GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

A putative male sea lamprey sex pheromone: its function, identity and potential application in the sea lamprey control

by:

Weiming Li, Ph.D.[2]

[2] Department of Fisheries and Wildlife
Michigan State University
East Lansing, MI 48824

January 2000

---

[1]Project completion reports of Commission-sponsored research are made available to the Commission's Cooperators in the interest of rapid dissemination of information that may be useful in Great Lakes fishery management, research, or administration. The reader should be aware that project completion reports have not been through a peer review process and that sponsorship of the project by the Commission does not necessarily imply that the findings or conclusions are endorsed by the Commission.

COMPLETION REPORT FOR GLFC RESEARCH

PROJECT TITLE

A putative male sea lamprey sex pheromone: its function, identity and potential application in the sea lamprey control

PRINCIPAL INVESTIGATOR AND AFFILIATION

Weiming Li, Ph.D
Department of Fisheries and Wildlife
Michigan State University

PROJECT DURATION

January 1, 1998 – December 31, 1999

January 31, 2000

A putative male sea lamprey sex pheromone: its function, identity and potential application in the sea lamprey control

Introduction

The principal objective of this study was to examine the function and identity of a potent odorant specifically released by spermiated male sea lampreys, and to determine whether this odor is a sex pheromone. We used the term putative pheromone because the function of this odor was to be determined in this study. The ultimate goal of this study is to develop new concepts and measures for sea lamprey control based on the premise that natural odorants could be used to alter or disrupt sea lamprey spawning behaviors or habitat selection. Several lines of evidence, from ecological, anatomical, behavioral and physiological studies, suggest that this strategy is feasible. At the spawning season, adult sea lampreys are congregated in a small portion of streams across the Great Lakes basin, and thus, can be targeted efficiently. These individuals have large and well-developed olfactory organs whose sensory epithelia are further enlarged with longitudinal folding (Kleerekoper 1972; Kleerekoper and van Erkel 1960; Thornhill 1967). Lamprey olfactory bulbs are exceptionally large compared to the size of their brain (Stoddart 1990). Further, functional studies on the lamprey olfactory sense demonstrate that this anatomically dominant system is indeed fundamental to prey searching, migration and mating of post-larval sea lampreys (cf. Kleerekoper and Mogensen 1959; 1963; Li et al. 1995; Li 1994; Teeter 1980). Clearly, a control strategy based on exploitation of the lamprey olfactory sense and odor-induced behaviors is likely to be effective, efficient and environmentally sound.

Mature male lampreys have long been suspected to release sex pheromones. In France, where lampreys are considered a delicacy, fishermen use mature male river lampreys to bait female lampreys to their basket traps (Fontaine 1938). When placed in a two-choice maze, mature females spent more time in the compartment where washings from sexually mature males had been introduced (Teeter 1980). This makes sense in the context of reproductive biology of sea lampreys because it is usually the male who initiates nest construction, a process joined by one or several females and males later (Applegate 1950). Recently, compelling evidence collected in electrophysiology studies demonstrated that spermiated male sea lampreys release a potent odor that could be a sex pheromone (Li 1994; Bjerselius et al. 1996). What was lacking, however, was direct evidence linking this odorant to a behavior or physiological action in conspecifics and knowledge of the chemical structure of the active component, or components.

Our primary hypothesis for this study was that the odorant released by spermiated male sea lampreys is a sex pheromone that modifies reproductive behaviors of conspecifics. To test this hypothesis, we proposed to examine the behavior responses of conspecifics to this odorant, and to characterize the molecular structure of its active component(s). Our results have confirmed that this odorant is a pheromone that

influences the behaviors of postovulatory females and suggests that its main component is a 472 Dalton steroid conjugated with a sulfate. These results will be reported in Part I and II, respectively. In addition, as a part of our initial effort to investigate the feasibility of applying this putative pheromone in the management of sea lamprey populations of the Great Lakes, we have confirmed that bisazir-sterilized and spermiated male sea lampreys release this odorant at a level that is virtually identical to that released by fertile spermiated males. These results will be reported in Part III.

Part I The Function of the Putative Male Lamprey Pheromone

Objective: Determine whether the odorant released by spermiated male sea lampreys elicit a behavior response from conspecifics.

Method: Preference/Avoidance Behavior Assays

Male and female adult sea lampreys were used as both test subjects and odor sources for behavioral tests. External characteristics were used to determine the sex of sea lampreys (Vladykov 1949). For each sex there were two sexual maturity classifications. For males, individuals that did not emit sperm after gentle pressure was applied to the abdomen were termed non-spermiated males. Males that did emit sperm were termed spermiated males. For females, individuals that did not release eggs after gentle pressure was applied to the abdomen were termed non-ovulated females. Females that did release eggs were termed ovulated females.

Mature male sea lampreys were induced to spermiate by holding them in separate tanks with 18 °C water and then injecting them intra-peritoneal with [D-Ala^6]-LH-RH(pGlu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2, Sigma Chemical Co., St Louis, MO USA) at a dose of 100 μg/Kg body weight (Li 1994). This injection was repeated in 48 hours and then again five days after the initial injection. Lampreys were checked every day until spermiation. Female lampreys were treated with the same LH-RH analog and at the same does to induce ovulation.

