GREAT LAKES FISHERY COMMISSION

2004 Project Completion Report[1]

Identifying and producing the sea lamprey migratory pheromone

by:

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April 5th, 2004

Completion Report for the Great Lakes Fishery Commission

Project Title: Identifying and producing the sea lamprey migratory pheromone.

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ABSTRACT

Previous field and laboratory studies conducted by the Sorensen laboratory and funded by the Great Lakes Fishery Commission (GLFC) have established that larval sea lamprey release a potent migratory pheromone that attracts migratory adults into streams for the purpose of spawning. These studies have found that this pheromone is comprised of the unique bile acid petromyzonol sulfate ('PS') and at least one other unidentified component with a molecular weight of 704 daltons ('704'). Our most recent (2003-2004) contract sought to characterize 704, determine its biological potency, and develop means to isolate it in bulk for field tests. We have good progress on all fronts. Several forms of mass spectrometry have succeeded in demonstrating that 704 has a mass of 704.3739 and two sulfates, suggesting a molecular formula of $C_{34}H_{60}N_2O_9S_2$. Preliminary analysis using nuclear magnetic resonance also suggests that this compound has a few aromatic or vinylic protons, several methine and or methylene protons, and several methyl groups. Efforts to identify this cue are progressing based on this information. Additionally, behavioral and electrophysiological studies have shown that 704 is active ($P<0.05$) by itself at concentrations ranging down to 10^{-14} Molar (approximately one gram in 100 billion liters of water). These experiments also elucidated the presence of a third previously unknown pheromonal compound with a molecular weight of 590 daltons ('590') which together with PS and 704 appear to account for the majority of the pheromonal activity of larval water. We are now in the process of identifying this cue. Lastly, XAD7HP resin has been found to quickly and easily extract PS, 704, and 590 from larval holding water with a high level of efficiency. This procedure has been developed and partially optimized for mass extraction so that extraction for field tests have been able to proceed.

Project Description and Objectives (paraphrased from the contract):

Field and laboratory studies have now clearly established that larval sea lamprey release a potent migratory pheromone which attracts migratory adults into streams for the purpose of spawning. Larval lampreys release this pheromone at a rate such that a single animal can activate at least 300 liters of river water in an hour. Further, a combination of behavioral, physiological, and biochemical studies have demonstrated that the pheromone is comprised of the unique bile acid petromyzonol sulfate ('PS') and at least one other unidentified component which the Sorensen laboratory isolated in 2002 and found to have a molecular weight of 704 daltons ('704'). The1-year contract (2003-2004) we report on here was designed to provide critical information and technologies needed to identify the pheromone and implement it in sea lamprey control. It had three objectives:

1. To characterize and identify a key unknown component ('704') in the larval lamprey migratory pheromone.
2. To determine the potency and role of 704 in the pheromone
3. To develop a method to extract large quantities of the entire pheromone from larval holding water at low cost.

Significant progress has been made addressing all three objectives and is discussed below.

Objective #1: To characterize and identify a key unknown component ('704') in the larval lamprey migratory pheromone.

Approximately 5,000 liters of larval holding water was collected from tanks of larval sea lamprey held at the Hammond Bay Biological Station (HBBS), extracted using XAD7HP resin (as described in Objective 3), and purified via two fractionation steps using two different methanol: water gradients by high performance liquid chromatography (HPLC) as described in the contract proposal. This process yielded approximately a milligram of partially pure 704 which was chemically characterized using 5 types of mass spectrometry (MS) as well as nuclear magnetic resonance (NMR). Briefly, these analyses, which are discussed in greater detail below, have shown that 704 has two sulfurs, a steroid-like structure and likely two nitrogen atoms. These findings are now being employed in ongoing structural analysis. We summarize a few of the key results from analyses conducted as part of our 2003-2004 contract below:

