GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

Potential Use of GnRH Analogs for Sterilizing Male Sea Lamprey's

by:

Dr. Stacia A. Sower[2], Lee Hanson[3], Caleb Slater[2], Bernie Schultz[2] and Jane Connolly[2]

[2] Department of Biochemistry and Molecular Biology
University of New Hampshire
Durham, New Hampshire 03824

[3]USFWS
120 South B Street
Cheboygan, Michigan 49721

April 2000

GREAT LAKES FISHERIES PROJECT FINAL REPORT

TO:

Randy L. Eshenroder
Great Lakes Fishery Commission
2100 Commonwealth Blvd., Suite 209
Ann Arbor, Michigan 48105

PROJECT TITLE:

Potential Use of GnRH Analogs for Sterilizing Male Sea Lampreys

PRINCIPAL INVESTIGATOR:

Dr. Stacia A. Sower
Department of Biochemistry and Molecular Biology
University of New Hampshire
Durham, New Hampshire 03824

CO-INVESTIGATOR:

Lee Hanson
USFWS (Retired)
120 South B Street
Cheboygan, Michigan 49721

OTHER PERSONNEL:

Caleb Slater, Post-Doctoral Research Associate,
Bernie Schultz and Jane Connolly, Graduate Students
Department of Biochemistry and Molecular Biology
University of New Hampshire

TIME PERIOD:

1997-2000

ACKNOWLEDGEMENTS:

We like to thank Roger Bergstedt and the personnel at Hammond Bay Biological Station as well as Mike Twohey and his crew for all of their assistance, facilities and lampreys that we used in these studies.

FINAL REPORT

OBJECTIVES:

The overall goal of this research program has been to investigate the biological effects of lamprey gonadotropin-releasing hormones (GnRH-I and -III) and analogs on the reproductive processes and behavior in adult male sea lampreys (*Petromyzon marinus*) to test the use of GnRH analogs for sterilizing lampreys. Previous studies have indicated that a lamprey GnRH analog (antagonist) may be useful as a method to sterilize male sea lampreys for use in a sterile-male-release program in the Great Lakes.

The specific objectives were 1). To determine the effects of injected lamprey GnRH-I and -III and analogs (antagonists) on gonadotropin concentrations and spermatogenesis in male sea lampreys; 2). To determine the effects of lamprey GnRH-I and -III analogs (antagonists) on nesting and spawning behavior of adult males (2nd and 3rd years) and on fertilization rates of eggs from females artificially spawned with treated males; and 3) To identify a pharmaceutical company that can make the microencapsulated GnRH analogs for testing during the 3rd year.

OVERALL SUMMARY:

Overall, there were significant differences between receptor binding and biological activity of certain GnRH analogs in lampreys compared to controls. The most promising GnRH analogs are those analogs synthesized with amino acid substitutions in the 2nd, 3rd and 6th position of lamprey GnRH-III. The most active analogs from our 1998 and 1999 studies were D-Arg^{6} lamprey GnRH-III, DPhe^{2}-DArg^{6}-DAla^{10} lamprey GnRH-II, DPhe^{2}-DArg^{6} lamprey GnRH-III and DPhe^{2}-Gly^{6} lamprey GnRH-I. Our results strongly support that synthesis of analogs with substitutions in the 2nd (i.e., Phe^{2}), 3rd positions (Pro^{3}) and D-Arg^{6} of lamprey GnRH-III are very promising GnRH analogs for use in lamprey male sterilization.

In 1994-1996 , we had successfully shown that microencapsulated GnRH analogs were effective. A private company, Aquapharm, had prepared these preparations. This company went out of business in the fall of 1997 and thus we had tried to secure the assistance other scientists. Because the microsphere encapsulation procedures were not available, we made our own microencapsulated peptides in 1998 and we had two companies make microencapsulated peptides in 1999. We tested two of the microencapsulated GnRH peptides before the lamprey season in 1999 and these preparations showed a slowed sustained release over a 3-week period both in *in vivo* and *in vitro* studies. We then had our newly synthesized peptides encapsulated by one of three methods. However, none of these microencapsulated preparations worked in the adult lampreys in 1998 or1999.

SIGNIFICANCE OF RESULTS TO THE COMMISSION'S SEA LAMPREY PROGRAM:

In lampreys, there is excellent promise from our studies that a lamprey GnRH analog may present a viable alternative and/or complement the use of bisazir for use in the sterile-male-release program. Thus, we propose that further testing of GnRH analogs will likely yield a method of sterilizing male lampreys for use in this program in the Great Lakes. However, before further testing of GnRH analogs is done, two systems have to be in place to optimize testing since there is only one time of year to test the analogs. The first is that a gonadotropin radioimmunoassay needs to be established in order to screen the analogs. Secondly, a microencapsulated process for the GnRH analogs has to be developed.

We propose that the use of GnRH analogs can be the most effective treatment for the following reasons:

1. The potential of using GnRH analogs (antagonist) is exciting because these compounds are proteins which are easily degraded within the organism, non-toxic to humans, easy to administer, low in cost, and relatively easy to synthesize. In other words, this compound can easily be injected into the lampreys in the field--we did have a delivery system (microencapsulation) that allows the GnRH analog to be released in the lamprey during the spawning season following a single injection. A new system has to be developed.

2. We have studied GnRH analogs because they are the most likely compound to be approved by the FDA. As examples, the use of GnRH analogs has already been approved for use in enhancing fish reproduction in aquaculture. An analog of GnRH is one of the leading chemical treatments for advanced prostate cancer in men and endometriosis in women. Other chemical compounds could also likely sterilize lampreys; however, a GnRH analog is probably the best candidate to be approved for use by the FDA. Initial discussions with senior personnel at the FDA indicated that there would likely be no problems with future approval.

3. New methods in molecular biology and in structural modeling of proteins will allow us to screen many potential GnRH analogs that we were not able to do previously. Once we have determined the structure of GTH in lampreys, we can then develop radioimmunoassays and by perifusion experiments of the pituitary, we can screen potential GnRH analogs. In addition, other techniques include the cloning of the GnRH receptor(s) in lamprey in order to do the molecular (mutagenic) studies. These procedures are currently being developed in my laboratory.

4. Lampreys are among the few vertebrates to clearly demonstrate roles for multiple GnRH molecules as neurohormones involved in pituitary-gonadal function. In mammals, the research to date has only shown one GnRH involved in pituitary-gonadal function. Studies have shown that GnRH can affect reproductive behavior in vertebrates . We have also shown that some GnRH-I analogs (but not GnRH-III) can influence the spawning behavior in lampreys --actually enhancing the spawning act rather than decreasing it. However, because lampreys have two GnRHs that act as neurohormones controlling the pituitary-gonadal axis and act in a differential manner, we propose that an analog to

lamprey GnRH-III can be developed in which the spawning behavior would not be affected, yet the lampreys would be sterilized.

LIST OF PEER-REVIEWED PUBLICATIONS SUPPORTED OR PARTIALLY SUPPORTED BY GLFC (1997-2000)

1997- Tobet, S.A., S.A. Sower and G.A. Schwarting. 1997. Gonadotropin-releasing hormone containing neurons and olfactory fibers during development: from lamprey to mammals. Brain Res. Bull. 44:479-486.

1998- Sower, S.A. 1998. Brain and Pituitary Hormones of lampreys, recent findings and their evolutionary significance. Amer. Zool. 38:15-38

1999- Nozaki, M., K. Ominato, A. Takahashi, H. Kawauchi, and S.A. Sower. 1999. Possible gonadotropin cells in the lamprey pituitary: colocalization of mammalian LH-like immunoreactivity and glycoconjugate in adult sea lampreys (*Petromyzon marinus*). Gen. Comp. Endocrinol. 113:23-31.

Slater, C.H., C.B. Schreck and S.A. Sower. 1999.Rapid clearance of D-Ala^{6}-Pro^{9}NEt mammalian GnRH (GnRHa) from chinook salmon plasma. North American Journal of Aquaculture 61:315-318.

2000- Gazourian, L., E.L. Evans, L. Hanson, C. Chase and S.A. Sower. 2000. The effects of lamprey GnRH analogs on steroidogenesis in the male sea lamprey (*Petromyzon marinus*). Aquaculture. In Press

Suzuki, K., R.L. Gamble, and S.A. Sower. 2000. Multiple transcripts encoding lamprey gonadotropin-releasing hormone-I precursors. J. Molec Endocrinol. In Press.

Nozaki, M., A. Gorbman and S.A. Sower. The distribution of lamprey GnRH-III in Brains of adult sea lampreys (*Petromyzon marinus*). Gen. Comp. Endocrinol. In Press.

Reed, K.L., MacIntyre, J.M., M. Nozaki, A. Gorbman, S.A. Sower and S. A. Tobet. The development of γ-aminobutyric acid neurons and their relationship to gonadotropin- releasing hormone neurons in the larval and adult sea lamprey, *Petromyzon marinus*. (In Revision)

MacIntyre, J.K, C.Chase, S. Done and S.A. Sower . The interrelationship of PMYand GnRH in the sea lamprey. Gen. Comp. Endocrinol. Accepted with revisions

Sower, S.A., K. L. Reed, M. O. Materne, J. Connolly and H. Kawauchi. The physiology

of reproduction in lampreys and applications for male lamprey. Can. J. Fish Aquat. Sci.

Sower, S.A. and E.L. Evans. Controlled release of D-Ala6, Pro9 Net mammalian GnRH in the sea lamprey (Petromyzon marinus). Aquaculture. Accepted—In Revision

Sower, S.A. and E.L. Evans. Steroid feedback in the male sea lamprey, *Petromyzon marinus*. Comp.Biochem Physiol. Accepted with revisions.

Sower, S.A., A. J. McGregor, O. Materne, C. Chase, I. Potter, and J.Joss. Evidence for lamprey GnRH-I and –III like molecules in the brains of the Southern Hemisphere lampreys. *Geotria australis and Mordacia mordax*. Gen. Comp. Endocrinol. Submitted.

EXPERIMENTS:

The experiments were done at the University of New Hampshire, May through July, 1998 and 1999 using land-locked lampreys shipped from Hammond Bay Biological Station to UNH and sea-run lampreys caught in nearby rivers in NH.

Experiment I: *In vivo* studies of microencapsulated GnRH in male lampreys (1998, 1999).

The objective of this study was to test the release rate and duration of a microencapsulated GnRH peptide (D-Ala^6-Pro^{9}NEt mammalian GnRH) in male lampreys. Thirty (1998) male lampreys (three groups of ten) and sixty (1999) male lampreys (six groups of ten) were injected with a single intramuscular 0.1 ml injection of microencapsulated D-Ala^6-Pro^{9}NEt mammalian GnRH on June 4, 1998 and June 1, 1999. Each injection contained 0.16 mg of microspheres in injection emulsion. One group (ten lampreys) was captured for sampling each week. At the time of sampling, a 1.0 ml blood sample was collected as previously described (Fahien and Sower, 1990). After centrifugation, the plasma from individual blood samples was stored at -20°C until assayed. Plasma samples were extracted and eluted on HPLC. The resulting fractions were assayed by a specific radioimmunoassay for D-Ala^6-Pro^{9}NEt mammalian GnRH.

Summary of Experiment I:

D-Ala^6-Pro^{9}NEt mammalian GnRH was not detected in any of the plasma samples collected in either 1998 or 1999. In 1998, the results indicated that the microspheres we had made either failed to incorporate the peptide, or they failed to release it in a time-release manner. In 1999, (see pages 8 to 11 of this report), none of the preparations made by SRI or Thies worked in these experiments. In our earlier work (1994-1996), we had demonstrated that microencapsulated GnRH made by Aquapharm was extremely effective in sustained release of GnRH. Microencapsulated GnRH analogs can be used for a variety of studies beyond what we have proposed. This will require the development of a microencapsulated process that is effective in lampreys.

Experiment II: *In vivo* studies on microencapsulated lamprey GnRH and analogs in male lampreys (Fig 1).

The objective of this study was to test the effects of microencapsulated lamprey GnRH III or analogs on brain GnRH levels. Sea run male lampreys were tested with a single 0.1 ml intramuscular injection of empty (control) microspheres or microspheres containing one the following peptides on June 4, 1998.

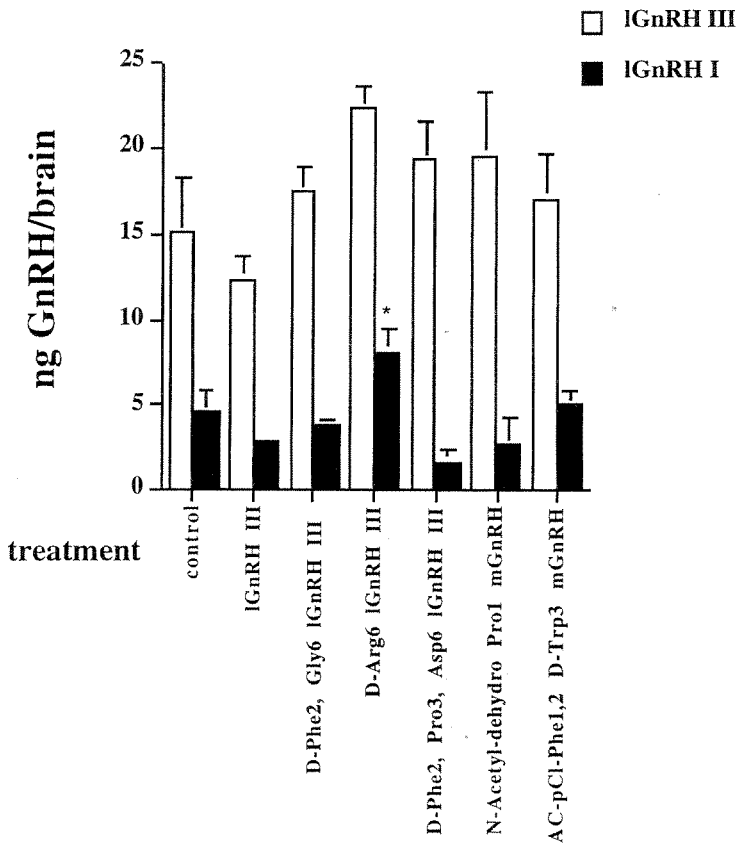
1) lamprey GnRH III
2) Phe^2 Gly^6 lamprey GnRH III
3) Phe^2 Pro^3 Asp^6 lamprey GnRH III
4) D-Arg^6 lamprey GnRH III

5) N Acetyl dehydro Pro'p Fluro D-Phe^{2} D-Trp^{3,6} mammalian GnRH

6) Ac D-pCl Phe^{1,2} D-Trp^{3}, D-Arg^{6} D-Ala^{10} mammalian GnRH

Six sea run lampreys were used for each treatment group. Lampreys were sampled at three weeks post-injection (June 24). At the time of sampling brains and pituitaries were collected. Tissues were frozen on dry ice and stored at -80°C. Brains were extracted, eluted on HPLC and the resulting fractions were assayed for lamprey GnRH I and lamprey GnRH III.