Preference tests were used to determine the attraction or repulsion behavior of sexually mature sea lampreys to odorants produced by conspecific adults of both sexes. In all, numerous tests were conducted representing all combinations of test subject and odor source. These tests were carried out using a two-choice (Y-maze) design (figure 1). Briefly, water was able to flow through these mazes, but sea lampreys were blocked from escaping by sealing the maze with flow-through plastic mesh. Odor chambers were located at the head of each side of the maze. The dimensions of each arm of a maze were identical. Flow was the same in each arm also. Two mazes were assembled in the raceways at LHBS (referred to as inside mazes). Another maze was constructed at a field site located on the Ocqueoc River, Presque Isle County, Michigan USA, a Lake Huron tributary (referred to as outside maze) to clarify responses to the spermiated male odor observed during inside tests. The Ocqueoc River is a historic sea lamprey spawning stream (Applegate 1950) in which a sea lamprey barrier was recently constructed to stop adults from migrating upstream to spawn. The absence of animals from our study area

assured that there was low background odor from other sea lampreys to interfere with our behavioral tests and yet offered water temperature and quality suitable for sea lamprey reproduction.

A single sea lamprey of a specific sex and maturity was chosen and introduced into the maze. The sea lamprey was allowed to acclimate for 10 minutes and then its behavior was video-recorded for 20 minutes. After this initial 20 minutes an odor from sea lampreys of a specific sex and maturity was introduced to one side of the maze chosen randomly by the toss of a coin. The odor was allowed to perfuse through the maze for 5 minutes, and the behavior of the lamprey was recorded again in the presence of the odor source for 20 minutes. The sea lamprey and odor source were then removed and the maze was allowed to flush free of odor for 10 minutes. Another test was then started. Each test cycle lasted about 65 minutes from acclimation to the end. Most tests were conducted between 0700 and 1700 hours in water temperatures that ranged from 7°C to 20°C for inside tests and 12°C to 24°C for outside tests. Videotapes were analyzed and the amount of time spent in the experimental side (side with odor) and the control side (no odor) of the maze before and after the odor was introduced was tallied. The variables for these values are as follows; B_e and B_c are the time in seconds spent in the experimental side and the control side before odor introduction, A_e and A_c are the time spent in each side after odor introduction respectively. Proportions of time spent before and after the odor was introduced were calculated for the experimental and control sides of the maze. The difference between the experimental proportion and the control proportion determined preference.

$$(A_e/B_e) - (A_c/B_c) = \text{Index of Preference}$$

A positive value indicated an overall attraction and a negative value indicated an overall repulsion for a single test. These proportions were set up to take into account the behavior before odor introduction. The assumption behind this analysis was that behavior wouldn't change from the first 20-minute recording period to the second 20-minute recording period if no odors were introduced, or if the behavioral response was neutral. Tests in which lampreys did not explore both sides of the maze in the 20 minutes before odor introduction and also tests in which lampreys did not explore both the left and right sides of the maze in the 20 minutes after odor introduction, were not used in analysis. This was done because proportions could not be generated from these situations. These situations occurred in approximately 25% of all tests. Actual fish (5 individuals used per test) and water in which lampreys were held (referred to as washings) were used for odor sources. A sample size of 12 was attempted for each test subject and odor source combination for the inside maze and a sample size of eight was attempted for the outside maze. When all tests were completed they were recorded in a spreadsheet according to test subject and odor source combination. A two-tailed Wilcoxon signed ranks test (calculated by hand, description in Rao 1998) was used on differences generated from the proportions of time spent in each arm of the maze before and after odor induction to determine the behavioral preference of sexually mature sea lamprey to odorous of conspecifics.

Results: Preference/Avoidance Behavioral Assays

Preference tests using the inside two-choice mazes constructed at LHBS were used as controls to define the behavioral preference responses of all test subject and odor source combinations (table 1). These tests showed that 14 of 14 ovulated female sea lampreys spent significantly more time in the experimental side of the maze containing spermiated male odor when actual lampreys were used as the odor source (P=0.0001). Preference responses were not observed in the other test combinations with three exceptions. First, 8 of 14 ovulated females tested spent more time in the side of the maze with ovulated female odor (P=0.05). Second, 15 of 18 spermiated males tested spent more time in the side of the maze with ovulated female odor (P=0.048). Lastly, 10 of 11 ovulated females tested spent more time in the control side of the maze (no odor) when exposed to non-spermiated male odor (P=0.002). Notably, 6 of 8 ovulated females spent more time in the side of the maze with spermiated male odor when washings were used as an odor source even though the statistical outcome was not significant.

Preference tests using the outside two-choice maze constructed on the Ocqueoc River were used to confirm the effect of spermiated male odor on adult sea lamprey and also the effect of non-spermiated male odor on ovulated females (table 2). These tests showed that 8 of 8 ovulated female sea lampreys spent significantly more time in the experimental side of the maze when actual spermiated male sea lampreys were the odor source (P=0.008). Also, 7 of 8 ovulated females tested spent more time in the side of the maze containing spermiated male odor when washings were used as the odor source (P=0.04). No other test subjects showed this same behavior towards the spermiated male odorant.

Methods: Searching Behavior Assays

The two-choice maze was also used to assess the effect of male odor on searching behavior of sexually mature sea lamprey. Searching behavior is defined as actively swimming at the head end of one arm of the maze. Characteristics of this behavior are pacing back and forth across the upstream block, increased swimming intensity and the rapid beating or vibration of the tail as the lamprey tries to overcome the upstream block. The videotapes of preference tests using spermiated males as an odor source and also those testing ovulated females with a non-spermiated male odor source were analyzed for this kind of behavior. The time spent involved in this activity was tallied for each side of the maze before and after odor introduction. Proportions and the differences were set up like in the preference test analysis to determine on which side the test subject spent more time actively searching. Again, tests in which lampreys did not show searching activity in both sides of the maze in the 20 minutes before odor introduction and also tests in which lampreys did not show searching activity in both the left and right sides of the maze in the 20 minutes after odor introduction, were not used in analysis. The differences were analyzed using a two-tailed Wilcoxon signed ranks test (Rao 1998) to determine whether there was significantly more searching activity taking place in the side of the maze with the spermiated male odor.