1) Negative ion electrospray ionization (ESI-MS). The negative ion ESI-MS of 704 yielded ions at 351, 703, and 725 m/z (Fig. 1). The isotope distribution peaks (at half mass intervals) for the first revealed that 351 is doubly charged, indicating that 703 is the $[M-H]^-$ ion. Careful analysis of the isotopomer ratios (especially of the M+2 intensity) further suggested that the compound contains two sulfur atoms. The presence of the 725 ion is consistent with an anion containing one sodium salt of a sulfate while the negative ion ESI-MS of PS (a model monosulfate) does not show an analogous $[M+22-H]^-$ ion. Together, these results suggest that 704 is a bis-sulfate or -sulfonate (Fig. 1)

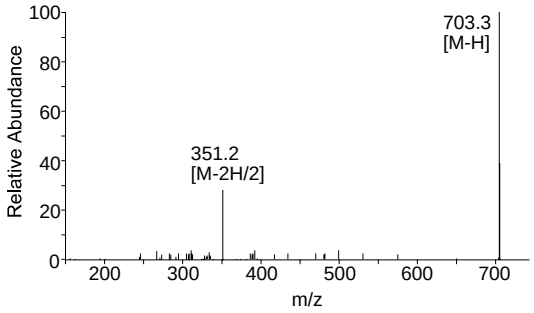

Fig. 1. Mass spectrometry of 704 using electrospray ionization with an ion trap in the negative ion mode. The [M-H]$^-$ ion can bee seen at 703.3 m/z; the dianion at 351.2 m/z. UNPUBLISHED DATA, PLEASE DO NOT CITE OR DISTRIBUTE

2) Negative ion electrospray mass spectrometry/mass spectrometry (MS/MS). When the 703.3 ion was collided with argon, it produced a dominant daughter ion at 605.5 (Fig. 2). The loss of 98 likely arises from fragmentation of sulfuric acid (H_2SO_4), and can be rationalized if the 703 ion contains: ^-O_3SO-R-OSO_3H or ^-O_3S-R-OSO_3H (i.e., bis-sulfate monoanion or mono-sulfonate/mono-sulfate monoanion).

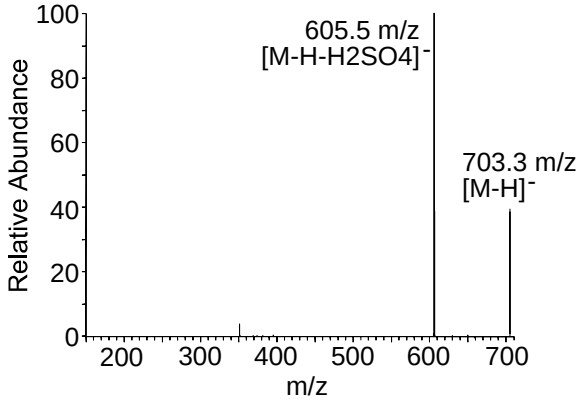

Fig. 2. Mass spectrometry/mass spectrometry of 704 using electrospray ionization with an ion trap in the negative ion mode at 30% collision energy. The [M-H]$^-$ ion is seen at 703.3 m/z; the fragment peak representing the loss of H_2SO_4 at 605.5 m/z. UNPUBLISHED DATA, PLEASE DO NOT CITE OR DISTRIBUTE

3) Positive ion electrospray ionization (ESI-MS). The positive ion ESI-MS yielded a positive ion at 705.4 m/z (data not shown). When the 705.4 ion was collided with argon, it produced a dominant daughter ion at 573.1 (data not shown). Interpretation of this fragment has not yet been possible although it will be useful in structural determination.

4) Matrix assisted laser desorption ionization time of flight (MALDI-TOF-MS).

The MALDI-TOF-MS in the positive ion mode was also informative. It showed two fragments. One with a molecular weight of 607 m/z reflecting the loss of one sulfate $[M+H-H_2SO_4]^+$, the other at 509 m/z reflecting the loss of two sulfates $[M+H-2H_2SO_4]^+$ (Fig. 3).

