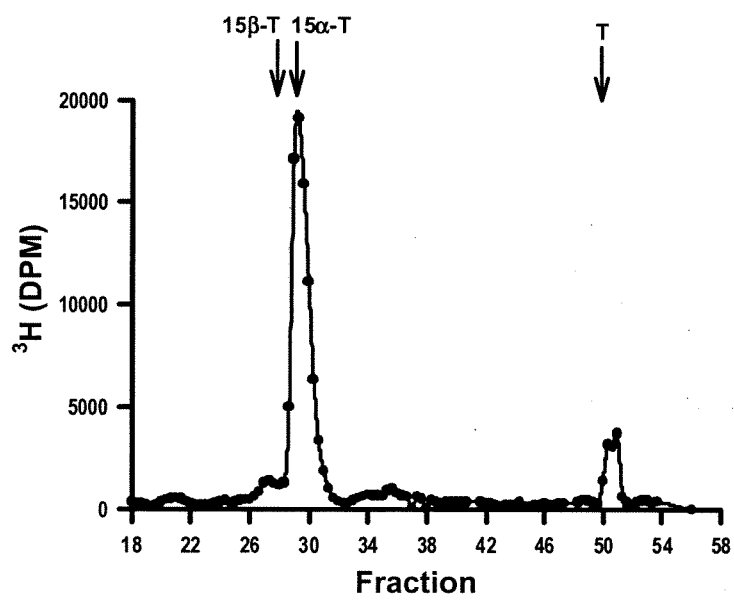
Figure 1

Figure 2

Figure 3
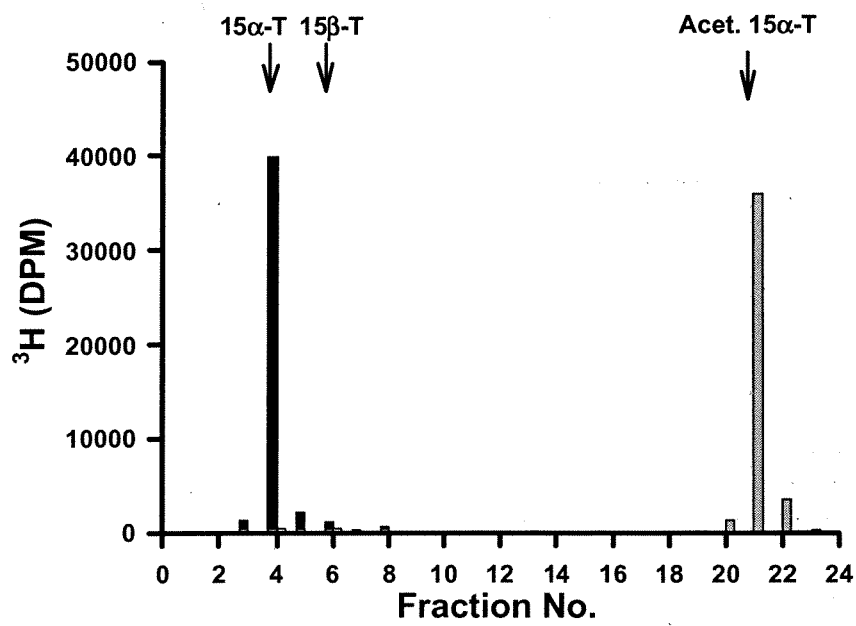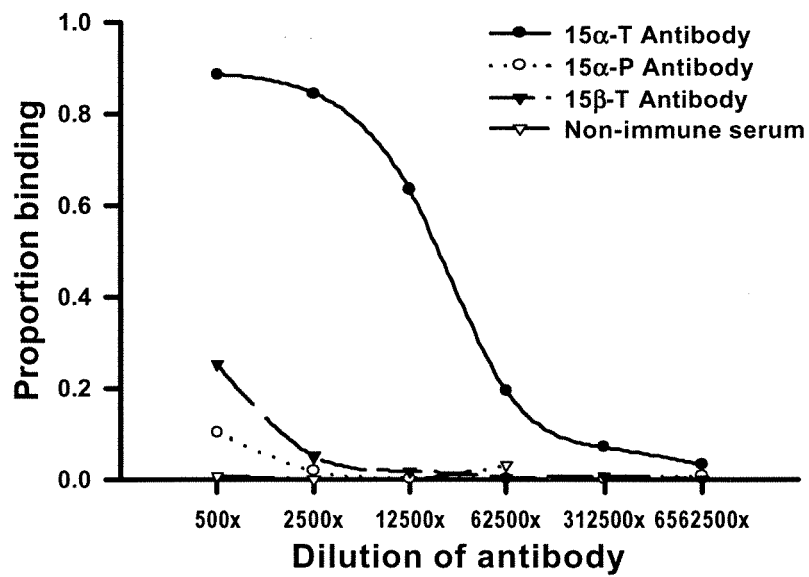