Results: Searching Behavior Assays

The videotapes of preference tests conducted in the inside and outside (table 3) mazes using spermiated males as an odor source and also those testing ovulated females with a non-spermiated male odor source were analyzed looking for this kind of behavior. Results showed that 7 of 7 (inside mazes) and 8 of 8 (outside mazes) ovulated female sea lampreys tested showed a significant increase in searching behavior in the side of the maze with spermiated male odorant (P=0.008). All other test subjects did not show similar searching behavior activity increases.

Methods: Radio-telemetry Tracking of Lamprey Behaviors in Spawning Streams

To assess preference in a natural setting ovulated and non-ovulated female sea lamprey behavior in response to spermiated and non-spermiated male odorant was observed in a known spawning stream. Tests were conducted between 0700 and 1700 hours in a water temperature between 12°C and 24°C. A 65 meter section of the Ocqueoc River was blocked off. This stream was chosen again for the reasons stated earlier. At the upstream portion of the study section, an island naturally divides the river (figure 2). Cages (1 m^3) for odor sources constructed with a wood frame encased in plastic mesh (~1.5 cm gap) were placed in the channels on each side of the island. A block net (~1.5 cm gap) was placed at the downstream end of the section to keep lampreys from escaping downstream. An acclimation cage (0.5 m^3) for test subjects constructed with the same material was placed at the downstream end.

A day in advance of testing a female sea lamprey (ovulated or non-ovulated) was fitted with a radio tag designed for external mount (Advanced Telemetry System, Isanti, Minnesota USA). The following morning sea lampreys being used for odor sources and the tagged female test subject were transported to the study site. The odor sources were placed in their respective cages at the upstream end of the site. Which side the odor sources were placed was chosen randomly by the toss of a coin. Ovulated female or non-ovulated female sea lampreys were the test subjects and spermiated male and non-spermiated male sea lampreys were used as the two odor sources. For both types of test subjects, one odor source was placed on one side of the upstream island and one on the other side. Simply, the test subject could choose to swim to spermiated males, non-spermiated males or none at all. The tagged lamprey was then placed in the acclimation cage and the downstream block net was installed.

The test subjects were allowed to acclimate, exposed to the two odor sources for two hours. The test subject was then released and its location was observed visually or by a radio receiver (Lotek Engineering Inc., Newmarket, Ontario, Canada) and recorded on a map grid of the site every five minutes. If test subjects failed to move from the release site within an hour they were removed. If tests subjects did move from the release site they were observed until (1) they reached an odor source and stayed there for an hour, (2) they swam past the odor sources, or (3) it was the end of the day (which usually was 4 hours from the start of the test). A contingency table was used to tally the behavior of female test subjects of different maturation. Categories of response consisted

of swimming to the spermiated male odor source, swimming to the non-spermiated male odor source, or not choosing an odor source (staying at an intermediate position within the stream section). A Fisher's exact test was used to analyze the data with SAS (1998 SAS Institute Inc., Cary, North Carolina USA).

Results: Radio-telemetry Tracking

This tracking study showed that the odorants released by spermiated male sea lampreys influenced the behavior of ovulated females. For the 13 ovulated female sea lampreys tested, 9 swam to, and then stayed at the cage containing spermiated males, 2 stayed at an intermediate position in the study area, and 2 did not move upstream from the acclimation cage. No ovulated female sea lampreys swam to the cage containing non-spermiated males. Among the 7 non-ovulated female sea lampreys tested, 1 swam to the cage containing spermiated males, 2 swam to the cage containing non-spermiated males, 3 stayed at an intermediate position in the study area, and 1 did not move upstream from the acclimation cage. A contingency table was set up to display and analyze the behavior of ovulated and non-ovulated female sea lampreys within the section of spawning stream (table 4). A Fisher's exact test indicated a significant difference in the choice of ovulated female and non-ovulated female sea lampreys on the side scented by spermiated male lampreys (P=0.024).

Conclusion

Ovulated female sea lampreys appear strongly attracted to odor from spermiated male sea lampreys. However, ovulated females did not show a significant preference for the odor of non-spermiated male and non-ovulated female sea lampreys. Also, even though statistically speaking ovulated females preferred the odor of ovulated females, only 8 of the 14 tested actually chose the side of the maze containing the odor. Spermiated males thus seem to secrete an odor that makes them attractive for ovulated females. This attraction response seems to be mediated by an increase in searching behavior brought about by exposure to the spermiated male odor. In addition, the amount of odorant released by spermiated male is enough to attract ovulated female sea lampreys in a natural spawning stream. Taken together, it is evident that spermiated male sea lampreys release a potent sex pheromone that specifically induces an increase in searching activities of ovulated females, a behavior that ultimately lead ovulated females to the source of this odorant, the spermiated males.

Part II Structural Characteristics of the Putative Pheromone

Objective: Extract and fractionate the spermiated male odor and to analyze the structure of the active components of this odor.

Methods

Washings from spermiated males were collected by holding spermiated males individually in buckets with 10 L of water for 4 hours, filtered with No. 3 Whatman filter

paper, extracted with C18 solid phase extraction (SPE) cartridges (Waters, Milford, MA) which was activated with 100% methanol. The cartridges were subsequently washed with water and eluted with methanol. The extracts were directly subjected to Fast Atom Bombardment Mass Spectrometry (FABMS) analyses at both negative and positive ionization modes by Dr. Douglas Gage, Department of Biochemistry and Michigan State University-National Institute of Health Mass Spectrometry Laboratory.