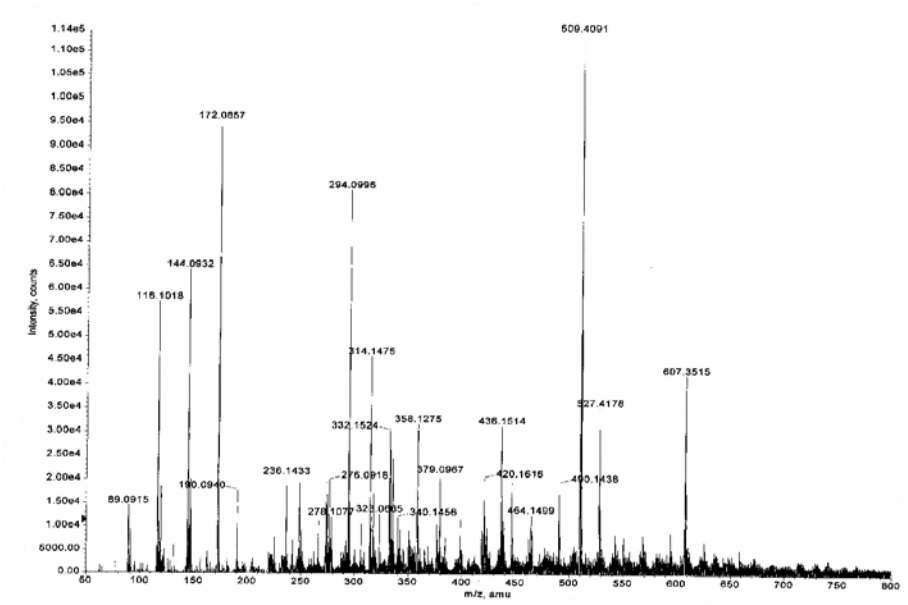

Fig. 3. Matrix assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF-MS) of 704 in the positive ion mode. UNPUBLISHED DATA, PLEASE DO NOT CITE OR DISTRIBUTE

5) Fourier transform ion cyclotron resonance (FT-ICR-MS).

High resolution data kindly collected by Dr. Muddiman at the Mayo Clinic, Rochester, MN allowed us to determine that 704 has a molecular mass of 704.3739, thereby permitting us to suggest it has a molecular formula of $C_{34}H_{60}N_2O_9S_2$ with an unsaturation number of 6. This work has since been confirmed using another high resolution mass spectrometer at the University of Minnesota (data not shown).

6) ^{1}H NMR of 704.

In addition to the MS work described above, we have obtained our first ^{1}H NMR spectrum of 704 (Fig. 4). Although interpretation of the structure at this point must be recognized as tentative because of the small amounts of compound employed and its modest purity, some suggestions can be made. Importantly, spectroscopically rich regions were in evidence. Briefly, there appeared to be few aromatic or vinylic protons (consistent with the lack of appreciable UV activity for active fractions of larval extract and with the relatively low unsaturation number (6) of the most likely formula), several methine and or methylene protons on sp^3-carbons bearing heteroatoms, and a complex array of resonances in the 1-3 ppm region. Much higher quality, and interpretable spectra are now being collected as part of our present contract and we are confident that we will be successful in identifying this compound.