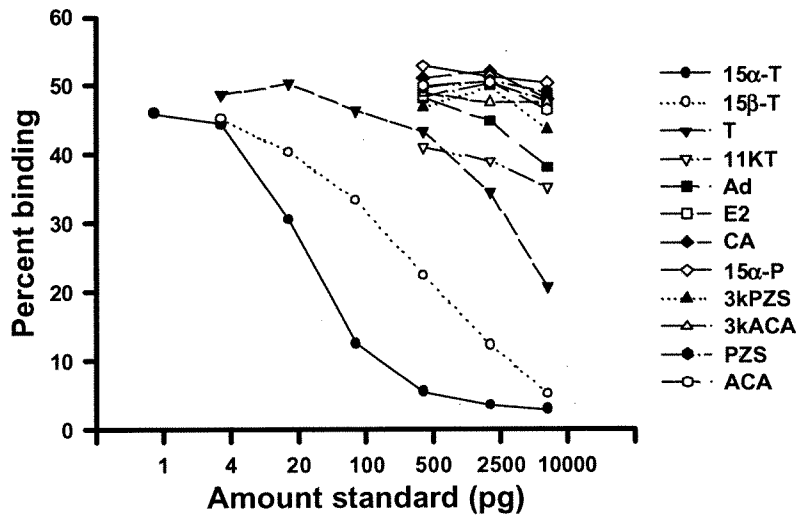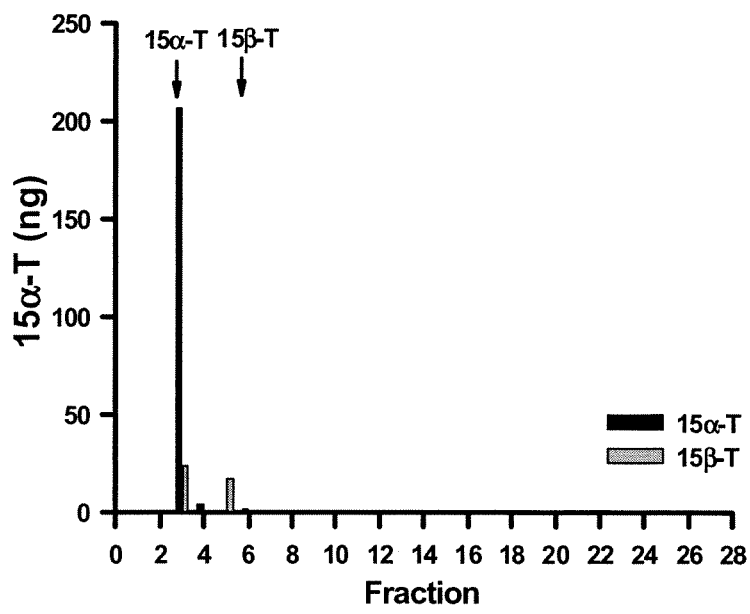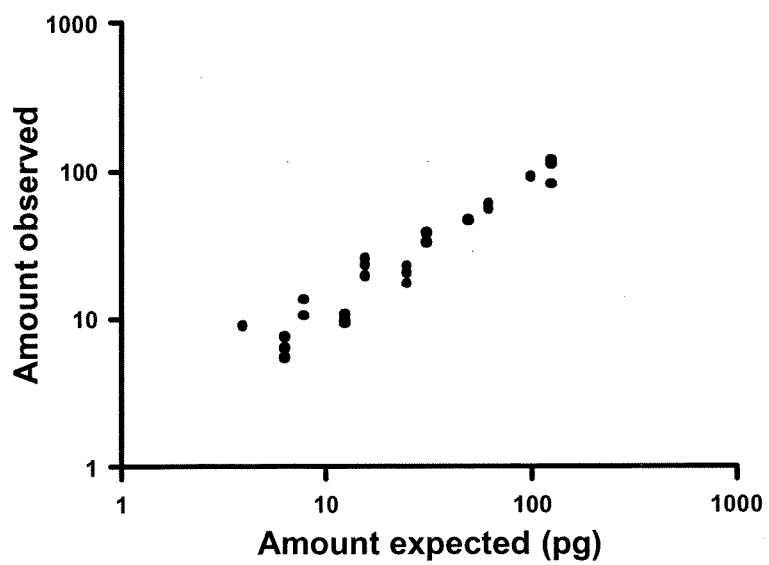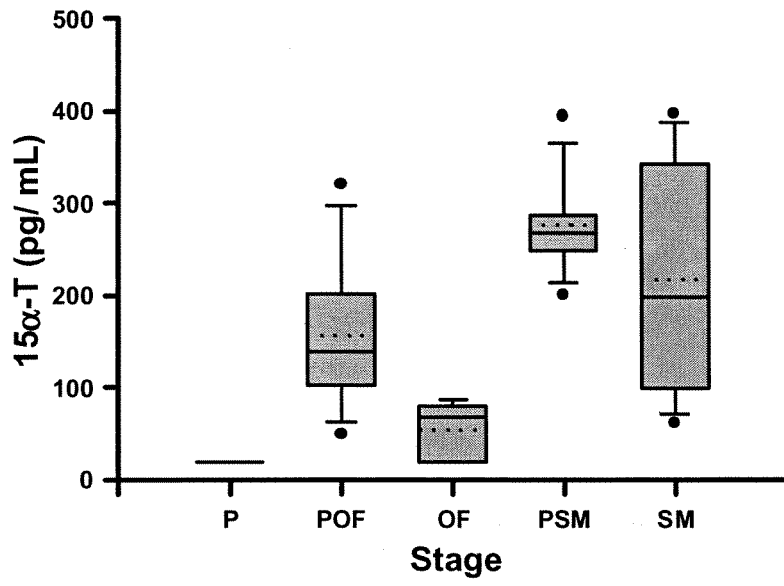