Crude extracts of spermiated male washings were also fractionated using a Gilson HPLC system, and later with a Waters HPLC system. Extracts were evaporated under a stream of nitrogen at 45 ^{o}C, reconstituted in 100 µl acetonitrile /water/trifluoroacetic acid (28/72/0.01; v/v/v) and then loaded onto an analytical reverse-phase HPLC column (Rainin Dynamax Microsorb; 5 µm octadecylsilane; 4.6mm x 25 cm; fitted with a 1.5 cm guard module). Two pumps were used to deliver solvents through the column at a rate of 0.5 ml/min. Solvent A was 0.01% Trifluoroacetic acid (TFA) in distilled water and solvent B was 70% acetonitrile and 0.01% TFA in distilled water: 0-10 min, 28.6% B; 10-60 min, 28.6-100% B; 60-80 min, 100% B; 80-82 min, 100-28.6% B; 82-100 min, 28.6% B. One-minute fractions were collected between 20 and 70 min. The fractions were subjected to FABMS and electro-olfactogram recording (EOG).

The olfactory potency of HPLC fractions was examined with EOG, which measures summated generator potentials of the olfactory neurons. The recording was conducted according to the procedures established by Li et al. (1995; 1997). Briefly, animals were anaesthetized with metomidate hydrochloride (Syndel, Vancouver, Canada, 3 mg/kg body weight), immobilized with Gallamine triethiodide (Sigma Chemical Co., 150 mg/kg body weight), and secured to a stand in a flow-through trough with water supplied to the gills. The olfactory lamellae were exposed. Differential electrical responses between the surface skin and the sensory epithelia were recorded using two Ag/AgCl electrodes.

Results

The FABMS analyses showed that the most abundant molecule in crude extracts of spermiated male sea lamprey washings has a molecular weight of 472 Dalton. This component is absent from crude extracts of other lampreys (figure 3). MS/MS analyses also confirmed that this molecule has two hydroxyl groups and is conjugated with a sulfate ester (figures too numerous to show here, but will be available upon request). When these extracts were fractionated with a Gilson HPLC system, fraction 46 was found to have the highest olfactory potency (figure 4). The retention time of this fraction coincided with that of steroids conjugated with sulfate. Subsequent FABMS analyses also found that this fraction contained mainly the same 472 Dalton molecule that is unique to extracts of spermiated male washings (figure not shown).

Conclusion

The male sea lamprey sex pheromone appears to be composed of mainly a conjugated steroidal molecule of 472 Dalton.

Part III Release of Sex Pheromone by Sterilized Male Sea Lamprey

Objective: Determine whether spermiated bisazir-sterilized male sea lampreys also release the putative pheromone.

Methods

Mr. Mike Twohey of the US Fish and Wildlife Service, Marquette Biological Station sterilized the sea lampreys. Briefly, bisazir (P,P-bis(1-azirindinyl)-N-methylphosphinothioic amide; Chemicals for Cancer Research, Burnsville, Minnesota) was dissolved in a 0.9% saline solution and administered intraperitoneally at a dosage of 100 mg/kg with an "auto-injector" produced by (Torben Rod, SPINO I/S, Gronlandsvej, Denmark). The injector positions the lamprey for injection at a point 46 percent of total length from the head. Lampreys will be held in 1,300 L tanks (water replenish rate: 23 l/min; 6 ^{0}C) for 48 hours after injection so that no detectable amounts of parent bisazir are present in tissue (Allen and Dawson 1987). Sterilized animals were subsequently injected with LH-RH analogs as described in Part I to induce spermiation.

Washings from spermiated males were collected as described in Method section of Part II, and immediately frozen at –80 °C for later analyses. EOG recordings were used as a bioassay to assess the potency of and compare odors from sterile spermiated males and non-sterile spermiated males to the olfactory organ of conspecifics. The recording procedure for this was the same as described in Part II. The results were analyzed with a paired Student t test. Also, we conducted preliminary experiments to assay the possible preference/avoidance responses of ovulated female sea lampreys to the sterile spermiated male odorant. These preference/avoidance experiments were conducted as described in the Method: Preference/Avoidance Behavior Assays section of Part I. In, addition extracts from sterile spermiated male washings were subject to FABMS to assess similarities and differences compared to extracts from non-sterile spermiated male washings.

Results

EOG results showed that the odorant released by sterile spermiated males induced responses from the olfactory epithelium of conspecific sea lampreys at a level that was virtually the same as those induced by non-sterile spermiated male odorant (figure 5, $P>0.10$). Also, Preference/avoidance behavioral assays showed that 5 of 5 (inside mazes) and 4 of 4 (outside maze) ovulated female sea lampreys spent more time in the side of the maze containing the sterile spermiated male odor. In addition, FABMS results showed that the most abundant molecule in crude extracts of sterile spermiated male sea lamprey

washings has a molecular weight of 472 Dalton, the same as extracts from non-sterile spermiated males.

Conclusion

Bisazir sterilized spermiated male sea lampreys appear to release the putative sex pheromone at a level that is virtually identical to that of non-sterile spermiated males. Behavioral results show that sterile spermiated males also elicit an attraction response from ovulated female sea lampreys. These results are virtually identical to results seen from using odors from non-sterile spermiated males. Extracts of sterile spermiated male washings appear to contain the same 472 Dalton molecule as extracts from non-sterile spermiated male washings. This suggests that the sterilization process doesn't effect the biosynthesis of this pheromone in male sea lampreys.

References

Applegate, V. C. and Smith, B. R. 1950. Sea lamprey spawning runs in the Great Lakes. U.S. Fish Wild. Serv. Spec. Sci. Rep. Fish. no. 61.