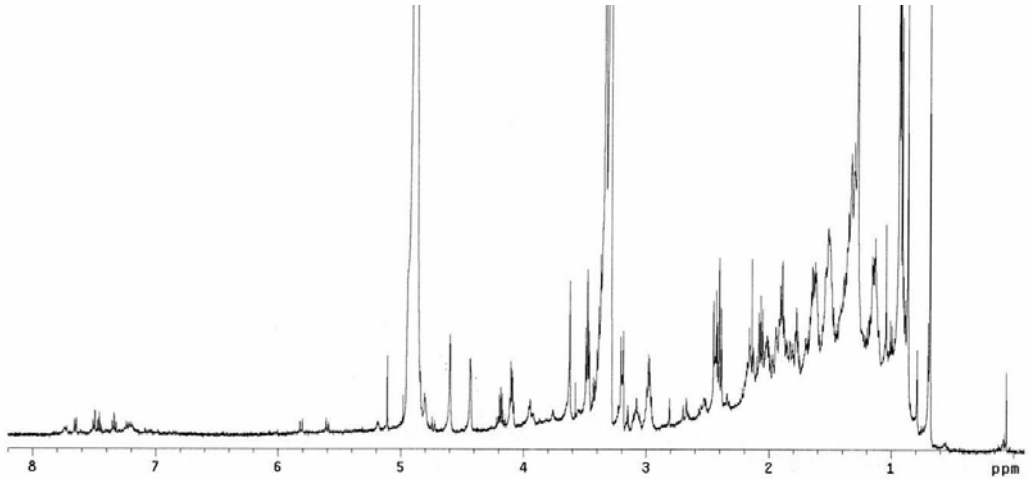

Fig. 4. Proton NMR of partially purified 704. It is clear that further purification is needed, however there appear to be few aromatic or vinylic protons, several methine and or methylene protons, a complex array of resonances in the 1-3 ppm region, and the presence of aliphatic methyl group resonances.
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Objective #2. To determine the potency and role of 704 in the pheromone.

704 was isolated from larval holding water as described above and aliquots quantified for use in behavioral and olfactory testing. In addition several other HPLC fractions were collected from larval holding water so that we could look for other pheromonal components that might exist in addition to 704. Samples of 704 were quantified by weighing purified compound on a microbalance (+/- 0.001 mg). This stock was also used to quantify larval water extracts using ESI-MS which found that the quantities of PS and 704 measured in samples increased in a linear, highly prescribed fashion. The olfactory and behavioral activity of 704 was greater than PS and perhaps the greatest described for a vertebrate pheromone. Key findings are described below.

1) Olfactory potency.

The olfactory activity of 704 was determined using electro-olfactogram recording (EOG), a technique which measures extracellular voltage transients from the olfactory epithelium which are thought to reflect receptor generator potentials. We followed protocols previously established by us for use on the sea lamprey (Li et al. 1995, Li & Sorensen 1997). Reponses to704 and PS were tested at concentrations ranging from 10^{-14} Molar (M) to 10^{-8} M and expressed relative to those elicited by a standard, 10^{-5} M L-arginine. The detection threshold to 704 was found to lie between 10^{-14} M and 10^{-13} M, about an order of magnitude lower than PS (Fig. 5).

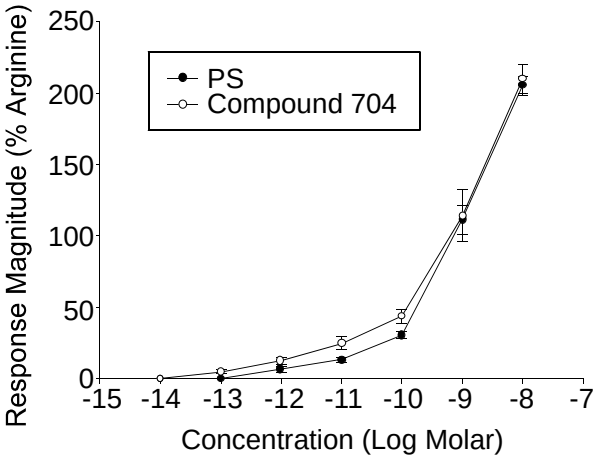

Fig. 5. Semi-log plots of the electro-olfactogram (EOG) dose-responses of adult sea lampreys (n=6) to Compounds 704 and petromyzonol sulfate (PS). Average responses are presented as a percentage of that elicited by the standard odorant, 10^{-5} Molar L-Arginine. Vertical bars represent one standard error. N=6 lampreys. UNPUBLISHED DATA, PLEASE DO NOT CITE OR DISTRIBUTE

To determine whether 704 is discriminated by independent receptor mechanisms in the lamprey olfactory system we also performed a simple cross-adaptation experiment. Saturating solutions of PS and 704 were introduced over the lamprey olfactory epithelium, and equipotent (10^{-10}M) concentrations of each odorant or 3keto-petromyzonol sulfate (3K-PS; the male sex pheromone) was then tested along with L-arginine control. When adapted to PS, responses to 704 were approximately 90% of normal (Table 1) while adaptation to 704 had little effect on responses to PS (Table 2). Clearly compounds 704 and PS stimulate totally independent receptor systems.