Fig. 1- Effects of microencapsulated GnRH analogs on brain GnRH-I and -III concentrations-Exp II at UNH.1998



Summary of Experiment II:

The results of experiment I demonstrated that the GnRH microspheres did not deliver significant quantities of peptide to injected animals. With one exception, there were no significant differences in brain lamprey GnRH I or lamprey GnRH III in animals treated with GnRH microspheres. However, there was a significant increase in brain levels of lamprey GnRH-I in the D-Arg^{6} lamprey GnRH III treated group (Fig. 1), suggesting an initial release of this peptide that was very potent.

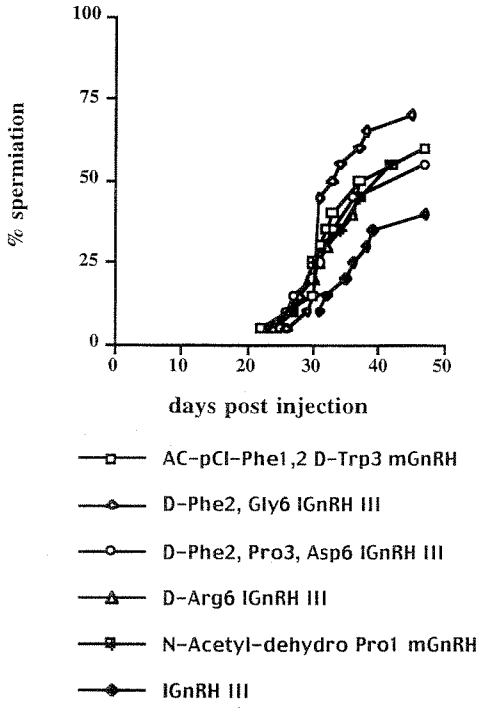
Experiment III: *In vivo* studies on microencapsulated lamprey GnRH and analogs in male lampreys.

The objective of this study was to test the effects of microencapsulated lamprey GnRH III or analogs on spermiation, sperm quality, egg fertilization rate, and embryo survival through the first eight days of growth. Groups of ten locked male lampreys were tested with a single 0.1 ml intramuscular injection of microspheres containing one the following.

1) lamprey GnRH III
2) Phe^2 Gly^6 lamprey GnRH III
3) Phe^2 Pro^3 Asp^6 lamprey GnRH III
4) D-Arg^6 lamprey GnRH III
5) N Acetyl dehydro Pro'p Fluro D-Phe^2 D-$Trp^{3,6}$ mammalian GnRH
6) Ac D-pCl $Phe^{1,2}$ D-Trp^3, D-Arg^6 D-Ala^{10} mammalian GnRH

On June 9, 1988 lampreys from the Cheboygan River were injected (10 per treatment group). On June 13, 1998 lampreys from the St. Mary's River were injected (10 per treatment group). The treated male lampreys were then placed in artificial spawning channels with untreated female lampreys. When spawning behavior was observed the male lamprey was removed from the spawning channel and used to fertilize the eggs (200) of an untreated female. Some eggs (200) were then also fertilized by an untreated male to act as a control. Eggs were then placed in glass battery jars (250 mm diameter) filled with 6 l of Lake Huron water. These jars were partially submerged in a constant temperature bath, held at 18.3 degrees C. After 6-10 days embryos were examined and survival rate and stage of development were recorded.

Fig. 2 Accumulative percent spermiation of lampreys treated with GnRH analogs at Hammond Bay Biological Station, Exp III. 1998.



Summary of Experiment III:

Treatment with microencapsulated GnRH analogs did not effect timing of spermiation (Fig. 2), egg fertilization rates or embryo survival. These data support our other studies in 1998 that the GnRH microspheres failed to deliver the GnRH peptide to treated lampreys.

Experiment IV: *In vivo* studies on lamprey GnRH and analogs in male lampreys in 1998.

The objective of this study was to test the effects of lamprey GnRH analogs on spermiation, sperm quality, egg fertilization rate, and embryo survival through the first eight days of growth. Lampreys were injected 11 times over a 35 day period (June 4- July 8). Lampreys were checked for spermiation 24 hours after injection. Lampreys were tested with 0.1 ml intraperitoneal injections of saline (control) or one of the following treatments.

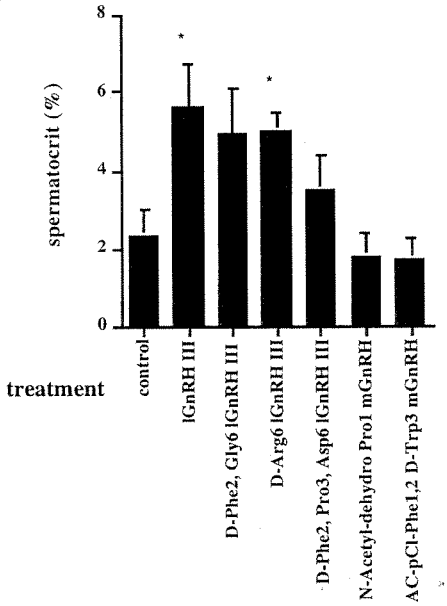
1) lamprey GnRH III
2) Phe^2 Gly^6 lamprey GnRH III
3) Phe^2 Pro^3 Asp^6 lamprey GnRH III
4) D-Arg^6 lamprey GnRH III
5) N Acetyl dehydro Pro'p Fluro D-Phe^2 D-$Trp^{3,6}$ mammalian GnRH
6) Ac D-pCl $Phe^{1,2}$ D-Trp^3, D-Arg^6, D-Ala^{10} mammalian GnRH

When a male lamprey was found to be spermiating, it was removed from the tank and artificially spawned with an untreated female. Spermatocrit was assessed and recorded. For determination of fertilization rates, eggs were placed in plastic petri dishes (50 mm in diameter) and held in a constant temperature incubator at 18.3°C, the optimum temperature for development of sea lamprey embryos. Fertilization rate was assessed at 24 hours post-spawning. Embryo survival to the head stage (7-8 days post fertilization) was also assessed and recorded.

Summary of Experiment IV:

As shown in Fig. 3, injection with some of the GnRH analogs advanced the timing of spermiation. D-Arg^6 lamprey GnRH-III, D-Phe^2, Gly^6 lamprey GnRH-III and lamprey GnRH-III advanced the timing of the spermiation response. Treatment with GnRH analogs did not significantly effect fertilization rate or egg viability through the head stage. The spermatocrit was increased significantly by treatment with D-Arg^6 lamprey GnRH-III and lamprey GnRH-III (Fig. 4). It is concluded from these studies, that fertilization can only be tested with females that demonstrate spawning behavior, in order to insure viable oocytes.

Fig. 3 Spermatocrit from lampreys treated with different GnRH analogs. UNH. 1998.



Experiment V: Binding specificity of GnRH binding in the pituitary of the male sea lamprey in 1998 and 1999.

The objective of this study was to determine the binding affinity of the GnRH analogs in the pituitary of the male sea lamprey to determine, in part, the structure-function biological activity of these analogs. Frozen pituitaries were embedded and 20_m sections were cut and thaw mounted on subbed slides. The slides were vacuum desiccated then incubated in triplicate with ^{125}I D-Ala^6-Pro^{9}NEt mammalian GnRH, and serial dilutions of radioinert test GnRH analogs (D-Arg^6 lamprey GnRH III, Phe^2 Pro^3 Asp^6 lamprey GnRH III, N Acetyl dehydro Pro'p Fluro D-Phe^2 D-Trp^{3,6} mammalian GnRH, and Ac D-pCl Phe^{1,2} D-Trp^3, D-Arg^6 D-Ala^{10} mammalian GnRH). Non-specific binding was obtained by incubating an extra set of slides with an excess of unlabelled D-Ala^6-Pro^{9}NEt mammalian GnRH.

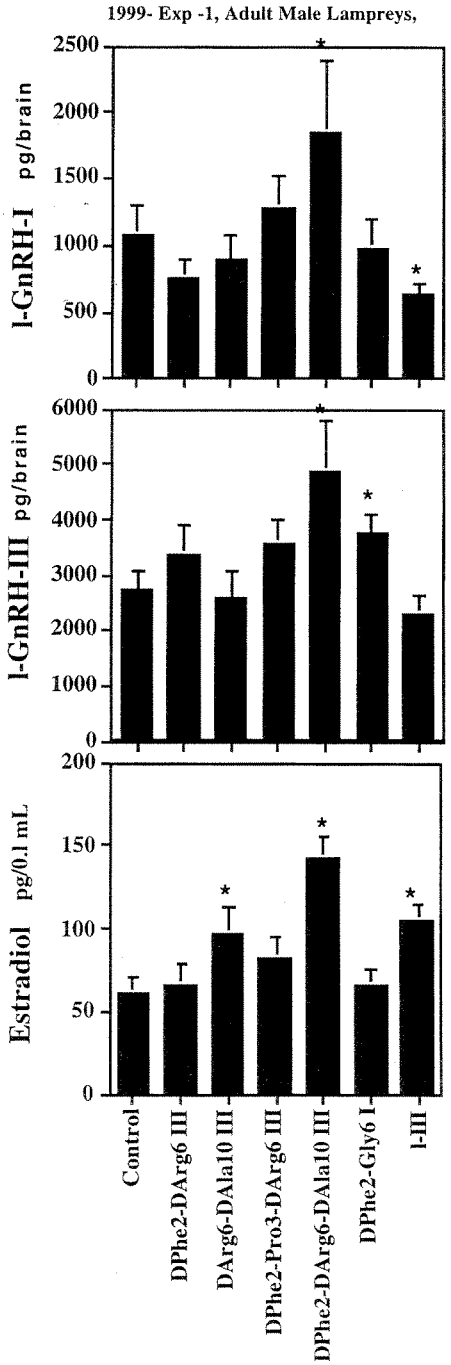
Summary of Experiment IV:

D-Arg^6 lamprey GnRH III showed high affinity for the GnRH receptors in lamprey pituitary (Fig. 4). The two mammalian analogs (N Acetyl dehydro Pro'p Fluro D-Phe^2 D-Trp^{3,6} mammalian GnRH, and Ac D-pCl Phe^{1,2} D-Trp^3, D-Arg^6 D-Ala^{10} mammalian GnRH) also demonstrated affinity for the receptors, while Phe^2, Pro^3, Asp^6 lamprey GnRH III demonstrated little affinity for the receptors (data not shown).

Experiments 1 and 2 -1999. *In vivo* studies of lamprey GnRH and analogs in male lampreys.

The objective of these studies were to test the effects of lamprey GnRH III or analogs on brain GnRH levels. Land-locked male lampreys were tested with two injections, three days apart

(12 sea-run-lampreys per treatment). There were two different experiments at two different temperatures 1) June 1 to 4 and 2) June 14-17. The blood was sampled 24 hrs after the 2nd injection and plasma collected into two containers (one for GTH and one for steroids). Brains pituitaries,and gonad samples were taken. Brains and pituitary were immediately frozen on dry ice and stored at –80C until assayed for GnRH and irGTH. The gonad samples were put into Bouin's solution and processed for histological examination. The treatments were 1) Control 2) D-Phe^2, Pro^3, D-Arg^6 lamprey III at 0.1 μg/g lamprey; 3) D-Arg^6, D-Ala^{10} lamprey III at 0.1 μg/g; 4) D-Phe^2, D-Arg^6, D-Ala^{10} lamprey III at 0.1 μg/g; 5) D-Phe^2,D-Arg^6 lamprey III at 0.1 μg/g; 6) D-Phe^2,Gly^6 lamprey I at 0.1 μg/g and 7) lamprey GnRH-III at 0.1 μg/g



Summary of Experiment 1 and 2, 1999: DPhe2-DArg6-DAla10 lamprey GnRH-III significantly elevated lamprey GnRH-I, -III and estradiol. DArg6-DAla10 GnRH-III, DPhe2-DArg6 GnRH-III and DPh2-Gly6 GnRH-I also showed significant effects either on lamprey GnRH or plasma estradiol concentrations (Exp 2 data not shown).

SUMMARY: These data provide exciting results suggesting that the most promising GnRH analog that can be used as an antagonist (or sterilant for male sea lampreys) are those analogs synthesized with amino acid substitutions in the 2nd, 3rd and 6th position of lamprey GnRH-III. The most active analog from our 1998 studies was D-Arg6 lamprey GnRH-III and from our 1999 studies was DPhe2-DArg6-DAla10 lamprey GnRH-III .

Microencapsulation Experiments, 1999

Objective: To develop a microsphere system which allows for the extended release of D-Ala^6-Des Gly ^{10} mGnRH, a GnRH analog, over a six week time period through both *in vitro* and *in vivo* release characterization.

Microspheres (definition): A spherical shell made of a biodegradable or nonbiodegradable polymer that has a diameter in the micrometer range. The polymer entraps and allows for the sustained release of a substance. (ie of D-Ala^6-Des Gly ^{10} mGnRH). *Functions*: Protection of the peptide from enzymatic degradation and control of the release of the analog as the hydrophobic polymer degrades. *History*: Microsphere technology developed in the late 1930's by Barrett Green of Moore Business Forms for use as "carbonless" copy paper.

Release Determination *in vitro:*

Procedure:

A. Preparation of incubation materials: Four preparations of microencapsulated GnRHa were received from Thies Technology (3/9/99). Each preparation initially began with 5 mg GnRHa and 100 mg of microspheres. They were as follows:

Non-biodegradable:
- Cellulose Acetate Butyional (CAB)
- Cellulose Acetate Propropional (CAP)

Biodegradable:
- Polypepsin (PP)
- Polylactic glycolic acid (PLGA)

B. Thies estimated a yield of approximately 40-60% . Actual yield was~20%.

C. A PLGA microsphere preparation was also received from Southern Research Institute (SRI) containing 5 mg of D-Ala^6-Des Gly ^{10} mGnRH in 333.3 mg of microspheres.