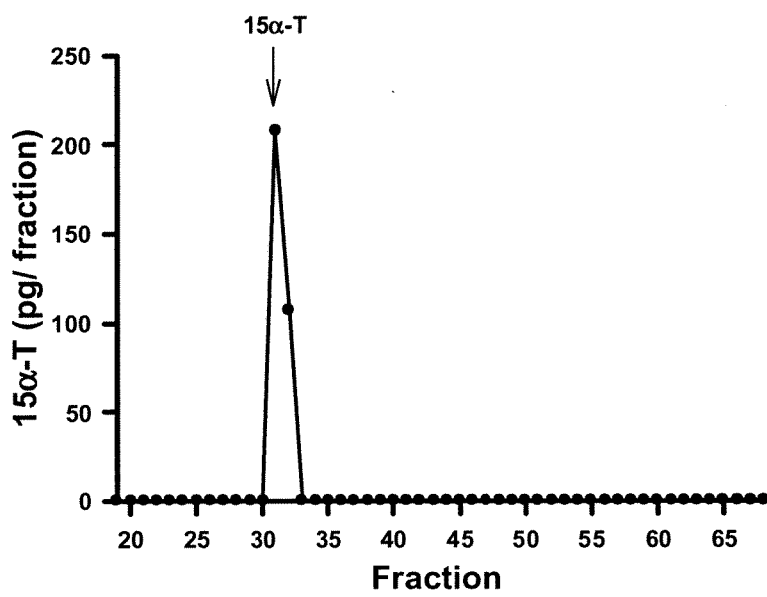
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Figure 4

Figure 5

Figure 6

Figure 7

Figure 8