TABLE 1. EOG responses of sea lamprey adapted to PS; n=3 fish

Odorant	Average PUR	Standard Deviation
Adapting PS	0	00
3k-PS	59.5	10.6
704	88.7	18.2

TABLE 2. EOG responses of sea lamprey adapted to 704; n=3 fish

Odorant	Average PUR	Standard Deviation
PS	93	10.6
3k-PS	94	21.9
704	0	0

2) Behavioral potency.

Behavioral experiments found 704 to possess remarkable behavioral activity. The behavioral activity of known quantities of purified 704 and related fractions were tested in a pair of mazes at HBBS following established protocols (Vrieze and Sorensen, 2001). Briefly, lake water (100L/min) was pumped down each side of a 1.4 x 7m two-choice maze along with a small amount of water (1%) from Nagel Creek which lacked lamprey. Experiments were conducted after sunset using groups of 4 sea lamprey which were released from cages positioned in the upper ends of these raceways. Their distribution was noted using cameras for 15 min. Three experiments were conducted.

First, we tested 704 at concentrations ranging from 10^{-15} - 10^{-12} M against blank water (lake/creek) water to determine the threshold for behavioral activity. These experiments established that 704 was highly attractive on it own ($P<0.05$ two –tailed tests) down to a concentration of 10^{-14} M (Fig. 6). This result was repeated.

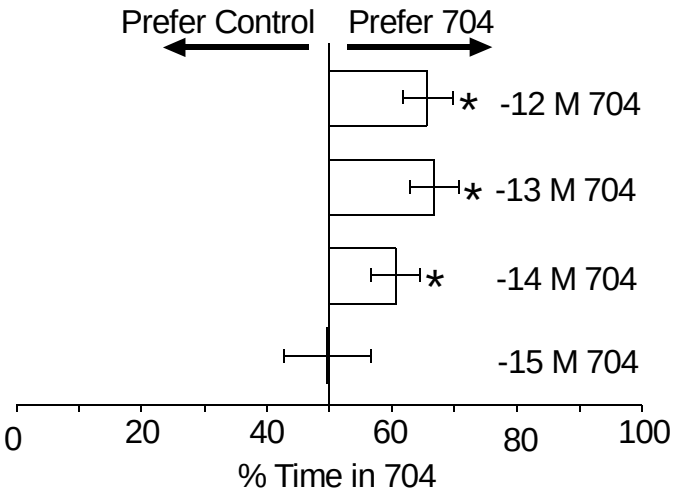

*Fig. 6. Mean percent time spent by adult sea lampreys in various log Molar (M) concentrations of purified 704 in a two-choice preference maze. The test was done twice at 10^{-14} M. N=13-14 groups of 4 lamprey for each test. Error bars are one standard error. Means were compared to a no-preference value of 50% using a student's T-test; * $p < 0.05$. UNPUBLISHED DATA, PLEASE DO NOT CITE OR DISTRIBUTE*

Second, we sought to determine whether a mixture of 704 and PS could account for all of the activity of larval water. To accomplish this, an aliquot of the XAD7HP pheromone extract of known concentration was tested against a mixture of PS and 704 in the HBBS raceways. This experiment demonstrated that although active, this mixture is not as active as whole larval extract (P>0.10; Fig 7.), suggesting that it does not constitute the complete pheromone. In an attempt to identify the 'missing' component(s), we combined adjacent Fractions # 9, 10,11 (Fraction 10 contained PS and 704) and tested this mixture against the whole pheromone extract. Notably, there was no significant difference (Fig. 7), suggesting that compounds found in these fraction do constitute the majority of the cue. Most recently (new contract), we have examined Fraction 9 and found a compound with a molecular weight of 590 which has strong olfactory and behavioral activity which we believe to be a key missing component. We are studying it as part of our ongoing GLFC contract.