D. 5.0 mg of each microsphere type were weighed out with great care.

1. 5.0 mg was added to 60 mL of Phosphate Buffer and divided into two 30 ml volumes

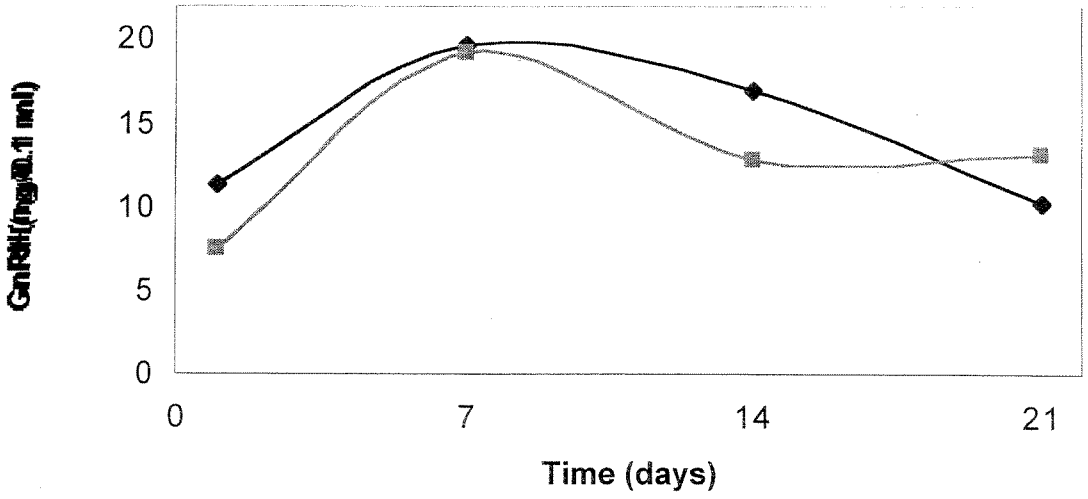
2. 5.0 mg was added to 60 mL of Hank's Buffered Saline Solution (HBSS) and divided into two 30 ml volumes.
3. SRI microspheres were not incubated in HBSS since it was shown that phosphate buffer, with its higher buffering capacity, maintained the pH better than HBSS.
4. Incubated at 17°C with gentle shaking.
5. pH of incubation media was measured every other day

E. Sampling of Incubation Media: Day 1, Week 1, Week 2, Weeks 3, Week 4
1. 1.0 mL of incubation buffer was sampled from each vial.
2. Microspheres were separated by centrifugation and returned to incubation vials resuspended in ~1.0 mL of fresh buffer.

F. Analysis of Samples: Extraction of GnRHa from sample and assessment of D-Ala^6-Des Gly ^{10} mGnRH concentration were determined as follows. The samples were extracted , eluted through C-18 seppak chromatography followed by radioimmunoassay and the Lowry Protein Assay.

Results of Microsphere Release *in vitro*

Results from the RIAs on the *in vitro* incubation of the microspheres received from Thies Technology were normalized and the total amount of analog in the incubation volume was determined. Duplicate incubations were averaged and the amount of the GnRH analog in the incubation solution was plotted versus time. The *in vitro* release of the PLGA microspheres received from SRI is shown below:



Summary of *in vitro* release

All four of the Thies microspheres showed release of the analog in a time dependent fashion (data not shown). Although somewhat constant for the first two weeks, the amount of D-Ala^6-Des Gly ^{10} mGnRH released from the microspheres increased beginning at week 2. In both incubation buffers the CAB microsphere preparation showed steadily increasing release of D-Ala^6-Des Gly ^{10} mGnRH, while the polypepsin microsphere incubation experienced a dramatic decrease in D-Ala^6-DesGly ^{10} mGnRH concentrations. This decrease is most likely due to nonenzymatic degradation of the analog and a slowing of the release rate from the microsphere preparation.

The SRI PLGA spheres (Figure shown above) also exhibited a time dependent release profile, with approximately 8 % of the entire D-Ala^6-Des Gly ^{10} mGnRH content of the spheres being released on the first day. This is a typical amount for the initial burst of peptide from the sphere. After a large increase of analog concentration at week 1, the levels of D-Ala^6-Des Gly ^{10} mGnRH decreased slowly and appeared to be slowing releasing by week 3. Attempts to employ the Lowry assay as a means of protein determination proved to be nonfeasible due to the lack of assay sensitivity and the small concentrations of protein in the sample.

IV. Experimental Design: Release Determination *in vivo* 1999

The objective of this study was to determine the release rates of the microencapsulated GnRH in fish prior to the lamprey season. In March, 1999, 40 red-fin shiners were placed in a6' flow-through tank at the Hatchery and maintained for several days prior to injection. (64-68°C). The microspheres from Thies were suspended in 2.1 mL of Microsphere Emulsion Media. On March 20, 1999 the shiners were anesthetized with MS222, injected and fin-clipped for identification as follows: The preparations used were CT990305A (CAB); CT990306B (CAP); CT990306A (and CT990306C (PLGA). Ten Fish were injected with 0.2 mL emulsion of each microsphere IP and returned to the 6' tank. Blood was sampled from each of the 40 fish on April 2 and plasma collected and stored at –80C until assayed. The samples were extracted , eluted through C-18 seppak chromatography followed by radioimmunoassay and the Lowry Protein Assay.

Results of *in vivo* release determination.

Initial analysis indicated that there was release of GnRH from the microencapsules. Further analysis demonstrated that only 3 of the 40 fish had measurable concentrations of D-Ala^6-Des Gly ^{10} mGnRH in the plasma (Data not shown). These data were in contrast to the in vitro release. The reasons for the lack of release are unclear at this time. Further studies employing these microencapsulation preparations and other preparations need to be done.

1998-1999: Heterologous Gonadotropin Hormone Double AntibodyAssay

As stated, one of our objectives was to be able to measure the direct actions of GnRH analogs. Because we have not yet identified the gonadotropin-like hormone, over a period of 8 months, we attempted to develop a double antibody heterologous radioimmunoassay to measure

immunoreactive gonadotropin of the lamprey using ovine LH antibody and ligand. By immunocytochemistry, we recently demonstrated ir-GTH-like immunoreactivity in the pituitary of the sea lamprey using antibodies to the β subunit of ovine luteinizing hormone (LH). Using this oLH antibody, attempts were made to develop a double antibody system to measure ir-lamprey gonadotropins. A variety of buffers, secondary antibodies and variations of the procedures were performed to optimize this assay for measurement of ir-GTH.

Initial Results We have shown that can measure ir-lamprey GTH in both plasma and extracted samples. Binding was as high as 31% with the veronal buffering system and 18% with the phosphate-gelatin buffering system. The oLH assay still needs further optimization including the determination of the ratio of normal rabbit serum, radioactive ligand, primary, and secondary antibodies before we can use it to measure the ir-GTH in lampreys. To aid with our methods in finalizing this assay, the PI (Stacia Sower) will be visiting Professor Karsch laboratory at the Univ. Michigan, Ann Arbor during the week of March 27 to 31, 2000. Professor Karsch is the scientist that developed the radioimmunoassay to measure ovine LH.

1999 Sterilization Study (GnRH Antagonists)

Lee Hanson and Stacia A. Sower

(These studies were done at Hammond Bay Biological Station).

Manistique River Lampreys (Outside Spawning Channel)

Male and female sea lampreys were captured in the Manistique River between May 30 and June 1, 1999 and held at the Hammond Bay sterilization facility. On June 2, we received 150 males and 93 females from the sterilization facility. Males were placed in a tank by the lunch room and females were placed in the outside spawning channel.

On June 7, we weighed, fin-clipped, and injected 70 males and placed them in the outside spawning channel. Groups of 10 males were injected with a single dose (1x) of GnRH antagonists. Injected males weighed between 175 and 274 grams.

Weights (in grams) of fin-clipped males

	(1x) SRIH793-102-00 1 Ant.	CT990522A 2 Ant.	CT990518B 3 Ant.	CT990521A 1 Post.	CT990519A 2 Post.	CT990517A 3 Post.	CT990518A 4 Post.
1	240	240	236	236	248	192	222
2	236	236	230	232	248	240	214
3	228	194	212	198	246	178	266
4	220	206	230	240	234	182	268
5	240	228	238	232	176	250	256
6	188	220	220	198	246	246	252
7	220	234	186	224	228	236	274
8	194	190	222	190	234	175	272
9	230	238	200	246	248	214	200
10	238	226	228	246	216	240	234
Ave	223	221	220	224	232	215	246
Range	188-240	190-240	186-238	190-246	176-248	175-250	200-274

On June 8, 75 uninjected (normal) males were placed in outside spawning channel.

From June 16 – 27 many males were observed building nests, but no spawning was observed. During this time, considerable mortality occurred, particularly among injected males. On June 27 all injected males were moved to the inside spawning channel where spawning was occurring.

Cheboygan River Lampreys (Inside Spawning Channel)

Male sea lampreys (about 380) were captured in the Cheboygan River on May 6, 1999 and held at Hammond Bay in a tank by the lunchroom. The males were stressed when received and 26 were dead the next day. Those remaining appeared fully recovered.

Females were captured in the Cheboygan and Manistique Rivers between May 4- 25 and held in a tank in the raceway room and a tank by the lunchroom. On June 2nd and 3rd, we placed 90 normal females in the inside spawning channel (50 from those captured on May 6 in the Cheboygan River and 40 taken from the Cheboyan and Manistique Rivers between May 4th - 25th.

On June 7, we weighed, fin-clipped and injected 60 males with a double dose (2x) of GnRH antagonists and placed them in the inside spawning channel. All males weighed between 176 and 240 grams.

Weights (in grams) of fin-clipped males

	CT990522B 1 Ant. 1 Post.	CT990523A 1 Ant. 2 Post.	CT990521B 1 Ant. 3 Post.	CT990519B 2 Ant. 1 Post.	CT990517B 2 Ant 2 Post.	(2x) SRIH793-102-00 2 Ant. 3 Post.
1	232	240	212	198	218	192
2	230	176	240	240	236	210
3	186	178	214	232	240	220
4	232	234	216	200	196	238
5	196	194	234	190	206	180
6	232	226	216	200	206	200
7	200	220	240	220	236	230
8	216	220	230	220	216	212
9	234	206	226	196	208	196
10	224					
11	240	186	240	206	236	234
Average	220	208	227	210	220	211
Range	186-240	176-240	212-240	190-240	196-240	180-238

On June 8, we placed 60 normal (uninjected) males in the inside spawning channel.

On June 21, nest building and spawning were observed for the first time (normal male and female). Slight drops in water temperature slowed spawning activity somewhat (see temperature charts and "Dead" tables), however spawning began in earnest on June 26. On June 27 all injected males from the outside spawning channel were transferred to the inside spawning channel.

Lampreys observed spawning were removed and artificially spawned. One portion of the eggs from each female was fertilized with sperm from an injected male; a second portion was fertilized with sperm from an untreated male to provide a control. Approximately 200 eggs were placed in 10-L glass battery jars (250 mm in diameter) containing about 6-L of Lake Huron water. The jars were partly immersed in a constant temperature water bath held at 18.3 degrees Celsius. After 5-7 days of incubation the

eggs were examined and the survival rates and stages of development were determined. The results are given in Table 1-7.

Table 1. D Phe^2 - DAla^6 1 GnRH III (SRIH793-102-00)

Batch No.	Group	SameFfemale used in Batch Number	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead
MANISTIQUE RIVER LAMPREYS (1 ANT.)(1X)											
32	Experimental	33-34	Yes	Yes	6-30	7-5	10's	Yes	203	96.6	3.4
34	Control	32-33	Yes	Yes	6-30	7-5	10's	Yes	199	97.5	2.5
54	Experimental	53-55	Yes	Yes	7-2	7-7	10's	Yes	193	91.2	8.8
55	Control	53-54	Yes	Yes	7-2	7-7	10's	Yes	200	95.5	4.5
79	Experimental	77-78-80	Yes	Yes	7-4	7-11	12's	Yes	201	92.5	7.5
80	Control	77-78-79	Yes	Yes	7-4	7-11	12's	Yes	199	84.9	15.1
89	Experimental	88-90-91	Yes	Yes	7-5	7-12	12's	Yes	200	98.5	1.5
91	Control	88-89-90	Yes	Yes	7-5	7-12	12's	Yes	200	97.5	2.5
13	Experimental	112-114	No	Yes	7-13	7-20	12's	Yes	206	73.8	26.2
14	Control	112-113	Yes	Yes	7-13	7-20	12's	Yes	200	1.0	99.0
CHEBOYGAN RIVER LAMPREYS (2 ANTS.-3 POST.)(2X)											
2	Experimental	1-3-4	Yes	Yes	6-23	6-30	12's	Yes	204	91.2	8.8
4	Control	1-2-3	Moving rocks	Yes	6-23	6-30	12's	Yes	190	93.2	6.8
17	Experimental	16-18	Yes	Yes	6-27	7-3	11's&12's	Yes	203	11.8	88.2
18	Control	16-17	Yes	Yes	6-27	7-3	11's&12's	Yes	205	97.1	2.9

26	Experimental	25-27-28-29	Yes	Yes	6-28	7-4	(11's&12's)	Yes	211	96.2	3.8
29	Control	25-26-27-28	Yes	Yes	6-28	7-4	(11's&12's)	Yes	199	90.5	9.5

Table 1. (Cont.)

