GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

Effects of Lamprey GnRH-I and -III on Reproductive Processes and Behavior of Male Sea Lampreys

by:

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FINAL REPORT

TO:

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

PROJECT TITLE:

Effects of lamprey GnRH-I and III on reproductive processes and behavior of male sea lampreys

OR

Potential Use of GnRH Analogs for Sterilizing Lampreys

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TIME PERIOD: Jan, 1994 to Feb, 1996

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FINAL REPORT

I. OVERALL SUMMARY

A. Objectives of Research:

The overall goal of this research program has been to investigate the biological effects of lamprey gonadotropin-releasing hormone (GnRH) and analogs on reproductive processes and behavior in adult male sea lampreys (*Petromyzon marinus*) to determine the possibilities of using GnRH analogs for sterilizing lampreys. The specific objectives included 1) to determine the effects of injected encapsulated lamprey GnRH-I and -III and analogs on steroidogenesis and spermatogenesis in male sea lampreys and 2) to determine the effects of lamprey GnRH-I and -III analogs (antagonists) on the nesting and spawning behavior of adult males and on the development of the eggs fertilized by treated males.

B. Summary:

In our first set of experiments, *in vivo* and *in vitro* studies were completed to test the effects of lamprey GnRH analogs on plasma steroid levels. The data from these experiments showed that there were significant differences in steroid responses to the various GnRH analogs tested. In a second set of experiments, we demonstrated that GnRH incorporated into microspheres and then injected intramuscularly is the most effective delivery system. This system allows a continuous slow release of GnRH in lampreys during the entire spawning season. In our last set of experiments, we determined the effects of two different GnRH analogs on the nesting and spawning behavior of adult males and on the development of the eggs fertilized by treated males. There were no significant differences of the survival of the eggs fertilized by treated males compared to controls.

C. 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 to 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.

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 have developed a delivery system that allows the GnRH analog to be released in the lamprey during the spawning season following a single injection.

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. We would have to clone the GnRH receptor(s) in lamprey in order to do the molecular (mutagenic) studies. These procedures are currently being developed in my laboratory. If this work was reconsidered for funding by the GLFC,we would propose that the structural modeling work would have to be done in collaboration with a private company on developing GnRH antagonists as a sterilant .

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 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 and act in a differential manner, we propose that an analog can be developed in which the spawning behavior would not be affected, yet the lampreys would be sterilized.

II. RATIONALE

GnRH is a hypothalamic decapeptide which regulates reproduction in vertebrates by stimulating the release of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which, in turn, stimulate gonadal activity. Since 1971, when the primary structure of mammal GnRH (or LHRH) was determined, over 7000 analogs have been made to GnRH and tested in hundreds of studies in mammals. Analogs are variants of the GnRH molecule with different substitutions of amino acids that can either make the GnRH a more potent agonist or an antagonist. In mammals there has been great success using various mammal GnRH analogs for sterilization, conception and other therapeutic and clinical applications. As an example, Lupron Depot, a GnRH antagonist, is now one of the leading chemical treatments for advanced prostate cancer and endometriosis. In humans, it is given as a monthly injection because it has been microencapsulated. Continuous treatment of lupron depot results in decreased levels of LH and FSH. In males, testosterone is reduced to castrate levels. In pre-menopausal females, estrogens are reduced to post-menopausal levels.. The most active synthetic agonists are found to be those with D-amino acid substitution in position 6. The most effective GnRH antagonists are those that also have substitutions in position 6 as well as substitution of amino acids in positions 1, 2 and 3.

We have identified the primary structure of GnRH-I (Sherwood et al.,1986) and GnRH-III (Sower et al., 1993) in the sea lamprey. This structural information combined with later immunocytochemical (Nozaki and Kobayashi, 1979; Nozaki et al., 1984; Crim et al., 1979; King et al., 1988; Wright et al., 1994; Tobet et al., 1995) and physiological studies (Review: Sower, 1990; Fahien and Sower, 1990; Sower and Larsen, 1991; Youson and Sower, 1991; Bolduc and Sower, 1992; Sower et al., 1993; Deragon and Sower, 1994; Sower et al., 1995) provide evidence for the regulatory influence of the hypothalamus on the pituitary-gonadal axis. However, further studies are needed to determine the function of lamprey GnRHs, particularly lamprey GnRH-III.