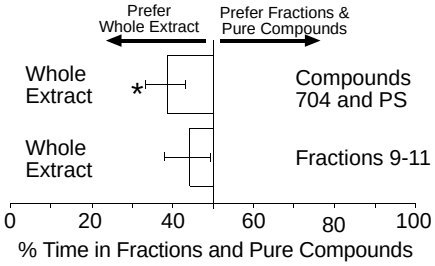

*Fig. 7. Mean percent time spent by adult sea lampreys in fractions and purified compounds when tested against whole larval extract in a two-choice preference maze. N=14 groups of 4 lamprey for each test. Error bars are one standard error. Means were compared to a no-preference value of 50% using a student's T-test; * $p < 0.05$. UNPUBLISHED DATA, PLEASE DO NOT CITE OR DISTRIBUTE*

Objective 3: To develop a method to extract large quantities of the entire pheromone from larval holding water at low cost.

To acquire enough pheromone for structural identification and field tests we have been developing methods to extract large amounts of the cue at low costs. These experiments have shown that XAD7HP resin can meet these needs. Key results are described below.

1) Confirming the ability of XAD7HP to extract the pheromone.

Ten liter aliquots of larval holding water from HBBS were extracted using a series of 5 XAD resins (Supelco; 75 ml/min), eluted with methanol and their activity tested against blank control. Only two worked well, XAD2000 and XAD7HP (Table 3). Because XAD7HP was easier to acquire we decided to pursue it further. Accordingly, XAD7HP extracts of larval holding water were compared directly against C18 (Sep-Paks; 1 L/hour) to determine if it extracted the entire pheromone as well as the latter procedure which we know from previous work to be 100% effective. We found XAD7HP to extract the entire pheromone (Fig. 8). Accordingly, we have recommended that HBBS use XAD7HP for their mass collection of pheromone extract for use in field trials. Presently, we are assisting them with this process.

TABLE 3. Results of tests screening for an XAD resin that extracts larval pheromone

XAD Resin	Larval water Extracted/test (l)	Sample size	Average Attraction	Standard Error	P-value t-test
XAD16HP	10	13	56.4	7.75	0.43
XAD2000	10	12	65.5	5.32	0.01
XAD2	50	13	48.9	6.34	0.84
XAD4	10	5	50.4	6.7	0.95
XAD7HP	10	14	65.8	4.99	0.007

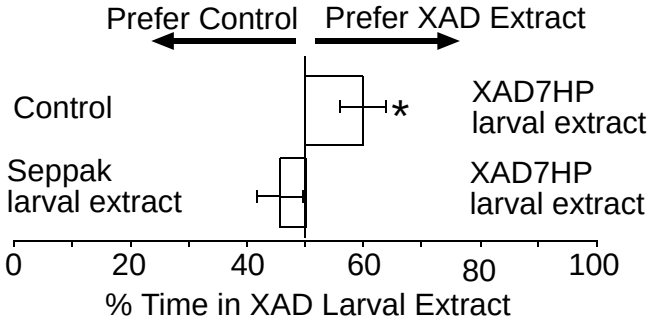

*Fig 8. Mean percent time spent by adult sea lampreys in larval holding water extracted with XAD7HP in a two-choice preference maze. N=12 or 13 groups of 4 lamprey. Error bars are one standard error. Means were compared to a no-preference value of 50% * $p < 0.05$. UNPUBLISHED DATA, PLEASE DO NOT CITE OR DISTRIBUTE*

2) Confirming the biochemical stability of pheromonal components.

To address the stability of the pheromone (a critical question for identification and field tests), a sample of larval extract (XAD7HP) was split into two sets of 5 aliquots. One of these was stored in the freezer at -25˚ C, the other maintained at room temperature (20˚ C). After one week, the content of PS, 704, and 590 in each was quantified by ESI-MS as described above. There was no measurable breakdown of PS and 590 at 20˚ C, but the quantity of 704 dropped by approximately 9% after a week at 20˚ C (data not shown). These experiments establish that 704 is stable enough that it can be shipped and used in the field with a little extra care (use of wet ice in coolers). More detailed information is presently being collected using NMR.

3) Optimizing XAD extraction for large scale use.