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number Eggs	Percentage Live	Percentage Dead	
36	Experimental	35-37	Yes	Yes	6-30	7-5	10's	Yes	209	93.8	6.2	
37	Control	35-36	Yes	Yes	6-30	7-5	10's	Yes	203	74.4	25.6	
51	Experimental	50-52	Yes	Yes	7-2	7-7	10's	Yes	200	99.0	1.0	
52	Control	50-51	Yes	Yes	7-2	7-7	10's	Yes	205	94.6	5.4	
71	Experimental	72-73	Yes	Yes	7-4	7-11	12's	Yes	200	96.0	4.0	
73	Control	71-72	Yes	Yes	7-4	7-11	12's	Yes	203	96.1	3.9	
77	Experimental	78-79-80	Yes	Yes	7-4	7-11	12's	Yes	233	19.9	90.1	Little fluid No rope Returned to channel
80	Control	77-78-79	Yes	Yes	7-4	7-11	12's	Yes	199	84.9	15.1	
82	Experimental	81-83	Yes	Yes	7-5	7-12	12's	Yes	196	94.9	5.1	
83	Control	81-82	Yes	Yes	7-5	7-12	12's	Yes	200	96.5	3.5	
84	Experimental	85-86-87	Yes	Yes	7-5	7-12	12's	Yes	200	98.5	1.5	
87	Control	84-85-86	Yes	Yes	7-5	7-12	12's	Yes	199	88.9	11.1	
122	Experimental	121-123	No	No	7-13	7-20	12's	Yes	206	88.3	11.7	Same male spawned again
123	Control	121-122	No	No	7-13	7-20	12's	Yes	206	98.5	1.5	

Samples

TABLE 2. D-Arg^{6}, D-Ala^{10} lamprey III (CT990522A&B)

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number Egss	Percentage Live	Percentage Dead
MANISTIQUE RIVER LAMPREYS (2 ANT.)(1X)											
23	Experimental	22-24	Yes	Yes	6-28	7-4	11's&12's	Yes	200	98.5	1.5
24	Control	22-23	Yes	Yes	6-28	7-4	11's&12's	Yes	195	61.5	38.5
27	Experimental	25-26-28-29	Yes	Yes	6-28	7-4	11's&12's	Yes	231	78.8	21.2
29	Control	25-26-27-28	Yes	Yes	6-28	7-4	11's&12's	Yes	199	90.5	9.5
38	Experimental	39-40	Yes	Yes	6-30	7-5	10's	Yes	207	68.6	31.4
40	Control	38-39	Yes	Yes	6-30	7-5	10's	Yes	200	74.5	25.5
50	Experimental	51-52	Yes	Yes	7-2	7-7	10's	Yes	210	98.6	1.4
52	Control	50-51	Yes	Yes	7-2	7-7	10's	Yes	205	94.6	5.4
98	Experimental	99-100	Yes	Yes	7-6	7-13	12's	Yes	200	95.5	4.5
100	Control	98-99	Yes	Yes	7-6	7-13	12's	Yes	179	94.4	5.6
101	Experimental	102-103	Yes	Yes	7-9	7-16	12's	Yes	201	90.5	9.5
103	Control	101-102	Yes	Yes	7-9	7-16	12's	Yes	202	89.6	10.4

110	Experimental	109-111	No	Yes	7-13	7-20	12's	Yes	194	36.6	63.4
111	Control	109-110	Yes	Yes	7-13	7-20	12's	Yes	174	97.1	2.9

Table 2 (Continued)

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (Plavis)	Normal Development (Yes-No)	Number Eggs	Percentage Live	Percentage Dead

CHEBOYGAN RIVER LAMPREYS (1 ANT.-1 POST.)(2X)

14	Experimental	13-15	Yes	Yes	6-27	7-3	11's&12's	Yes	196	87.2	12.8	
15	Control	13-14	Yes	Yes	6-27	7-3	11's&12's	Yes	200	85.0	15.0	
20	Experimental	19-21	Yes	Yes	6-28	7-4	11's&12's	Yes	196	88.3	11.7	
21	Control	19-20	Yes	Yes	6-28	7-4	11's&12's	Yes	202	99.0	1.0	
41	Experimental	42-43	Yes	Yes	7-1	7-6	10's	Yes	207	96.6	3.4	
43	Control	41-42	Yes	Yes	7-1	7-6	10's	Yes	201	95.0	5.0	
62	Experimental	63-64	Yes	Yes	7-3	7-10 7-13	Many Abnormal 13's	No Yes	201 109	54.2 75.2	45.8 24.8	Live held for 3 more days
64	Control	62-63	Yes	Yes	7-3	7-10 7-13	Many abnormal 13's	No Yes	199 127	63.8 78.7	26.2 21.3	
63	Experimental	62-64	Yes	Yes	7-3	7-10 7-13	35 abnormal 13's	No Yes	205 105	51.2 59.0	48.8 41.0	Live held for 3 more days
64	Control	62-63	Yes	Yes	7-3	7-10 7-13	Many abnormal 13's	No Yes	199 127	63.8 78.7	36.2 21.3	
69	Experimental	68-70	Yes	Yes	7-3	7-10	12's	Yes	200	98.0	2.0	
70	Control	68-69	Yes	Yes	7-3	7-10	12's	Yes	200	99.0	1.0	
75	Experimental	74-76	Yes	Yes	7-4	7-11	12's	Yes	205	1.0	99.0	Clear fluid-no sperm

76	Control	74-75	Yes	Yes	7-4	7-11	12's	Yes	202	97.5	2.5

Table 2 (Continued)

Batch No.	Group	Same Female used in Batch Number	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead
81	Experimental	82-83	Yes	Yes	7-5	7-12	12's	Yes	203	96.1	3.9
83	Control	81-82	Yes	Yes	7-5	7-12	12's	Yes	200	96.5	3.5
102	Experimental	101-103	Yes	Yes	7-9	7-16	12's	Yes	201	94.5	5.5
103	Control	101-102	Yes	Yes	7-9	7-16	12's	Yes	202	89.6	10.4
115	Experimental	116-117	No	No	7-13	7-20	11's&12's	Yes	205	2.0	98.0
117	Control	115-116	No	No	7-13	7-20	12's	Yes	208	42.3	57.7
19	Experimental	118-120	No	No	7-13	7-20	12's	Yes	212	43.9	56.1
20	Control	118-119	No	No	7-13	7-20	12's	Yes	197	46.7	53.3

Table 3. D-Phe^2, Pro^3, D-Argb lamprey III (samples CT990518B and CT990523A)

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead
MANISTIQUE RIVER LAMPREY (3 ANT.)(1X)											
19	Experimental	20-21	Yes	Yes	6-28	7-4	11's&12's	Yes	200	99.0	1.0
21	Control	19-20	Yes	Yes	6-28	7-4	11's&12's	Yes	202	99.0	1.0
CHEBOYGAN RIVER LAMPREYS (1 ANT.-2 POST.)											
8	Experimental	9-10	Yes	Yes	6-26	7-3	12's	Yes	202	42.6	57.4
10	Control	8-9	Yes	Yes	6-26	7-3	12's	Yes	202	34.7	65.3
28	Experimental	25-26-27-29	Yes	Yes	6-28	7-4	11's&12's	Yes	186	97.8	2.2
29	Control	25-26-27-28	Yes	Yes	6-28	7-4	11's&12's	Yes	199	90.5	9.5
47	Experimental	48-49	Yes	Yes	7-1	7-6	10's	Yes	200	96.0	4.0
49	Control	47-48	Yes	Yes	7-1	7-6	10"s	Yes	203	97.0	3.0
48	Experimental	47-49	Yes	Yes	7-1	7-6	10's	Yes	200	95.5	4.5
49	Control	47-48	Yes	Yes	7-1	7-6	10's	Yes	203	97.0	3.0
60	Experimental	59-61	Yes	Yes	7-3	7-10	12's	Yes	203	99.0	1.0
61	Control	59-60	Yes	Yes	7-3	7-10	12's	Yes	202	97.5	2.5
72	Experimental	71-73	Yes	Yes	7-4	7-11	12's	Yes	200	97.5	2.5
73	Control	71-72	Yes	Yes	7-4	7-11	12's	Yes	203	96.1	3.9

Table 3 Cont.

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date eggs examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead	
95	Experimental	96-97	Yes	Yes	7-6	7-13	12's	Yes	204	98.5	1.5	
97	Control	95-96	Yes	Yes	7-6	7-13	12's	Yes	202	97.0	3.0	
105	Experimental	104-106	Yes	Yes	7-10	7-16	11's&12's	Yes	204	91.7	8.3	
106	Control	104-105	Yes	Yes	7-10	7-16	11's&12's	Yes	197	94.9	5.1	
112	Experimental	113-114	No	Yes	7-13	7-20	12's	Yes	200	72.0	28.0	
114	Control	112-113	Yes	Yes	7-13	7-20	12's	Yes	200	1.0	99.0	
121	Experimental	122-123	No	No	7-13	7-20	12's	Yes	204	30.4	69.6	Little fluid
123	Control	121-122	No	No	7-13	7-20	12's	Yes	206	98.5	1.5	

Samples

TABLE 4. D-Phe^2 , D-Arg^6 lamprey III (CT990521A&B)

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage Development (Piavis)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead
CHEBOYGAN RIVER LAMPREYS (1 ANT. – 3 POST.)(2X)											
3	Experimental	1-2-4	Yes	Yes	6-23	6-30	12's	Yes	202	84.7	15.3
4	Control	1-2-3	Only Moving Rocks	Yes	6-23	6-30	12's	Yes	190	93.2	6.8
35	Experimental	36-37	Yes	Yes	6-30	7-5	10's	Yes	212	93.4	6.6
37	Control	35-36	Yes	Yes	6-30	7-5	10's	Yes	203	74.4	25.6

Table 4 (Continued)

Batch No.	Group	Same Female used in Batch Number	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (19990	Date Eggs Examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead
56	Experimental	57-58	Yes	Yes	7-3	7-10	12's	Yes	198	79.8	20.2
58	Control	56-57	Yes	Yes	7-3	7-10	12's	Yes	200	94.0	6.0
59	Experimental	60-61	Yes	Yes	7-3	7-10	12's	Yes	200	99.0	1.0
61	Control	59-60	Yes	Yes	7-3	7-10	12's	Yes	202	97.5	2.5
78	Experimental	77-79-80	Yes	Yes	7-4	7-11	12's	Yes	204	81.4	18.6
80	Control	77-78-79	Yes	Yes	7-4	7-11	12's	Yes	199	84.9	15.1
88	Experimental	89-90-91	Yes	Yes	7-5	7-12	12'S	Yes	202	98.5	1.5
91	Control	88-89-90	Yes	Yes	7-5	7-12	12's	Yes	200	97.5	2.5
92	Experimental	93-94	Yes	Yes	7-6	7-13	12's	Yes	200	68.0	32.0
94	Control	92-93	Yes	Yes	7-6	7-13	12's	Yes	206	19.9	80.1
104	Experimental	105-106	Yes	Yes	7-10	7-16	11's& 12's	Yes	212	91.5	8.5
106	Control	104-105	Yes	Yes	7-10	7-16	11's& 12's	Yes	197	94.9	5.1
107	Experimental	108	Yes	Yes	7-10	7-16	12's	Yes	202	96.0	4.0
108	Control	107	Yes	Yes	7-10	7-16	12's	Yes	196	100.0	0.0

Samples

TABLE 5. D-Phe^{2}, D-Arg^{6}, D-Ala^{10} lamprey III (CT990519A&B)

Batch No.	Group	Same Female used in Batch Number	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (female)	Normal Development (yes-no)	Number Of Eggs	Percentage Live	Percentage Dead
MANISTIQUE RIVER LAMPREYS (2 POST) (1X)											
22	Experimental	23-24	Yes	Yes	6-28	7-4	11's & 12's	Yes	195	91.8	8.2
24	Control	22-23	Yes	Yes	6-28	7-4	11's & 12's	Yes	195	61.5	38.5
25	Experimental	26-27-28-29	Yes	Yes	6-28	7-4	11's& 12's	Yes	206	89.3	10.7
29	Control	25-26-27-28	Yes	Yes	6-28	7-4	11's& 12's	Yes	199	90.5	9.5
33	Experimental	32-34	Yes	Yes	6-30	7-5	10's	Yes	200	96.0	4.0
34	Control	32-33	Yes	Yes	6-30	7-5	10's	Yes	199	97.5	2.5
66	Experimental	65-67	Yes	Yes	7-3	7-10	12's	Yes	192	96.9	3.1
67	Control	65-66	Yes	Yes	7-3	7-10	12's	Yes	200	87.5	12.5
85	Experimental	84-86-87	Yes	Yes	7-5	7-12	12's	Yes	200	100.0	0.0
87	Control	84-85-86	Yes	Yes	7-5	7-12	12's	Yes	199	88.9	11.1
118	Experimental	119-120	No	No	7-13	7-20	-	-	204	0.0	100.0
120	Control	118-119	No	No	7-13	7-20	12'S	Yes	197	46.7	53.3
CHEBOYGAN RIVER LAMPREYS (2 ANT-1POST) (2X)											
42	Experimental	41-43	Yes	Yes	7-1	7-6	10's	Yes	200	96.5	3.5
43	Control	41-42	Yes	Yes	7-1	7-6	10's	Yes	201	95.0	5.0
44	Experimental	45-46	Yes	Yes	7-1	7-6	10's	Yes	209	97.1	2.9
46	Control	44-45	Yes	Yes	7-1	7-6	10's	Yes	208	97.6	2.4
57	Experimental	56-58	Yes	Yes	7-3	7-10	12's	Yes	200	95.0	5.0
58	Control	56-57	Yes	Yes	7-3	7-10	12's	Yes	200	94.0	6.0

Table 5 Continued

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead
90	Experimental	88-89-91	Yes	Yes	7-5	7-12	12's	Yes	200	98.5	1.5
91	Control	88-89-90	Yes	Yes	7-5	7-12	12's	Yes	200	97.5	2.5
93	Experimental	93-94	Yes	Yes	7-6	7-13	12's	Yes	191	67.0	33.0
94	Control	92-93	Yes	Yes	7-6	7-13	12's	Yes	206	19.9	80.1
96	Experimental	95-97	Yes	Yes	7-6	7-13	12's	Yes	203	98.0	2.0
97	Control	95-96	Yes	Yes	7-6	7-13	12's	Yes	202	97.0	3.0
99	Experimental	98-100	Yes	Yes	7-6	7-13	12'S	Yes	209	87.6	12.4
100	Control	98-99	Yes	Yes	7-6	7-13	12's	Yes	179	94.4	5.6
109	Experimental	110-111	No	Yes	7-13	7-20	12's	Yes	202	98.0	2.0
111	Control	109-110	Yes	Yes	7-13	7-20	12's	Yes	174	97.1	2.9

(Samples)

TABLE 6. D-Arg[6] lamprey III (CT990517A&B)