Applegate, V.C. 1950. Natural history of the sea lamprey (*Petromyzon marinus*) in Michigan. U.S. Fish Wild. Serv. Spec. Sci. Rep. Fish. Serv. 55: 237.

Bjerselius, R. Li, W. et al. 1996. Spermiated Male Sea Lamprey Release A Potent Sex Pheromone. Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish, editors. P. Thomas and F. Goetz, Austin, TX, pp 271.

Fontaine, M. 1938. La lamproie marine. Se peche et son iconomique. Bull. Soc. Ocianogr. Fr. 17: 1681-1687

Kleerekoper, H. 1972. The sense organs. In: The Biology of Lampreys, vol. 2, edited by: Hardistry, M. W. and I. C. Potter, Academic Press, New York, pp.373-404.

Kleerekoper, H. & Mogensen, J. 1959. The chemical composition of scent of fresh water fish with special references to amines and amino acids. Zeitsch. Vergl Physiol. 42: 492-500.

Kleerekoper, H. and Mogensen, J. 1963. Role of olfaction in the orientation of *Petromyzon marinus*. I. Response to a single amine in prey's body odor. Physiol. Zool. 36: 347-360.

Kleerekoper, H. and van Erkel, G. A. 1960. The olfactory apparatus of *Petromyzon marinus* L. Can. J. Zool. 36: 347-360.

Li, W. 1994. The olfactory biology of adult sea lamprey (*Petromyzon marinus*). Ph. D thesis. University of Minnesota.

Li, W. and Sorensen, P. W. 1997. Four independent olfactory receptor sites for bile acids, putative migratory pheromones, in the adult sea lamprey (*Petromyzon marinus*). J. Comp. Physiol. A. 180(4): 429-438.

Li, W. et al. 1995. The olfactory system of migratory adult sea lamprey (*Petromyzon marinus*) is specifically and acutely sensitive to unique bile acids released by conspecific larvae. J. Gen. Physiol. 105: 567-587.

Rao, P. V. 1998. Statistical Research Methods in the Life Sciences. Brooks/Cole Publishing Company, Pacific Grove, California. pp. 193-197.

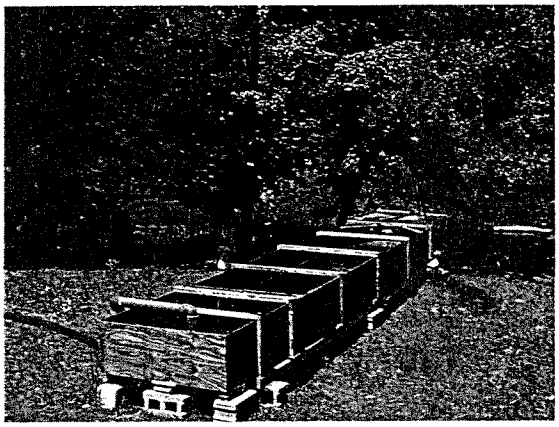
Stoddart, D. M. 1990. The scented ape: the biology and culture human odor. Cambridge University Press. Cambridge.

Teeter, J. 1980. Pheromone communication in sea lampreys (*Petromyzon marinus*): Implications for population management. Can. J. Fish. Aquat. Sci. 37: 2123-2132.

Thornhill, R.A. 1967. The ultrastructure of the olfactory epithelium of the lamprey *Lampetra fluviatilis*. J. Cell Sci. 2: 591-602.

Vladykov, V. D. 1949. Quebec lampreys. 1.-List of species and their economical importance. Dept. Fish., Prov. of Quebec, Contrib. 26. 67 pp.

List of Figures



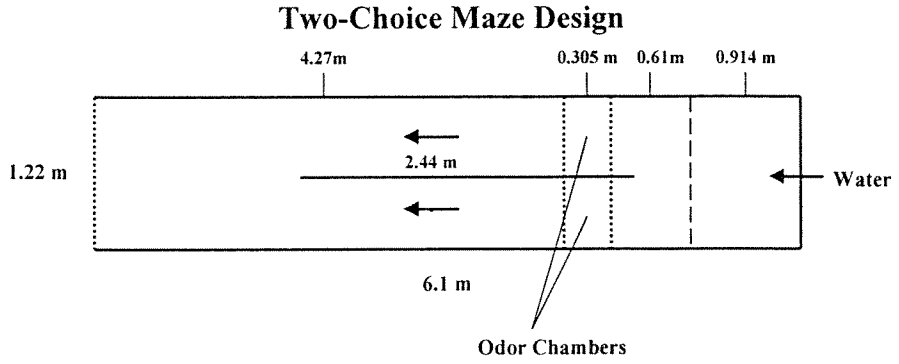


Figure 1. Experimental apparatus (two-choice maze) used to examine the behavioral responses of adult sea lampreys to pheromones. The top figure shows the actual device and its arrangement in experimental sites. The bottom figure illustrates the design of the device. The dotted lines represent meshes, solid lines represent wooden board and the dashed line represents a laminar flow device. Odor donors are placed in the odor chambers. Test subjects are released in the down stream part of the test area and their behaviors recorded with a video camera. Water flow is indicated by the arrows.

Figure 2. Section of the Ocqueoc River used in radio-telemetry tracking experiments. Shown is the upstream portion of the study section including an island that divides the river and the odor cages used in experiments. Ovulated and non-ovulated female sea lampreys were fitted with radio tags and their behavior monitored in response to spermiated and non-spermiated male odors.