The prospect of collecting pheromone on a large scale for field application using extraction was a daunting task so we sought to optimize it. Initial efforts have focused on: a) how much pheromone can be extracted using a single column, b) how much methanol needs to be used to extract it; and c) how many times the resin can be re-used. We have addressed these issues and developed a practical method for HBBS to employ for mass-pheromone extraction. These issues are described below.

a) Determining how much pheromone an XAD7HP column could extract

To employ XAD7HP resin for extraction, HBBS needed to know exactly how much larval water could be extracted before the resin loses its efficiency. To establish this, 760 liters of larval holding water that held 4,500 larvae for 24 hours were passed through a large (1.3 M) XAD7HP column at a rate of 0.5 L/min. We collected 5 liter sub-samples of larval water both before filtering, and of the filtrate at the start and after 50, 75, and 100% of the water had passed through the column. These sub-samples were then extracted with C18 solid-phase extraction cartridges, eluted with methanol, and later analyzed by ESI-MS to determine if and when un-extracted PS, 704, and 590 started to 'break through' the XAD without being captured. This experiment was repeated three times so that means and standard deviations could be calculated. PS was not detectable in the filtrate while 704 and 590 were detectable in the filtrate at about 1% of the levels found in control, demonstrating that the pheromone did not break through the column (Fig. 9). HBBS now follows this protocol for their mass collection of pheromone extract for use in field trials during the spring of 2004.

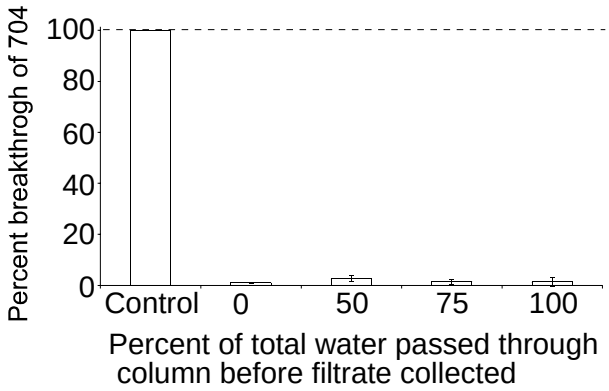

Fig. 9. Quantities of 704 which were not retained by XAD7HP extraction as increasing volumes of larval holding water were extracted. 704 was quantified by ESI-MS.

b) Determining the minimum amount of methanol to elute XAD7HP

XAD7HP needs to be eluted with methanol to reclaim the pheromone. Over the course of a year's extraction this potentially represents a large quantity of flammable methanol to ship, store, and concentrate. This experiment sought to determine the minimal amount of methanol needed to extract. We extracted three 760 L batches of larval holding water using the large XAD7HP column described above and eluted them with 4 L of methanol, collecting sequential 1 liter sub-samples of the eluate. These were then analyzed by ESI-MS. Approximately 90% of Compounds 704, 590 and PS were eluted in the first two liters, demonstrating that 4 L was not necessary (Fig. 10; data for 704 only). HBBS staff now uses 2.5 L methanol for column elution while collecting larval extract for field trials.

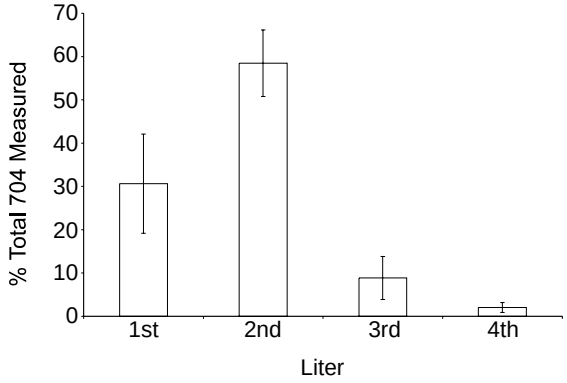

Fig 10. Quantities of 704 that were measured in each successive liter of methanol as XAD7HP resin was eluted. N=3 extractions. Bars are average % total 704 measured, error bars are one standard deviation. Results for PS and 590 were similar.

c) Determining how many times XAD7HP resin can be re-used.