Batch No.	Group	Same Female used in Batch Number	Male observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (female)	Normal Development (Yes-No)	Number Of Eggs	Percentage Live	Percentage Dead	
CHEBOYGAN RIVER LAMPREYS (2 ANT-2 POST) (2X)												
1	Experimental	2-3-4	Yes	Yes	6-23	6-30	12'S	Yes	200	88.0	12.0	1 twin
4	Control	1-2-3	Moving rocks only	Yes	6-23	6-30	12's	Yes	190	93.2	6.8	
5	Experimental	6-7	Yes	Yes	6-26	7-3	12's	Yes	206	71.4	28.6	
7	Control	5-6	Moving rocks only	Yes	6-26	7-3	12's	Yes	200	76.5	23.5	
6	Experimental	5-7	Yes	Yes	6-26	7-3	12's	Yes	204	28.4	71.6	
7	Control	5-6	Moving rocks only	Yes	6-26	7-3	12's	Yes	200	76.5	23.5	
9	Experimental	8-10	Yes	Yes	6-26	7-3	12's	Yes	201	59.7	40.3	
10	Control	8-9	Yes	Yes	6-26	7-3	12's	Yes	202	34.7	65.3	
11	Experimental	12	Yes	Yes	6-26	7-3	12's	Yes	204	99.5	0.5	
12	Control	11	Yes	Yes	6-26	7-3	12's	Yes	209	84.7	15.3	
13	Experimental	14-15	Yes	Yes	6-27	7-3	11's& 12's	Yes	201	83.6	16.4	
15	Control	13-14	Yes	Yes	6-27	7-3	11's& 12's	Yes	200	85.0	15.0	
16	Experimental	17-18	Yes	Yes	6-27	7-3	11's& 12's	Yes	206	96.1	3.9	
18	Control	16-17	Yes	Yes	6-27	7-3	11's& 12's	Yes	205	97.1	2.9	
30	Experimental	31	Yes	Yes	6-29	7-5	11's& 12's	Yes	203	94.6	5.4	
31	Control	30	Yes	Yes	6-29	7-5	11'S& 12'S	Yes	200	97.0	3.0	
39	Experimental	38-40	Yes	Yes	6-30	7-5	10's	Yes	200	72.0	28.0	
40	Control	38-39	Yes	Yes	6-30	7-5	10's	Yes	200	74.5	25.5	

Table 6 (Continued)

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date Eggs Examined	Stage of Development (Piavis)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead
45	Experimental	44-46	Yes	Yes	7-1	7-6	10's	Yes	200	88.5	11.5
46	Control	44-45	Yes	Yes	7-1	7-6	10's	Yes	208	97.6	2.4
86	Experimental	84-85-87	Yes	Yes	7-5	7-12	12's	Yes	200	99.0	1.0
87	Control	84-85-86	Yes	Yes	7-5	7-12	12's	Yes	199	88.9	11.1

Samples

Table 7, D-Phe^2, gly^6 lamprey I (CT990518A)

Batch No.	Group	Same Female used in Batch Number:	Male Observed Spawning (Yes-No)	Female Observed Spawning (Yes-No)	Date Spawned (1999)	Date eggs Examined	Stage of Development (Yes-No)	Normal Development (Yes-No)	Number of Eggs	Percentage Live	Percentage Dead

MANISTIQUE RIVER LAMPREYS (4 POST)(1X)

53	Experimental	54-55	Yes	Yes	7-2	7-7	10's	Yes	200	95.0	5.0
55	Control	53-54	Yes	Yes	7-2	7-7	10's	Yes	200	95.5	4.5
65	Experimental	66-67	Yes	Yes	7-3	7-10	12's	Yes	206	96.6	3.4
67	Control	65-66	Yes	Yes	7-3	7-10	12's	Yes	200	87.5	12.5
68	Experimental	69-70	Yes	Yes	7-3	7-10	12's	Yes	200	99.0	1.0
70	Control	68-69	Yes	Yes	7-3	7-10	12's	Yes	200	99.0	1.0
74	Experimental	75-76	Yes	Yes	7-4	7-11	12's	Yes	200	96.0	4.0
76	Control	74-75	Yes	Yes	7-4	7-11	12's	Yes	202	97.5	2.5
116	Experimental	115-117	No	No	7-13	7-20	--	--	203	0.0	100.0
117	Control	115-116	No	No	7-13	7-20	12's	Yes	208	57.7	42.3

S = observed spawning and artificially spawned

Dead in Inside Spawning Channel

Date 1999	90 unclipped females	60 unclipped males	2X 1 Ant. 1 Post.	1 Ant. 2 Post.	1 Ant. 3 Post.	2 Ant. 1 Post	2X 2 Ant. 2 Post.	2 Ant. 3 Post.
6-13		1			1			
6-16	1							
6-19	1	1						
6-20						1		
6-23	S	S			S		S	S
6-24		1						
6-25	3							
6-26	S S S	S S S		S			S S S S	
6-27	1 S S	S S	S			1	S S	S
6-28	S S S	S S S	S	S				S
6-29	1 S	S					S	
6-30	S S 1	S S 2			S		S	S
7-1	S S 1 S	S S S	S	S S		S S	S	
7-2	S 1 S	S S						S
7-3	S S S S	S S S 1 S S	S S S	S	S S	S		
7-4	S 2 S S	S S S	S	S	S			S S
7-5	S S 2 S	S S S	S		S	S	S	S S
7-6	S S 1	S S S		S	S	S S S		
7-7	1	1						
7-8	3	1						
7-9	S 2	S	S					
7-10	3			S	S S			
7-11	7	1						
7-12	3							
7-13	2		S S	S S		S		
7-14								
			11	10	10	10	11	9

S = observed spawning and artificially spawned

Dead in Outside Spawning Channel

Date 1999	93 unclipped females	77 unclipped males	1 Ant.	1X 2 Ant.	3 Ant.	1 Post	2 Post.	1X 3 Post.	4 Post.
6-8	1								
6-15	3								
6-16	3								
6-17	1				1 some hemorrhage around injection site				
6-18	1							1 sick for several days	
6-19					1			1	1
6-20	2							1	1
6-21	4	1			1	1			1
6-22		2				3 open wound 1/8" at injection site on one	1		
6-23	2	1						4	
6-24				1		2			
6-25	2	1	1		1	1		1	
6-26	3	1	1		2		1		
6-27	2	3	1			1			1
6-27	Survivors		7 (6?)	9	4	2	8	2	6
Moved all clipped females to inside spawning channel									
6-27	⇓ outside ⇓		1						
6-28	1	1		S S 1	S		S S 1	1	
6-29	1	3			1				1
6-30	3	4	S	S 1	1	1	S		
7-01	5	3					1		
7-02	3	3	S	S	1				S
7-03		2					S		S S
7-04	4	7	S						S
7-05	7	2	S				S		
7-06				S					
7-09				S					
7-13			S	S			S		S
			9 SS	10	10	9	10	9	10

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Aquaculture 61231 (2000) xxx

Aquaculture

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The effects of lamprey GnRH-I, -III and analogs on steroidogenesis in the sea lamprey (*Petromyzon marinus*)

Lee Gazourian [a], Everett L. Evans [b], Lee Hanson [c], Cindy F. Chase [a], Stacia A. Sower [a,*]

[a] *Department of Biochemistry and Molecular Biology, University of New Hampshire, Rudman Building, Durham, NH 03824, USA*

[b] *Department of Zoology, University of New Hampshire, Durham, NH 03824, USA*

[c] *Lake Huron Biological Station, Millersburg, MI, USA*

Received 22 July 1998; received in revised form 30 June 1999; accepted 28 January 2000

Abstract

The objective of this study was to examine the in vivo and in vitro effects of lamprey gonadotropin-releasing hormone (GnRH)-I, -III or analogs on steroidogenesis in the adult sea lamprey for the purpose of identifying putative potent agonists and antagonists. In the in vivo studies, the effect on steroid production was examined by injecting males with lamprey GnRH-I, -III and analogs at 8°C and 16°C. The following peptides and analogs were tested: lamprey GnRH-I and -III, [D-Glu^6] lamprey GnRH-I, cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I, cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I, [Gly^6] lamprey GnRH-I, [D-Phe^{2,6}, Pro^3] lamprey GnRH-I, [Phe^2] lamprey GnRH-I, [Trp^3] lamprey GnRH-I, and [Gly^6] lamprey GnRH-III. All peptides tested in vivo, except [Trp^3] lamprey GnRH-I, effectively stimulated plasma oestradiol after 4 h in lampreys held at 8°C or 16°C. In the in vitro studies, lamprey GnRH-I and -III significantly stimulated the pituitary to release a putative gonadotropin capable of stimulating the ovaries to release oestradiol when incubated at 18°C. [D-Glu^6] lamprey GnRH-I at all doses suppressed the putative pituitary response on the testis at 14°C, whereas cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I only suppressed the pituitary at a dose of 100 and 1000 ng/ml. It is suggested from these studies that the actions and differences between the in vivo and in vitro studies on lamprey GnRH-I and -III and analogs are dependent on temperature and/or stage of reproduction likely reflecting differences in metabolic turnover or degradation rates of GnRH, GTH, and/or their receptors.

* Corresponding author. Tel.: +1-603-862-2103; fax: +1-603-862-3784.

PII: S0044-8486(00)00327-6

From these studies, proposed putative agonists/antagonists have been identified that may be used to enhance reproduction in lampreys. Agonists/antagonists will be tested further to determine their ability to inhibit spermatogenesis without destroying the mating competitiveness of males. This would be a valuable tool in a sterile-male release program in the Great Lakes.

Keywords: Gonadotropin-releasing hormone; Steroidogenesis; Sea lamprey

1. Introduction

Over the past 15 years a considerable amount of research has been devoted to the effects of GnRH and analogs on reproduction in fish. Almost all of the research to date has been focused on GnRH-based spawning induction therapy in a number of commercially important species (Zohar, 1989). Brood females of salmon and other valuable species will spawn in captivity, but have difficulties in their spawning and the timing of spawning. By implanting a GnRH agonist into a brood female, a fish farmer can ensure that the female will ripen at the proper time, thus preventing potentially costly guesswork. Progress for induction of spawning using GnRH compounds has been made with such fish species as coho salmon, *Oncorhynchus kisutch* (Sower et al., 1982; Crim and Glebe, 1984), seabass, *Lates calcarifer* (Harvey et al., 1985), common sole, *Solea solea* L. (Ramos, 1986), sablefish, *Anoplopoma fimbria* (Solar et al., 1987), seabream, *Sparus auratus* (Zohar et al., 1995) and many others. Few researchers have examined the ability of GnRH antagonists to sterilize male fish, due to its lack of applications in the field of aquaculture. However, a new method of sterilization would be very useful in the field of sea lamprey control in the Great Lakes. Male sea lampreys are currently being sterilized by an injection of bisazir, a mutagenic chemical. Bisazir is extremely hazardous to humans, therefore a special facility was constructed at the Lake Huron Biological Station, Michigan, expressly for the use of this chemical. In a 1992 Sex Determination/Differentiation Workshop (Sower and Hanson, 1992), sponsored by the Great Lakes Fishery Commission, the identification of a less hazardous method for sterilization was given a high priority.

The potential is present for using GnRH analogs to sterilize male sea lampreys. However, putative lamprey GnRH analogs must first be tested to determine which are reproductively active in the sea lamprey. Reproductive activity can be evaluated by measuring the GnRH analogs' ability to stimulate or inhibit plasma steroid levels in vivo. In addition, a pituitary perifusion method can be used to evaluate pituitary response to various GnRH analogs. Lamprey gonadotropins have yet to be isolated, making it necessary to use indirect measurements of pituitary responsiveness. Oestradiol release from testes sections which were incubated in the pituitary perifusion effluent was used as an indirect measure of pituitary response. Plasma levels of oestradiol and progesterone have been used as indicators of reproductive activity in response to lamprey GnRH injections in both male and female lampreys (Sower, 1989, 1990a,b; Sower et al., 1985a,b). Previous physiological studies in male lampreys (Katz et al., 1982; Fukayama and Takahashi, 1985; Sower, 1989; Sower et al., 1985a,b) and the

demonstrated absence of androgen receptors in the lamprey testis (Ho et al., 1987) suggest that testosterone may not have a role during the final spermatogenic phases in adult male lampreys. As reviewed in Sower (1990a,b, 1997), oestradiol is considered to be one of the major steroids associated with reproductive activity in male sea lampreys. The role of progesterone in male reproductive activity has yet to be determined, although progesterone levels were demonstrated to be significantly higher in males compared to females during final reproductive stages (Linville et al., 1987). Thus, lamprey GnRH analogs that appear to be reproductively active would then be subject to further testing to determine their potential as sterilants.

The primary sequences of two forms of GnRH have been identified in the sea lamprey, lamprey GnRH-I (Sherwood et al., 1986) and lamprey GnRH-III (Sower et al., 1993). Both lamprey GnRH-I and GnRH-III have been demonstrated to act as neurohormones that stimulate the pituitary-gonadal axis in the adult sea lamprey. Ovulatory, spermiation, and steroidogenic responses to lamprey GnRH-I have been well documented in the sea lamprey (Sower, 1989, 1990a,b; Sower et al., 1987). Recent studies testing lamprey GnRH-III have also shown biological activity of this form as determined by increased levels of plasma steroids (Sower et al., 1993; Deragon and Sower, 1994; Gazourian et al., 1997). In lampreys, physiological and immunocytochemical data have clearly shown that lamprey GnRH-I and -III act at the pituitary-gonadal axis (for review see Fahien and Sower, 1990; Sower, 1990a,b; Sower and Larsen, 1991; Sower et al., 1993; Youson and Sower, 1991; Bolduc and Sower, 1992). These data currently suggest that both GnRHs are neurohormones involved in the reproductive processes of the sea lamprey. However, further studies are necessary to elucidate the differential expression and function of lamprey GnRH-I and -III.

The effects of mammalian and lamprey GnRH analogs have been examined in the female sea lamprey. Injections of a synthetic agonist of mammalian GnRH ([D-Ala^6, Pro^9] NEt mammalian GnRH) significantly elevated plasma oestradiol and advanced ovulation by at least several weeks (Sower et al., 1983). In this same study, a mammalian GnRH antagonist ([Ac-3 Pro^1, 4-FD-Phe^2, D-Trp^{3,6}] mammalian GnRH), which is a competitive inhibitor of GnRH in mammalian systems, had no apparent effect on plasma oestradiol concentrations or on timing of ovulation. These data confirm that the receptors for GnRH in the sea lamprey are specific and can distinguish between variants in this molecule. The results of this study were supported by the findings of Sower et al. (1985b), where both female and male sea lampreys were injected with these same analogs. While plasma oestradiol concentrations were elevated in both sexes compared to controls, total androgens were not affected (Sower et al., 1985b). [D-Phe^{2,6},Pro^3] lamprey GnRH was one of the first GnRH analogs tested in lamprey and found to be a putative antagonist. It inhibited ovulation in mature female lampreys, and inhibited spermiation and reduced plasma progesterone levels in the male sea lampreys (Sower, 1989; Sower et al., 1987).