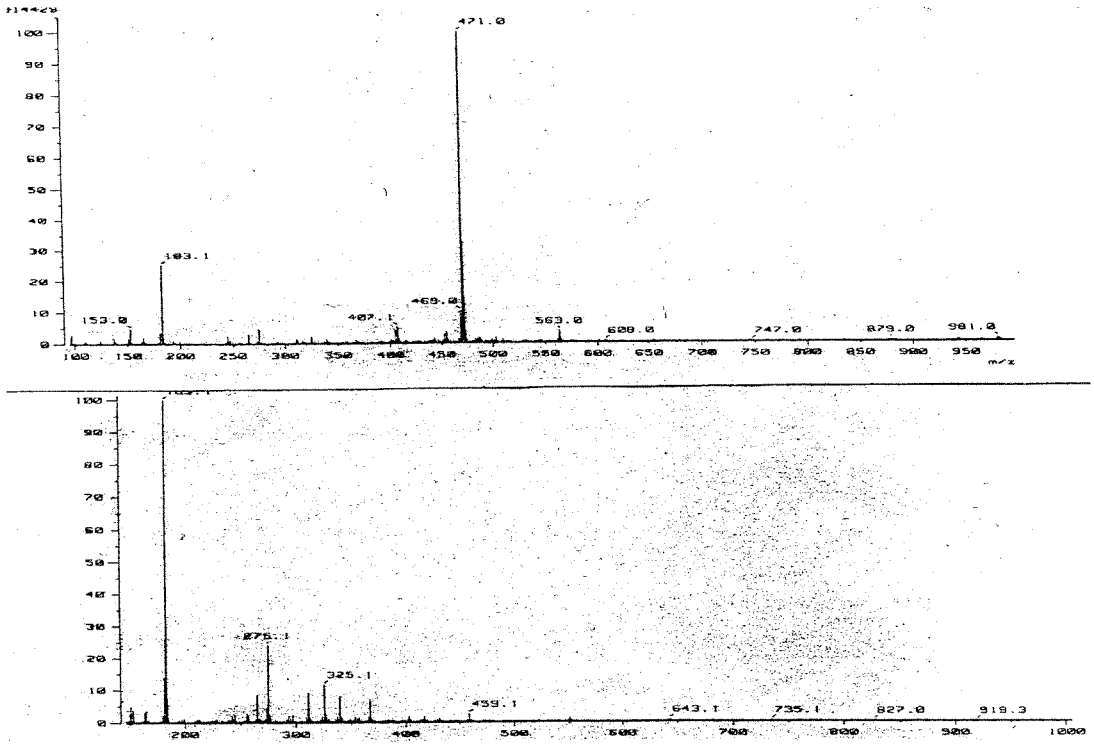


Figure 3. Fast Atom Bombardment Mass Spectrometry to show that the 472 Dalton molecule (m/z value of 471) is the most abundant molecule in extracts of spermiated male washings (top graph) but is absent from the non-spermiated male washings (bottom graph). Please notice the difference in scale of the glycerol peak (matrix; m/z value of 183) which could serve as a reference for relative quantities.