Although XAD7HP is re-usable and our experience had shown that it can be re-used at least 5 times, it was highly desirable to know the limits so that resins can be changed when needed. Accordingly, we repeatedly extracted and eluted a single sample of 40 liters of larval holding water using a small XAD7HP column (40 cm length x 25 mm diameter), saving all the eluate after 1, 10, 20, and 40 elutions. The quantity of Compounds 704, 590 and PS in these samples was measured by ESI-MS to determine when pheromone extraction rates started to decline with re-use. This experiment was repeated 3 times. After 40 extraction/elutions, the ability of XAD7HP to extract Compounds 704, 590, and PS was not reduced (Fig. 11). Although it is likely XAD7HP can be used more than 40 times, we have instructed the staff at HBBS to replace the XAD7HP in their columns every 40 uses to be safe as XAD7HP is inexpensive and easy to obtain.

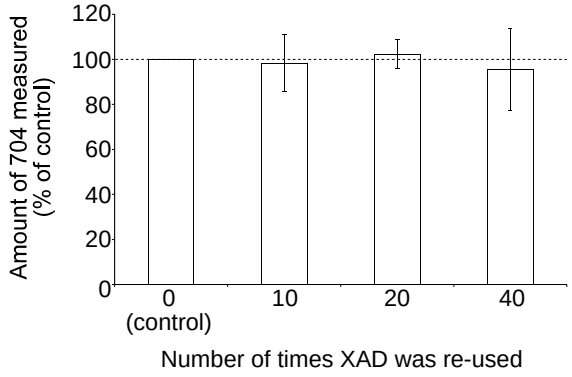

Fig. 11. A measure of the ability of XAD7HP to repeatedly extract 704 from larval holding water. Larval water was passed through XAD7HP either 10, 20 or 40 times, and the resultant 'used' XAD was than compared to new 'unused' XAD. The amount of 704 was quantified in each extract using ESI-MS and expressed as a percentage of 704 in the control. Bars are an average of 3 separate extractions, error bars are one standard deviation. Results for 590 were similar.

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Li, W., and Sorensen, P. W. 1997. Highly independent receptor sites for naturally occurring bile acids in the sea lamprey, *Petromyzon marinus*. *J. Comp. Physiol. A.* 180: 429-438.

Vrieze, L. A. and Sorensen, P. W. 2001. Laboratory assessment of the role of a larval pheromone and natural stream odor in spawning stream localization by migratory sea lamprey (*Petromyzon marinus*). *Can. J. Fish. Aquat. Sci.* 58: 2374-2385.

Presentations Given

Sorensen, P.W., Vrieze, L.A., and Fine, J.M. May, 2003. A multi-component migratory pheromone in the sea lamprey. Seventh International Symposium on the Reproductive Physiology of Fish. Mie, Japan.

Fine, J.M., and Sorensen, P.W. July, 2003. Partial identification of a multi-component migratory pheromone used by the sea lamprey. Chemical Signals in Vertebrates X. Corvallis, Oregon.

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Fine, J.M., Sherman, M., and Sorensen, P.W. January, 2004. The possible use of pheromones in invasive fish control: common carp & sea lamprey. Annual Retreat for the Minnesota Invasive Biology Research Consortium. Chanhassen, MN.

Fine, J.M., and Sorensen, P.W. March, 2004. Chemical Fractionation Demonstrates that the Sea Lamprey Migratory Pheromone is Comprised of Several Bile Acid-Like Components. (Poster). Annual Meeting of the Minnesota Chapter of the American Fisheries Society. St. Cloud, MN.

Publications

Sorensen, P.W., Vrieze, L.A., and Fine, J.M. 2004. A multi-component migratory pheromone in the sea lamprey. *Fish Physiology and Biochemistry* (in press).

Fine, J.M., and Sorensen, P.W. Biologically Relevant Concentrations of the Sea Lamprey Migratory Pheromone Measured in Stream Water. in Mason, R., Muller-Schwarze, D., and Lemaster, M. (eds.). Advances in Chemical Signals in Vertebrates. Kluwer Academic/Plenum Publishers, New York (in press).

Fine, J.M., Vrieze, L.A., and Sorensen, P.W. 2004. Evidence that Petromyzontid lampreys employ a common migratory pheromone which is at least partially comprised of bile acids. *Journal of Chemical Ecology*. Submitted.