Temperature has been considered an important environmental factor for the final maturational processes in adult sea lampreys (Fahien and Sower, 1990; Bolduc and Sower, 1992). Therefore, one objective of this study was to determine the effects of different temperatures on pituitary responsiveness to lamprey GnRH-I, -III and analogs. Sea lampreys usually do not spawn until the water temperature reaches at least 15°C

(Hanson and Manion, 1978). In the in vivo study, the effects of lamprey GnRH-I, -III and analogs on plasma oestradiol concentrations in the male sea lamprey were examined at 8°C and 16°C. In the in vitro studies the structure–activity relationships of lamprey GnRH-I, -III and analogs were studied at 14°C and 18°C to assess the ability of the pituitary and gonads to distinguish between variant forms of the molecule and to cover the optimal range of temperature of sea lampreys.

2. Materials and methods

2.1. Collection of lampreys

For the in vivo studies conducted during the summers of 1994 and 1995, 150 landlocked lampreys, which averaged 900 g in body weight, were captured from a trap on the Cheboygan River in early June, transported to the Lake Huron Biological Station in Millersburg, Michigan and maintained in cement raceways supplied with flow-through lake water at an ambient temperature range of 8–18°C.

For the in vitro studies, adult sea run lampreys, which averaged 900 g in body weight, were collected in a trap located at the top of the fish ladders at the Cocheco River in Dover, NH, in May 1994 during their upstream spawning migration from the ocean. The animals were transported to the Anadromous Fish and Aquatic Invertebrate Research laboratory in Durham, NH, where they were maintained in an artificial spawning channel supplied with flow-through reservoir water at an ambient temperature range of 13–20°C under natural photoperiod. A total of 40 lampreys was used in these experiments.

2.2. Peptides

Synthetic lamprey GnRH-I was purchased from Peninsula Labs (Belmont, CA). Synthetic lamprey GnRH-III and [Gly^6] lamprey GnRH-III were purchased from American Peptide (Sunnyvale, CA). The lamprey GnRH-I analogs [D-Glu^6] lamprey GnRH-I, cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I, cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I, [Gly^6] lamprey GnRH-I were obtained from Dr. Goodman (UC San Diego) (Sower et al., 1995). [D-$Phe^{2,6}$, Pro^3] lamprey GnRH-I, [Phe^2] lamprey GnRH-I and [Trp^3] lamprey GnRH-I were obtained from Dr. Marshak (Cold Spring Harbor Laboratory) (Sherwood et al., 1986).

2.3. In vivo studies (1994 and 1995)

Twice during the reproductive season, groups of 10 male adult lampreys each were injected with a single dose of either 0.05 or 0.1 μg peptide/g lamprey or 0.6% saline (control). All peptides were dissolved in saline 30 min prior to injection. The following peptides were tested in 1994: lamprey GnRH-I (0.1 μg/g lamprey), lamprey GnRH-III (0.1 μg/g lamprey), lamprey GnRH-I and lamprey GnRH-III combined (0.05 and 0.05 μg/g lamprey), [Phe^2] lamprey GnRH-I (0.05 or 0.1 μg/g lamprey), [Gly^6] lamprey

GnRH-III (0.05 or 0.1 μg/g lamprey) and cyclo-[Glu^6-Trp^7-Lys^8] lamprey GnRH-I (0.1 μg/g lamprey). Two more analogs, [Gly^6] lamprey GnRH-I (0.05 or 0.1 μg/g lamprey) and [Trp^3] lamprey GnRH-I (0.05 or 0.1 μg/g lamprey), were also tested. The following lamprey GnRH analogs were tested in 1995: [Gly^6] lamprey GnRH-III (0.05 or 0.1 μg/g lamprey), [Phe^2] lamprey GnRH-I (0.05 or 0.1 μg/g lamprey) and [D-Glu^6] lamprey GnRH-I (0.05 or 0.1 μg/g lamprey). A 0.5 or 1.0 ml blood sample was collected at 4 and 24 h as previously described by Sower et al. (1985b). After centrifugation, the plasma from individual blood samples was stored at −20°C until assayed for oestradiol and progesterone by radioimmunoassay. Samples of testis were also taken from control lampreys for histological examination (Sower et al., 1985b). The reproductive maturity of each lamprey was assigned to one of seven stages: Stage I, primary spermatocytes; Stage II, primary and dividing primary spermatocytes; Stage III, primary spermatocytes through spermatids; Stage IV, spermatids and immature sperm; Stage V, immature sperm; Stage VI, immature and mature sperm; Stage VII, mature sperm (Fahien and Sower, 1990).

2.4. In vitro procedures

2.4.1. Pituitary and gonad tissue preparation

On the morning of sampling, four lampreys were removed from the spawning channel; the length of each lamprey was measured and blood samples were taken via cardiac puncture as above.

Immediately after blood sampling, the lampreys were decapitated and the pituitaries were removed and immediately placed in Hank's balanced salt solution (HBSS) (Sigma, St. Louis, MO) at pH 7.0 with 25 mM HEPES at 4°C. The total transfer time from pituitary removal to their placement in the perifusion system was less than 45 min. Concurrent with pituitary dissection, the ovaries or testes were removed from the region just posterior to the liver and placed in a petri dish containing HBSS held on ice. The gonads in HBSS were cut into approximately 120 pieces of about 10 mg mass each. The ovary and testes of the lamprey develops in a synchronous manner such that each of the pieces used was in the same reproductive stage reflecting relatively similar steroidogenic potency. The pieces were transferred to fresh HBSS and allowed to preincubate for 2 h at 14°C or 18°C. Fractions of pituitary perifusate collected from the perifusion system (see below) were transferred to a 24-well plate. One gonad section was then added to each well and allowed to incubate for 20 h on a shaker table in an incubator held at 14°C or 18°C.

Another gonad sample was immediately placed in Bouin's solution for histological preparation and examination as described by Sower et al. (1985b). The ovaries were examined and staged according to the method of Bolduc and Sower (1992). Stages were classified as: Stage I, close association of the follicular envelope and oocyte; Stage II, initial separation of the follicular envelope and oocyte; Stage III, complete separation of the follicle layers from the oocyte; Stage IV, oocyte is no longer associated with the follicle cells. The testes were examined and stages identified based on morpology as described by Fahien and Sower (1990).

analogs. Peptides tested included lamprey GnRH-I and -III at concentrations of 1000 ng/ml; and analogs, [D-Glu^6] lamprey GnRH-I, cyclo [Glu^6, Trp^7, Lys^8] lamprey GnRH-I, cyclo [D-Glu^6, Trp^7, Lys^8] lamprey GnRH-I, [Gly^6] lamprey GnRH-I, [Gly^6] lamprey GnRH-III, [D-Phe^{2,6}, Pro^3] lamprey GnRH-I, and [Phe^2] lamprey GnRH-I at concentrations of 10, 100 and 1000 ng/ml. Each injection of GnRH or analogs into the perifusion system was followed by a rinse with 50 μl reservoir buffer to remove residual GnRH from the injection ports before the next injection. All experiments were conducted at temperatures of 14°C and 18°C. The flow rate of buffer through the system was adjusted so that six 400-μl fractions were collected every 6 min.

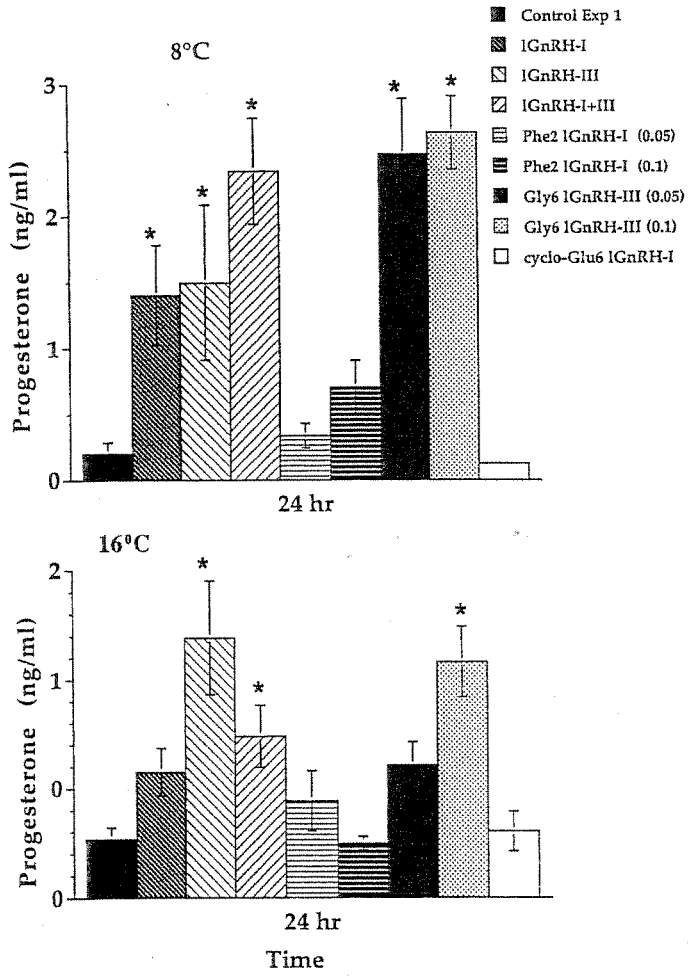

Fig. 2. Plasma progesterone levels (ng/ml) at 24 h of male lampreys injected during the 1994 season with 0.6% saline (control), lamprey GnRH-I, lamprey GnRH-III, lamprey GnRH-I + GnRH-III, [Phe^2] lamprey GnRH-I, [Gly^6] lamprey GnRH-III or cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I. Individual lampreys received a dose of 0.05 or 0.1 μg peptide/g lamprey. Lampreys were maintained in holding tanks at 8°C (top) and 16°C (bottom). Bars depict ± SEM. * Denotes significance at $P < 0.05$.

2.5. In vitro perifusion system

An Acusyst-S multiperifusion system was used to deliver medium at the same rate and temperature to six chambers of constant volume (400 μl). One pituitary was placed into each of chambers 3, 4, 5, and 6 on a steel screen with chambers 1 and 2 acting as controls. A continuous flow of HBSS buffer from the reservoir was pumped for 2 h to obtain the basal rate of hormone secretion before subjecting the pituitaries to GnRH or

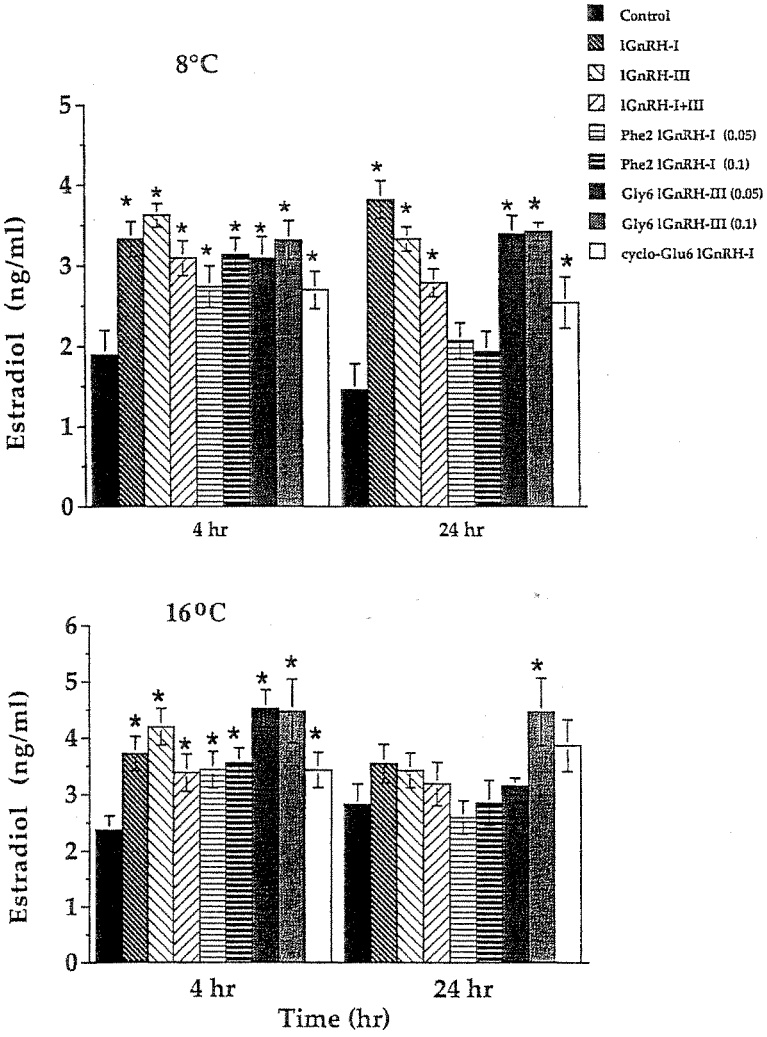

Fig. 1. Plasma oestradiol levels (ng/ml) of male lampreys injected during the 1994 season with 0.6% saline (control), lamprey GnRH-I, lamprey GnRH-III, lamprey GnRH-I + GnRH-III, [Phe^2] lamprey GnRH-I, [Gly^6] lamprey GnRH-III or cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I. Individual lampreys received a dose of 0.05 or 0.1 μg peptide/g lamprey. Lampreys were maintained in holding tanks at 8°C (top) and 16°C (bottom). Plasma samples were taken 4 and 24 h after injection. Bars depict ± SEM. * Denotes significance at $P < 0.05$.

Control experiments were performed to measure the baseline responsiveness of the pituitary to injections of HBSS buffer only (in the absence of GnRH). In addition, tests were performed in which GnRH was injected at a range of doses, in the absence of pituitary, to evaluate the ability of GnRH to stimulate the gonads directly.

2.6. *Radioimmunoassay*

Plasma oestradiol was measured from duplicate 100-μl plasma aliquots by RIA as described by Sower et al. (1983). The lower limit of sensitivity was 78 pg/1.0 ml, with antibody binding efficiencies ranging from 41% to 53% (1994 in vivo) and 44.7–58.9% (1994 in vitro) and from 48% to 53% (1995 in vivo). Plasma progesterone was measured from duplicate 100-μl plasma aliquots by RIA as described by Linville et al. (1987). The lower limit of detection was 78 pg/1.0 ml, with antibody binding efficiencies ranging from 48% to 50%.

2.7. *Statistical analysis*

Differences in hormone concentration were analyzed by Fisher PLSD after preliminary analysis of variance. In all tests, the level of significance for differing groups was $P < 0.05$.