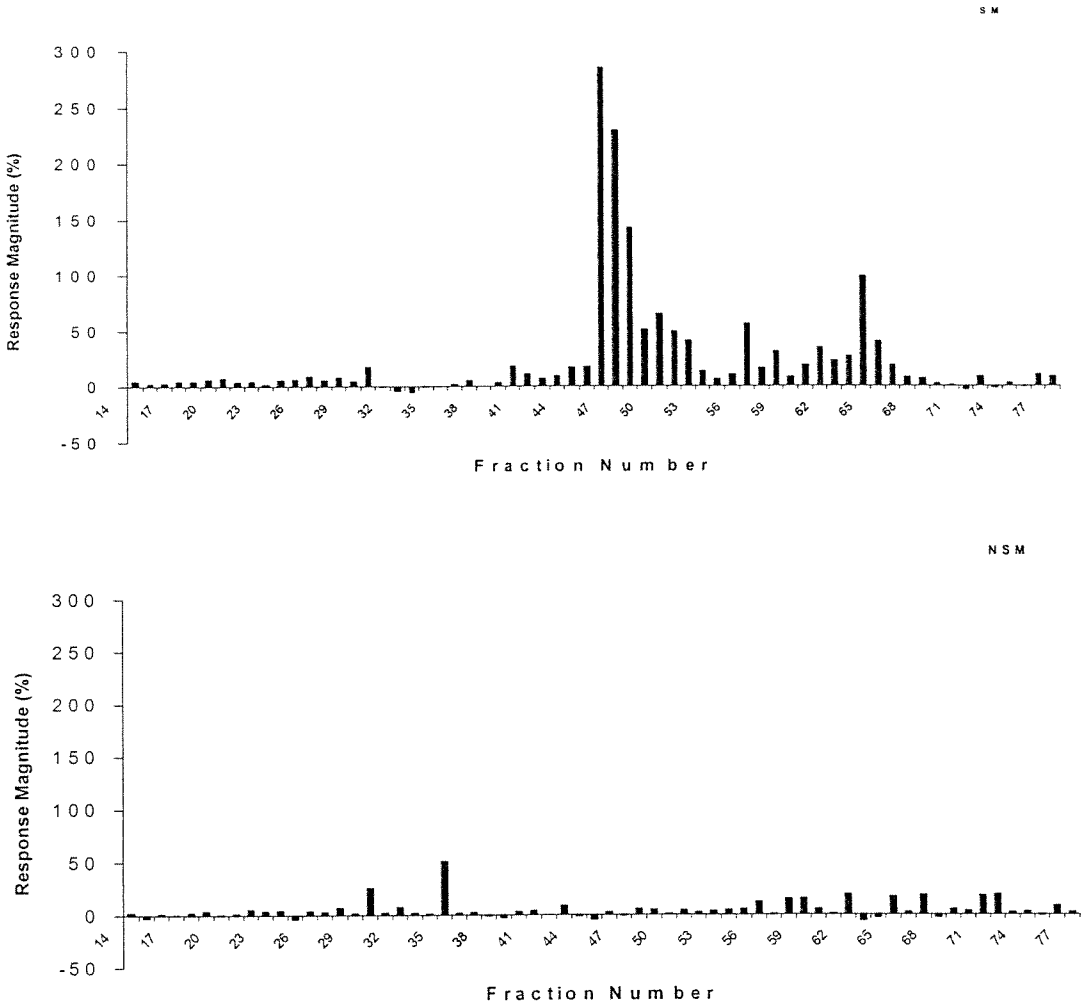


Figure 4. The Olfactory potency, as measured by electro-olfactogram recording, of extracted washings from spermiated (top figure) and non-spermiated (bottom figures) males fractionated with a HPLC using a preparative C-18 column. The response magnitude is the average of responses measured on 2 animals and is represented as a percentage of that of the standard stimulant, 10^{-4}M L-arginine. SM: fractions of extracted spermiated male washings. NSM: fractions of extracted non-spermiated male washings.

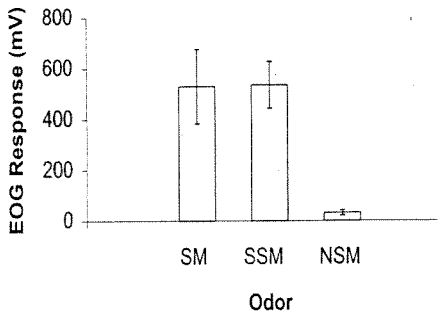


Figure 5. Olfactory responses to spermiated, sterile spermiated and non-spermiated male washings. Washings from the spermiated males (SM) and sterile spermiated males (SSM) elicited EOG responses from ovulated females at virtually the same magnitude. These responses are larger than that to non-spermiated males (NSM). Vertical bars, one standard error. Sample size, 3.

List of Tables

Inside Raceway Behavioral Tests

Odor Source	Test Subject	n	Experimental Side	Control Side	p value
SM	OF	14	14	0	0
SM	NOF	16	4	12	NS
SM	SM	12	6	6	NS
SM	NSM	11	6	5	NS
SMW	OF	8	6	2	NS
NSM	OF	11	1	10	0.002
NSM	NOF	29	12	17	NS
NSM	SM	18	8	10	NS
NSM	NSM	11	4	7	NS
OF	OF	14	8	6	0.05
OF	NOF	11	7	4	NS
OF	SM	18	15	3	0.0482
OF	NSM	11	5	6	NS
NOF	OF	13	6	7	NS
NOF	NOF	11	5	6	NS
NOF	SM	13	8	5	NS
NOF	NSM	11	4	7	NS
C	OF	13	6	7	NS
C	NOF	14	6	8	NS
C	SM	12	7	5	NS
C	NSM	11	7	4	NS

Table 1. Table summing the preference responses of all combinations of test subjects and odor sources for inside mazes constructed in the Lake Huron Biological Station. Notably, ovulated females spent significantly more time in the side of the maze containing spermiated males. Abbreviations are as follows; ovulated females (OF), non-ovulated females (NOF), spermiated males (SM), non-spermiated males (NSM), control or no odor (C), water collected from spermiated males (SMW). P values were generated from a Wilcoxon Signed Ranks Test (2-tailed).

Outside Raceway Behavioral Tests

Odor Source	Test Subject	n	Experimental Side	Control Side	p value
SM	OF	8	8	0	0.008
SM	NOF	8	5	3	NS
SM	SM	10	6	4	NS
SM	NSM	13	6	7	NS
SMW	OF	8	7	1	0.04
NSM	OF	10	7	3	NS

Table 2. Table summing the preference responses of test subjects to a spermiated male odor source for the outside maze constructed on the Ocqueoc River. Ovulated females spent significantly more time in the side of the maze containing spermiated males and spermiated male washings. Abbreviations are as follows; ovulated females (OF), non-ovulated females (NOF), spermiated males (SM), non-spermiated males (NSM), water collected from spermiated males (SMW). P values were generated from a Wilcoxon Signed Ranks Test (2-tailed).

Inside Searching Behaviour

Odor Source	Test Subject	n	Exp. Side Searching	Con. Side Searching	P-value
SM	OF	7	7	0	0.008
SM	NOF	13	7	6	NS
SM	SM	9	4	5	NS
SM	NSM	10	6	4	NS
SMW	OF	7	7	0	0.008
NSM	OF	9	4	5	NS

Outside Searching Behaviour

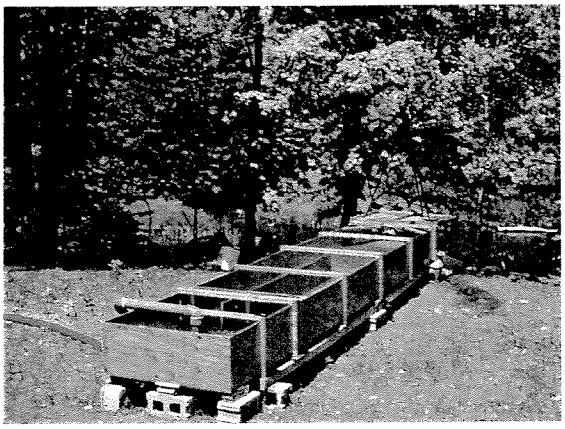
Odor Source	Test Subject	n	Exp. Side Searching	Con. Side Searching	P-value
SM	OF	8	8	0	0.008
SM	NOF	6	3	3	NS
SM	SM	10	6	4	NS
SM	NSM	8	2	6	NS
SMW	OF	7	7	0	0.008
NSM	OF	7	4	3	NS

Table 3. Display of inside maze searching behavior activity for inside (top table) and outside (bottom table) mazes in response to spermiated male odor. Ovulated females showed significantly more searching behavior in the side of the maze containing spermiated males and their washings. Abbreviations are as follows; ovulated females (OF), Non-ovulated females (NOF), spermiated males (SM), and Non-spermiated males (NSM). P values generated using a Wilcoxon Signed.