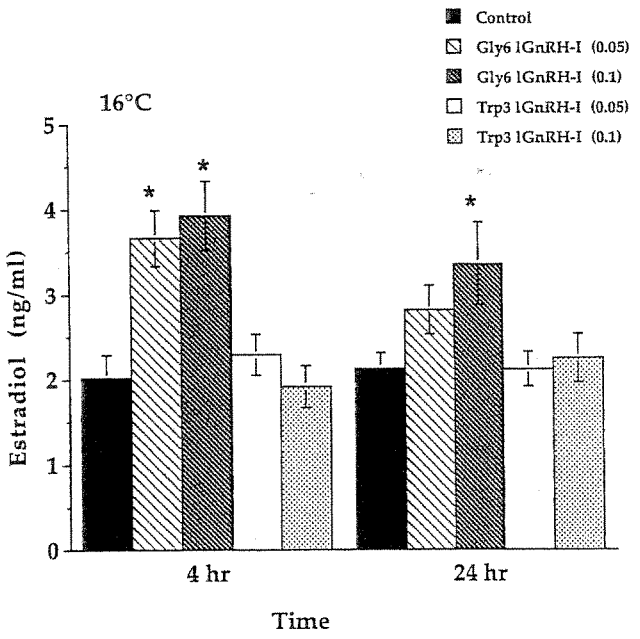

Fig. 3. Plasma oestradiol levels (ng/ml) of male lampreys injected during the 1994 season with 0.6% saline (control), [Gly^6] lamprey GnRH-I or [Trp^3] lamprey GnRH-I. Individual lampreys received a dose of 0.05 or 0.1 μg peptide/g lamprey. Lampreys were maintained in holding tanks at the AFAIR laboratory at 16°C. Plasma samples were taken 4 and 24 h after injection. Bars depict ± SEM. * Denotes significance at $P < 0.05$.

3. Results

3.1. In vivo studies 1994

The water temperature at the first sampling date was 8.0°C and male lampreys were in reproductive stages I and II. All tested GnRH analogs increased plasma oestradiol concentrations significantly compared to controls after 4 h (Fig. 1). After 24 h, only lampreys injected with [Phe^2] lamprey GnRH-I at both concentrations did not show significantly increased plasma oestradiol levels compared to controls. The plasma samples obtained after 4 h did not have sufficient volume to complete progesterone assays, so only samples obtained after 24 h were assayed. After 24 h, only lampreys injected with [Phe^2] lamprey GnRH-I at both concentrations and cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I (0.1 μg/g) did not show significantly increased plasma progesterone concentrations compared to controls (Fig. 2).

The water temperature at the second sampling date was 16°C. Male lampreys were in reproductive stages V and VI. All tested GnRH analogs, with the exception of [Trp^3]

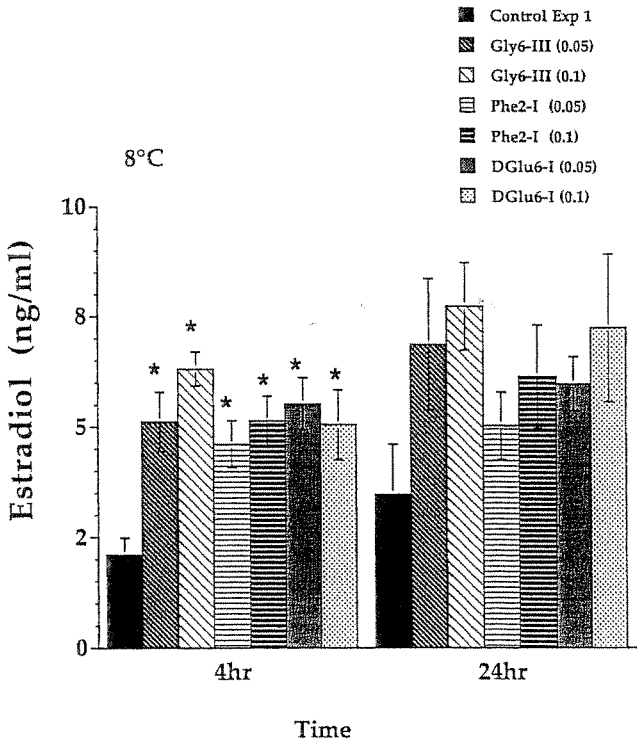

Fig. 4. Plasma oestradiol levels (ng/ml) of male lampreys injected during the 1995 season with 0.6% saline (control), [Gly^6] lamprey GnRH-I, [Phe^2]lamprey GnRH-III or [D-Glu^6] lamprey GnRH-I. Individual lampreys received a dose of 0.05 or 0.1 μg peptide/g lamprey. Lampreys were maintained in holding tanks at 8°C (top) and 16°C (bottom). Plasma samples were taken 4 and 24 h after injection. Bars depict ± SEM. * Denotes significance at $P < 0.05$.

lamprey GnRH-I at both concentrations, increased plasma oestradiol significantly after 4 h compared to controls (Figs. 1 and 3). After 24 h, only the lampreys treated with [Gly^6] lamprey GnRH-I or -III at a dose of 0.1 μg/g lamprey still had significantly elevated plasma oestradiol levels. After 24 h, only the lampreys treated with lamprey GnRH-III, lamprey GnRH-I and -III combined, or [Gly^6] lamprey GnRH-III at a dose of 0.1 μg/g lamprey still had significantly elevated plasma progesterone concentrations (Fig. 2).

3.2. In vivo studies 1995

In 1995, the water temperature at the first sampling date was 8.0°C. Male lampreys were in reproductive stages III through V. All treatment groups had significantly

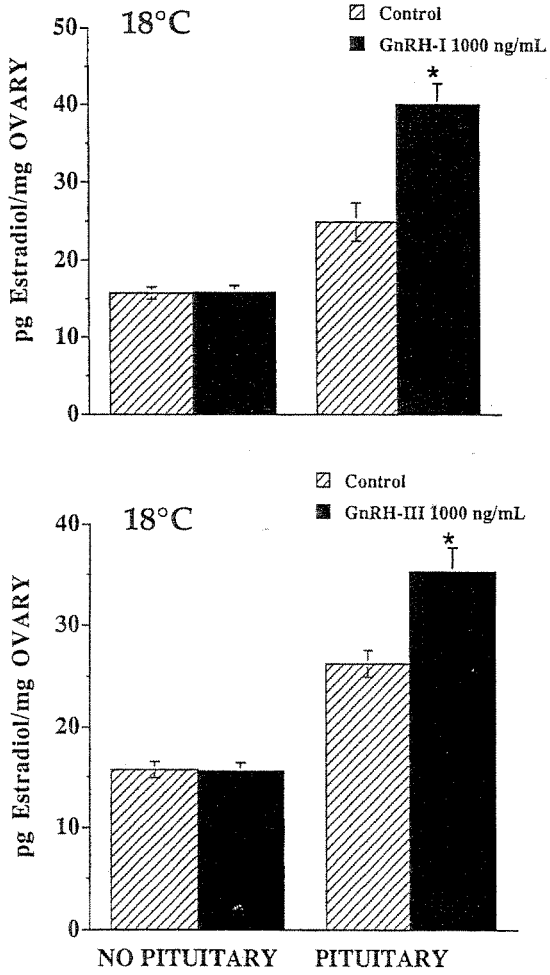

Fig. 5. Ovarian responsiveness to pituitary perifusion effluent at 18°C following injections of 1000 ng/ml lamprey GnRH-I (top) and 1000 ng/ml lamprey GnRH-III (bottom). No pituitary represents the direct effects of GnRH on the ovary and pituitary represents the effects of GNRH on the pituitary. Bars depict ± SEM. * Denotes significance at $P < 0.05$.

increased plasma oestradiol concentrations compared to controls after 4 h (Fig. 4). After 24 h, there were no significant differences in plasma oestradiol levels observed.

The water temperature at the second sampling date was 16°C. Male lampreys were in reproductive stages V and VI. All tested GnRH analogs elevated plasma oestradiol significantly after 4 h compared to controls (Fig. 4). After 24 h, only the lampreys injected with [Gly^6] lamprey GnRH-III (0.05 or 0.1 μg/g lamprey) and [D-Glu^6] lamprey GnRH-I (0.05 μg/g lamprey) had significant increases in plasma oestradiol compared to controls.

3.3. In vitro studies 1994

The response of the pituitary, as measured by an increase of oestradiol release by ovarian tissue incubated at 18°C, was significantly enhanced by lamprey GnRH-I and

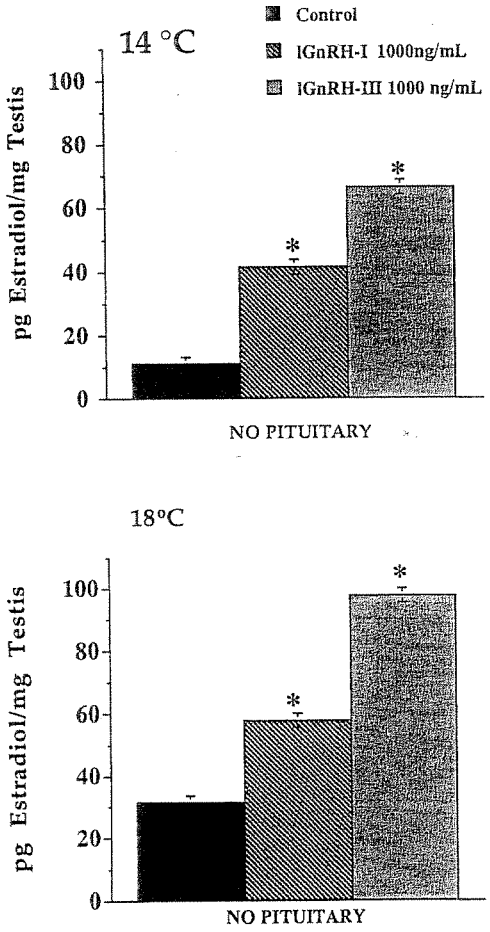

Fig. 6. Testis responsiveness to perifusion effluent at 14°C (A) and 18°C (B) following injections of 1000 ng/ml lamprey GnRH-I and 1000 ng/ml lamprey GnRH-III in the absence of pituitary, which represents the direct effect on the testis. Bars depict ±SEM. * Denotes significance at $P < 0.05$.

-III at 1000 ng/ml ($P < 0.05$) (Fig. 5). In addition, lamprey GnRH-III at 1000 ng/ml directly stimulated the ovaries incubated at 14°C ($P < 0.05$) (data not shown).

In the absence of a pituitary, both lamprey GnRH-I and lamprey GnRH-III at 1000 ng/ml demonstrated a direct effect on the testis incubated at 14°C ($P = 0.0077$ and $P = 0.0004$, respectively) and at 18°C ($P = 0.0013$ and $P = 0.0011$, respectively) (Fig. 6).

A significant decrease of oestradiol release from testis incubated at 14°C was noted following pituitary perifusion with [D-Glu^6] lamprey GnRH-I at 10, 100, and 1000 ng/ml ($P < 0.05$). Cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I at 100 ng/ml and 1000

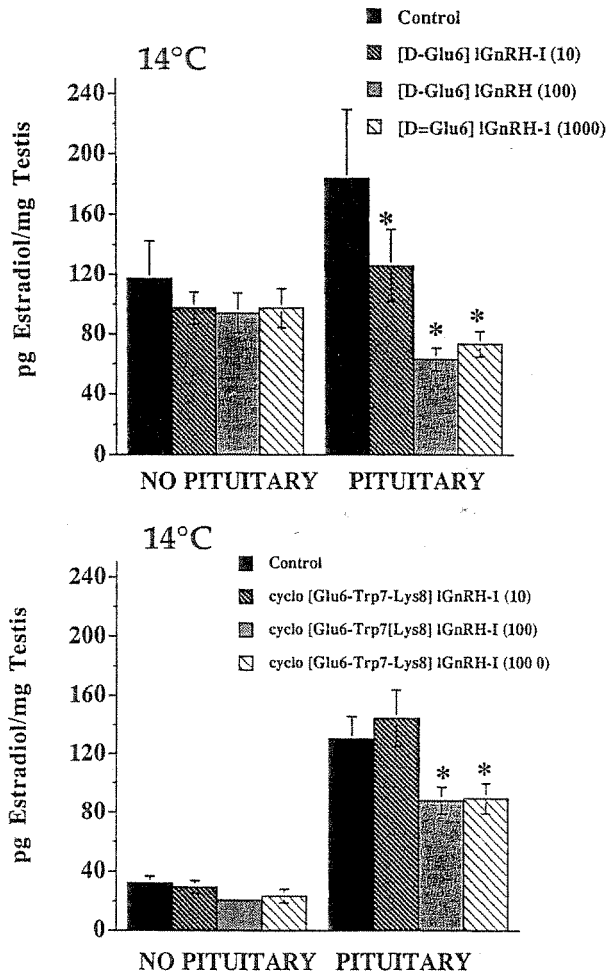

Fig. 7. Testis responsiveness to pituitary perifusion effluent at 14°C following injections of Control (0.6% saline) or 10, 100 or 1000 ng/ml [D-Glu^6] lamprey GnRH-I (top) or cyclo [Gly^6-Trp^7-Lys^8] lamprey GnRH-I (bottom). No pituitary represents the direct effects of GnRH on the testis and pituitary represents the effects of GnRH on the pituitary. Bars depict ±SEM. * Denotes significance at $P < 0.05$.

Table 1
Direct effects of GnRH analogs at 14°C
Testis responsiveness to perifusion effluent in the absence of pituitary at 14°C following injections of Control (0.6% saline) or 10, 100 or 1000 ng/ml [D-Glu^6] lamprey GnRH-I, cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I, cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I, [Gly^6] lamprey GnRH-I, [Gly^6] lamprey GnRH-III, [D-Phe^{2,6}, Pro^3] lamprey GnRH-I and [Phe^2] lamprey GnRH-I.

	Control	10 ng/ml	100 ng/ml	1000 ng/ml
[D-Glu^6] lamprey GnRH-I	117.3 ± 25.3	97.4 ± 10.6	94.1 ± 13.3	97.2 ± 13.1
Cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I	32.0 ± 4.8	29.7 ± 4.2	20.9 ± 2.0	23.5 ± 4.7
Cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I	26.3 ± 4.1	88.0 ± 29.6*	41.8 ± 7.1	74.0 ± 8.0*
Gly^6 lamprey GnRH-I	14.8 ± 2.0	77.0 ± 9.7*	63.4 ± 10.1*	102.0 ± 19.6*
Gly^6 lamprey GnRH-III	12.6 ± 3.0	19.8 ± 2.7	26.8 ± 3.4*	65.2 ± 10.7*
D-Phe^{2,6}, Pro^3 lamprey GnRH-I	22.7 ± 6.6	17.4 ± 2.7	8.1 ± 1.8	32.7 ± 11.5
Phe^2 lamprey GnRH-I	2.1 ± 0.5	3.3 ± 0.6	4.6 ± 1.7	34.2 ± 12.2*

* Denotes significance at $P < 0.05$.

ng/ml also significantly diminished pituitary responsiveness of the testis incubated at 14°C ($P < 0.05$) (Fig. 7).

In addition, cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I at 10 and 1000 ng/ml, [Gly^6] lamprey GnRH-I at 10, 100, and 1000 ng/ml, [Gly^6] lamprey GnRH-III at 100 and 1000 ng/ml, and [Phe^2] lamprey GnRH-I at 1000 ng/ml directly stimulated the testis incubated at 14°C ($P < 0.05$) (Table 1). [D-Glu^6] lamprey GnRH-I at 100 and 1000 ng/ml, [Gly^6] lamprey GnRH-I at 10, 100, and 1000 ng/ml, [Gly^6] lamprey GnRH-III at 1000 ng/ml, [D-Phe^{2,6}, Pro^3] lamprey GnRH-I at 10 and 1000 ng/ml and [Phe^2] lamprey GnRH-I at 1000 ng/ml directly stimulated the testis incubated at 18°C ($P < 0.05$) (Table 2). The differences in the oestradiol concentrations of the controls between the two tables likely reflect the differences in the incubation temperatures of the testes.

Table 2
Direct effects of GnRH analogs at 18°C
Testis responsiveness to perifusion effluent in the absence of pituitary at 18C following injections of Control (0.6% saline) or 10, 100 or 1000 ng/ml [D-Glu^6] lamprey GnRH-I, cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I, cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I, [Gly^6] lamprey GnRH-I, [Gly^6] lamprey GnRH-III, [D-Phe^{2,6}, Pro^3] lamprey GnRH-I and [Phe^2] lamprey GnRH-I.

	Control	10 ng/ml	100 ng/ml	1000 ng/ml
[D-Glu^6] lamprey GnRH-I	17.7 ± 4.3	24.7 ± 2.2	37.8 ± 6.6*	52.5 ± 11.8*
Cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I	174.0 ± 36.0	128.7 ± 24.4	151.4 ± 46.2	114.0 ± 28.2
Cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I	35.1 ± 12.0	45.1 ± 11.9	57.2 ± 26.0	73.0 ± 15.0
Gly^6 lamprey GnRH-I	5.7 ± 1.0	22.3 ± 4.2*	25.3 ± 4.8*	31.3 ± 5.9*
Gly^6 lamprey GnRH-III	84.3 ± 9.2	135.0 ± 20.8	115.9 ± 22.4	212.9 ± 26.0*
D-Phe^{2,6}, Pro^3 lamprey GnRH-I	26.0 ± 5.3	57.6 ± 7.6*	33.3 ± 6.9	54.5 ± 9.2*
Phe^2 lamprey GnRH-I	43.9 ± 4.9	57.1 ± 4.9	96.9 ± 14.2	158.1 ± 52.1*

* Denotes significance at $P < 0.05$.

4. Discussion

The aim of this study was to examine the effects of lamprey GnRH-I, -III and analogs on steroidogenesis in the adult sea lamprey. The results of these data suggest that the third and sixth positions of lamprey GnRH-I and the sixth position of lamprey GnRH-III are important for function, because they affect the secretion of steroids from gonads. The actions of lamprey GnRH-I and -III and analogs appeared to be dependent on temperature and/or stage of reproduction likely reflecting differences in metabolic turnover or degradation rates of GnRH, GTH, and/or their receptors. From these studies, potential or putative agonists/antagonists have been identified that can be used to enhance reproduction in lampreys, as well as to be further tested for use in inhibiting spermatogenesis for eventual use in a sterile-male release program in the Great Lakes.

Two forms of GnRH have been characterized and sequenced in the sea lamprey, lamprey GnRH-I and -III (Sherwood et al., 1986; Sower et al., 1993). Unlike in most other vertebrate species, there is compelling immunocytochemical and physiological evidence which indicates that both lamprey GnRH-I and -III act through the hypothalamic-pituitary-gonadal axis to modulate reproductive processes in the sea lamprey (Fahien and Sower, 1990; Sower, 1990a,b; Sower and Larsen, 1991; Sower et al., 1993; Youson and Sower, 1991; Bolduc and Sower, 1992; Deragon and Sower, 1994). Previous studies have shown that GnRH analogs do affect reproductive behavior directly and indirectly, and it is likely that lampreys have differential regulation of GnRH on reproduction and behavior (Sower et al., 1992). Thus, it is necessary to assess the activities of various GnRH analogs and determine whether an analog can inhibit spermiation without affecting reproductive behavior in males.

In the current in vivo study, the effects of lamprey GnRH-I, -III and analogs on plasma oestradiol in male landlocked lamprey were determined at different temperatures and different stages of reproduction. Both lamprey GnRH-I and lamprey GnRH-III significantly elevated plasma oestradiol levels for 24 h at 8°C, but not at 16°C. This is consistent with a previous study, where injections of lamprey GnRH-I significantly elevated plasma oestradiol levels in male sea lampreys for up to 48 h at a low temperature, 10°C (Sower, 1989). In female sea lampreys, it was found that plasma oestradiol remained significantly elevated for 24 h after injections of lamprey GnRH-I and -III at 13°C, but not at 19°C (Gazourian et al., 1997). These combined data suggest a greater metabolic turnover or degradation of lamprey GnRH, GTH or their respective receptors at higher temperatures or later stages of reproductive maturity. In the in vitro study, lamprey GnRH-I and -III significantly stimulated the pituitary to release a putative gonadotropin capable of stimulating the ovaries to release oestradiol when incubated at 18°C. [D-Glu^6] lamprey GnRH-I at all doses suppressed pituitary response on the testis at 14°C, whereas cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I only suppressed the pituitary at a dose of 100 and 1000 ng/ml. As stated earlier, it was expected that the cyclized analogs would assume the active binding conformation of the mammalian GnRH peptide. It is proposed that the constrained analogs may interact with the pituitary GnRH receptor that may inhibit putative gonadotropin release or cause the release of a substance capable of inhibiting steroidogenesis in the lamprey testis.

As stated earlier, lamprey GnRH-I and lamprey GnRH-III are the only vertebrate GnRHs with amino acid substitutions in the sixth position, Glu and Asp, respectively (Sower et al., 1993). Thus, in earlier studies, cyclized GnRH analogs were examined to test whether the close proximity of the N and C terminus is important for binding of GnRH to its receptor in lampreys. Sower et al. (1995) determined the in vivo effects of two lamprey GnRH-I analogs with substitutions of D-glutamate and glycine in the sixth position of the molecules, [D-Glu^6] lamprey GnRH-I and [Gly^6] lamprey GnRH-I, respectively. Two additional analogs, cyclo-[Glu^6-Trp^7-Lys^8] lamprey GnRH-I and cyclo-[D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I, with their respective R groups linked by amide bonds at position six and eight were also studied to determine how restricting the flexibility of the molecule would influence its activity. The lamprey forms are the only members of the vertebrate GnRH family which do not have glycine in the sixth position. In the Sower et al. (1995) study, [Gly^6] lamprey GnRH-I acted antagonistically by delaying ovulation by 3 weeks as compared to controls, while [D-Glu^6] lamprey GnRH-I advanced ovulation. All GnRH analogs tested significantly elevated plasma oestradiol levels compared to controls suggesting that the sixth position of the lamprey GnRH peptide is important for its function. The suggested active conformation of mammalian GnRH contains a type IIβ-bend at the level of Gly^6–Leu^7 which brings the putative binding sites on the amino and carboxy termini into proximity (Struthers et al., 1985). The small R-group (a single hydrogen atom) of the sixth position glycine is at the inside of this β-bend, therefore a bulkier R-group would sterically force the conformation of the molecule out of the putative active position (Gupta et al., 1993). Lamprey GnRH-I and lamprey GnRH-III have glutamate and aspartate in the sixth position, respectively. Therefore it is possible that lamprey GnRH has a different conformation compared to the putative conformation of the other members of the vertebrate GnRH family.

Even though gonadotropins have not yet been isolated from lamprey pituitaries, there is substantial direct and indirect evidence of pituitary responsiveness to lamprey GnRH. The first direct evidence of GnRH stimulating the pituitary was provided by Knox et al. (1994) in which the lamprey pituitary was shown to contain two high-affinity binding sites for GnRH. In lampreys, GnRH is considered to diffuse from the neurohypophysis to the anterior pituitary controlling pituitary-gonadal function and does not travel via the systemic circulation. This is supported by studies in which lamprey GnRH-I and -III have not been detected in plasma (Millar and King, 1987; Fahien and Sower, 1990; Sower, unpublished), nor by anatomical diffusion studies (Nozaki et al., 1994). However, the question remains as to whether there is a GnRH-like factor produced in gonads and whether GnRH administered intraperitoneally has potentially any direct effect on the gonads. Gazourian et al. (1997) showed that lamprey GnRH-III at 100 and 1000 ng/ml stimulated oestradiol secretion from both lamprey ovaries and testis in vitro. This same study also provided evidence for the presence of a high affinity/high capacity GnRH binding site in the gonads of the adult sea lamprey. These studies suggest that GnRH or a GnRH-like factor may be produced locally in the gonads of the adult sea lamprey and act in a paracrine/autocrine fashion to modulate gonadal function.

The effects of lamprey GnRH analogs on steroidogenesis would be expected to differ from the effects of the native molecules for several reasons. Substitutions in the native molecule could increase resistance to enzyme degradation, which would lead to an

extended half-life and increased potency. Substitutions of novel amino acids could also affect the conformational structure of the molecule. Structural modifications in the GnRH molecule can affect molecule/receptor interactions in many ways. These changes in structure may promote the conformation necessary for receptor interaction, or the changes may lead to an inactive conformation, which is unable to bind to and/or activate the receptor.

In this study GnRH analogs which had modifications in the second and third positions of the native molecule were tested. The putative binding domains of the mammalian GnRH molecule are considered the amino and carboxy termini (Struthers et al., 1985), therefore substitutions of amino acids in these termini may affect receptor binding and/or activation. It has been found that potent mammalian GnRH antagonists usually contain substitutions in the second and/or third positions (Heber and Swerdloff, 1984). In the present study, the activity of [Phe^2] lamprey GnRH-I, [Trp^3] lamprey GnRH-I and others was examined. [Phe^2] lamprey GnRH-I injected in vivo elevated plasma oestradiol levels after 4 h, but had no effect after 24 h. In the in vitro studies, [Phe^2] lamprey GnRH-I only stimulated oestradiol production with 1000 ng/ml at 14°C and 18°C. Since this analog initially had a stimulatory effect on plasma oestradiol levels and acted directly on the testis, it apparently was able to bind, and subsequently activate, the GnRH receptor. The inability of this analog to sustain elevated plasma oestradiol levels for 24 h suggests that this analog was susceptible to enzymatic degradation which shortened its plasma half-life. The presence of an endopeptidase capable of degrading mammalian GnRH analogs at the His^2–Trp^3 position has been suggested (Brudel et al., 1994); however, it is not known whether this enzyme is active in the lamprey system. Lamprey GnRH-I is the only member of the vertebrate GnRH family to have an amino acid other than tryptophan in the third position. In the present study, replacement of the native Tyr^3 of lamprey GnRH-I with tryptophan rendered the analog completely inactive, suggesting that the third position of lamprey GnRH-I is critical for binding and/or activation of the receptor.

In the present study, the effects of [Gly^6] lamprey GnRH-I, [Gly^6] lamprey GnRH-III, [D-Glu^6] lamprey GnRH-I and cyclo-[Glu^6-Trp^7-Lys^8] lamprey GnRH-I were examined in the male sea lamprey. Substitution of the native sixth-position amino acid of lamprey GnRH-I or -III with glycine resulted in increased potency of the analogs for 24 h in vivo. Cyclo-[Glu^6-Trp^7-Lys^8] lamprey GnRH-I or [D-Glu^6] lamprey GnRH-I also stimulated plasma oestradiol in vivo. In the in vitro studies, [Gly^6] lamprey GnRH-I at all doses and [Gly^6] lamprey GnRH-III at 100 and 1000 ng/ml directly stimulated oestradiol production in the testis of the male lamprey incubated at 14°C. At 18°C, only 1000 ng/ml of [Gly^6] lamprey GnRH-I and -III directly elevated oestradiol production. These data support the in vivo data with both [Gly^6] lamprey GnRH-I and -III elevating oestradiol levels. In addition, cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I at 10 and 1000 ng/ml directly stimulated the testis at 14°C, whereas [D-Glu^6] lamprey GnRH-I at 100 and 1000 ng/ml significantly stimulated oestradiol production at 18°C. Sower et al. (1995) also showed that cyclo [D-Glu^6-Trp^7-Lys^8] lamprey GnRH-I and [D-Glu^6] lamprey GnRH-I elevated oestradiol levels in vivo. The lower activity of [Gly^6] lamprey GnRH-I and -III at 18°C, as compared to 14°C, may be due to increased enzymatic degradation of the peptide or the inability of the peptide to interact with the receptor,

which may be enhanced at lower temperatures. In the in vivo studies, it is possible that the noted increase in oestradiol was due to both the direct activation of the GnRH analogs on steroidogenesis in the testis of the lamprey and the action of the lamprey GnRH analog acting through the pituitary-gonadal axis in the lamprey.

It is proposed that the substitution of Gly^6 may have modified the structure of the molecule, possibly promoting the conformation required for receptor interaction, or that the substitution of Gly^6 augmented the resistance of the molecule to enzymatic degradation. Enzymatic degradation of both mammalian GnRH and salmon GnRH primarily results in cleavage of the Tyr^5–Gly^6 or Gly^6–Leu^7 bond (Goren et al., 1990). If these enzymes are present in the sea lamprey, a substitution of the less bulky glycine in the sixth position should have resulted in increased degradation and decreased activity of the molecule. Since the Gly^6 substituted analogs consistently acted as the more potent analogs, this suggests that there may be different enzymes at work in the sea lamprey compared to other vertebrates.

In summary, all the GnRH analogs tested are likely candidates for further testing as potential sterilants for use in the sterile male release program. Based on these and other mammalian and teleost studies, our data suggest that other analogs with substitutions of bulky aliphatic amino acids in the second, third and sixth position of lamprey GnRH-I and -III should also be tested.

5. Uncited reference

Abrams, 1991

Acknowledgements

This research was supported by NSF MCB 9722765, Great Lakes Fisheries Commission, a summer undergraduate research fellowship to Lee Gazourian and a scientific contribution No. 1987 from the New Hampshire Agricultural Experiment Station.

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Fish. Phys. Biochem.