Behavior of Female Sea Lamprey In a Spawning Stream

	Behavioral Choice		
Maturation	SM	NSM	Int.
OF	9	0	4
NOF	1	2	4

Table 4. Choices of female sea lampreys in a spawning stream with the presence of spermiated and non-spermiated males. Fisher's Exact Test (2-Tail), P value = 0.024. Ovulated female (OF) sea lampreys swam significantly more to spermiated males (SM) than non-spermiated males (NSM). Non-ovulated females (NOF) were not attracted to spermiated males.



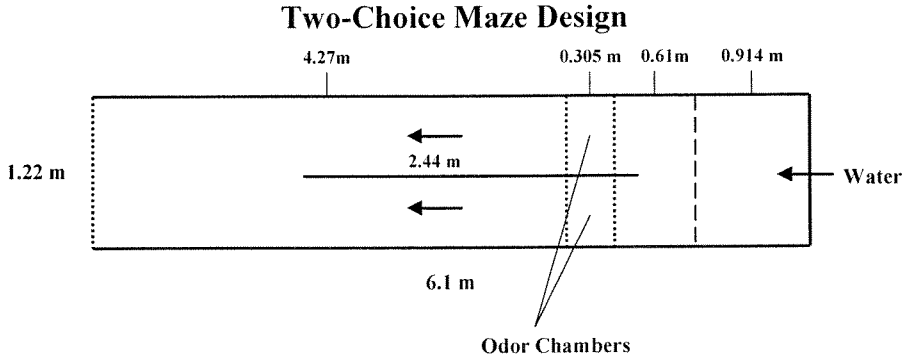
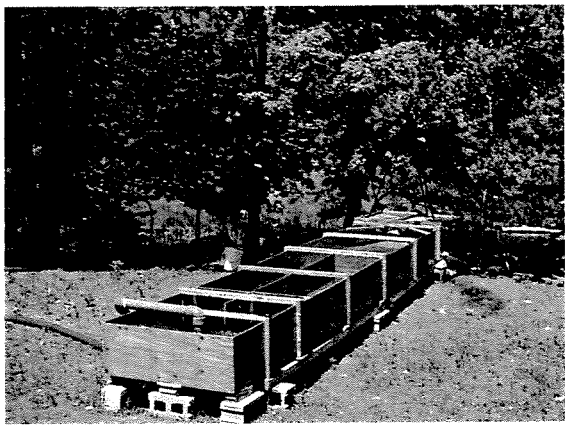


Figure 1. Experimental apparatus (two-choice maze) used to examine the behavioral responses of adult sea lampreys to pheromones. The top figure shows the actual device and its arrangement in experimental sites. The bottom figure illustrates the design of the device. The dotted lines represent meshes, solid lines represent wooden board and the dashed line represents a laminar flow device. Odor donors are placed in the odor chambers. Test subjects are released in the down stream part of the test area and their behaviors recorded with a video camera. Water flow is indicated by the arrows.

Figure 2. Section of the Ocqueoc River used in radio-telemetry tracking experiments. Shown is the upstream portion of the study section including an island that divides the river and the odor cages used in experiments. Ovulated and non-ovulated female sea lampreys were fitted with radio tags and their behavior monitored in response to spermiated and non-spermiated male odors.



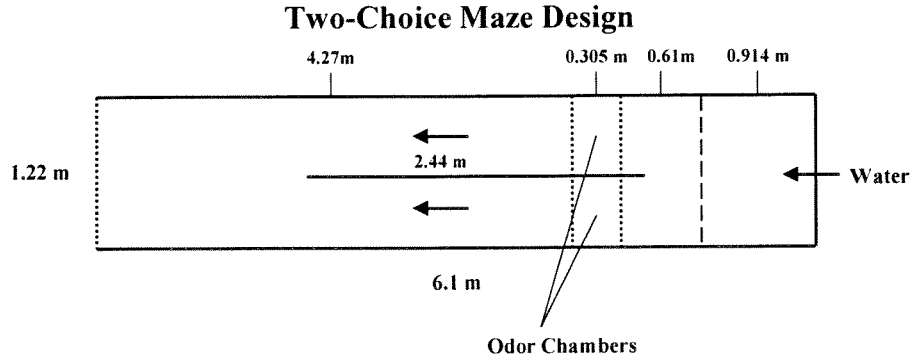


Figure 1. Experimental apparatus (two-choice maze) used to examine the behavioral responses of adult sea lampreys to pheromones. The top figure shows the actual device and its arrangement in experimental sites. The bottom figure illustrates the design of the device. The dotted lines represent meshes, solid lines represent wooden board and the dashed line represents a laminar flow device. Odor donors are placed in the odor chambers. Test subjects are released in the down stream part of the test area and their behaviors recorded with a video camera. Water flow is indicated by the arrows.

Figure 2. Section of the Ocqueoc River used in radio-telemetry tracking experiments. Shown is the upstream portion of the study section including an island that divides the river and the odor cages used in experiments. Ovulated and non-ovulated female sea lampreys were fitted with radio tags and their behavior monitored in response to spermiated and non-spermiated male odors.