Similar to research in mammals, GnRH and analogs have been used in various applications in fish, including aquaculture and fish management. We propose that lamprey GnRH analogs can eventually be used for sterilizing lampreys in a sterile-male-release program in the Great Lakes. In the Great Lakes, lampreys are considered a major deterrent to fish populations because of the lamprey's parasitic-phase in which it feeds on other fish with its suctorial mouth and extracts body fluid often causing high mortalities. The extraordinary amount of damage to the fishery of the Great Lakes caused by the invasion of the sea lamprey has resulted in one of the largest and most intensive efforts to control a vertebrate predator that has ever been attempted (Strategic Vision of the Great Lakes Fishery Commission for the Decade of the 1990s, Feb. 1992). The estimated damage by the lamprey to the sport and commercial fisheries in the Great Lakes has been estimated at over two billion dollars during the past ten years (Gavin Christe, GLFC, personal communication). The lampreys are believed to have invaded the Upper Great Lakes starting with the opening of the Erie Canal in 1819 or the Welland Canal in 1829 which allowed the movement of fish from Lake Ontario into the Upper Great Lakes (Lamsa et al., 1980). By the 1930's the lampreys had established themselves in all the Great Lakes. The Great Lakes Fishery Commission was established in 1955 by a treaty between Canada and the U.S. The two major responsibilities of this Commission were and continue to be: 1) to develop coordinated programs of research in the Great Lakes, and 2) to formulate and implement programs to eradicate or minimize sea lamprey populations in the Great Lakes. In the 1950s, the nonindigenous sea lamprey, among other pressures, had all but decimated the sport and commercial fisheries of the Great Lakes (Lamsa et al., 1980). In 1957, a chemical (TFM) was discovered that killed larval lampreys without harming other organisms. Since that time, the use of selective toxicants to kill larval lampreys in streams has successfully controlled lamprey populations in the Great Lakes. However, there is increasing

concern about increasing numbers of lampreys and the continued use of chemicals in the environment. Therefore, the Commission, which directs the sea lamprey control program, is looking for other control methods which will reduce or eliminate dependency on lampricides as a control method. The sterility method of pest control is currently being used on Lake Superior and the St. Mary's River as a supplemental control method. Bisazir is a chemical compound that has been researched (Hanson and Manion 1978; 1980; Hanson, 1981) and is currently being used to sterilize males in a sterile-male-release program. Because this compound is extremely hazardous to humans, a special facility was constructed at the Hammond Bay Biological Station, Michigan, for use of this chemical. The GLFC would like to terminate the use of bisazir and develop other chemosterilants that are non-hazardous. The potential of using GnRH analogs as sterilants 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 addition, GnRH analog should easily be approved by the FDA because this agency has now approved the use of GnRH analogs in food fish to enhance reproduction in aquaculture conditions. Thus, there is excellent promise from our studies that a lamprey GnRH analog may present a viable alternative to 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.

III. EXPERIMENTS

A. 1994 and 1995 Experiments

The *in vivo* experiments were done at the Hammond Bay Biological Station by Lee Hanson, Everett Evans (graduate student) and Stacia Sower and the *in vitro* experiments were done at the University of New Hampshire by Lee Gazourian (undergraduate student) and Stacia Sower during May through July, 1994. In 1995, experiments were conducted at Hammond Bay Biological Station (HBBS) by Lee Hanson and Everett Evans. Lampreys were captured from a trap on the Cheboygan River in early June, transported to HBBS, and maintained in raceways with flow-through lake water at ambient temperature ranging from 7 to 19°C.

Experiment I: *In vivo* studies on lamprey GnRH and analogs in male lampreys

The objective of this study was to test the effects of lamprey GnRH analogs on plasma steroid levels at two different water temperatures of 8°C and 16°C. In 1994, lampreys were tested with a single 0.1 ml intraperitoneal injection of saline (control) or one of the following treatments: lamprey GnRH-I (0.1μg/g Body Weight); lamprey GnRH-III (0.1μg/g BW); lamprey GnRH-I+III (0.05 + 0.05 μg/g BW); Phe^2 lamprey GnRH-I (0.05 μg/g BW); Phe^2 lamprey GnRH-I (0.1 μg/g BW); Gly^6 lamprey GnRH-III (0.05 μg/g BW); Gly^6 lamprey GnRH-III (0.1 μg/g BW); or cyclo-[Glu^6-Trp^7-Lys^8] lamprey GnRH-I (0.1 μg/g BW).

In 1995, lampreys were tested with a single 0.1 ml intraperitoneal injection of saline (control) or one of the following treatments: Gly^6 lamprey GnRH-III (0.05 μg/g BW); Gly^6 lamprey GnRH-III (0.1 μg/g BW); Phe^2 lamprey GnRH-I (0.05 μg/g BW); Phe^2 lamprey GnRH-I (0.1 μg/g BW); D-Glu^6 lamprey GnRH-I (0.05 μg/g BW); or D-Glu^6 lamprey GnRH-I (0.1 μg/g BW). Ten lampreys were used for each treatment group. Lampreys were sampled for blood at 4 and 24 hours after injection. The plasma samples were stored at -20°C until assayed by radioimmunoassay for estradiol.

Ten lampreys were used for each treatment group. Lampreys were sampled 4 and 24 hours after the injection. 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 for estradiol and progesterone.

Some of the steroid data are shown in Figs 1 and 2. The data are presented as means of each treatment group with standard error bars. In summary, all analogs tested effectively stimulated plasma estradiol in lampreys held at 8°C or 16°C at 4 hrs. At 24 hrs, all GnRH analogs, except Phe^2 lamprey GnRH-I (1994 and 1995) and DGlu^6 lamprey GnRH-I at 0.1 (1995), significantly increased plasma estradiol in lampreys held at 8°C.

Experiment 2: *In vitro* studies on lamprey GnRH and analogs in male lampreys

The objective of this study was to examine the effects of lamprey GnRH-I and -III and analogs on the hypothalamus-pituitary axis using an Acusystem-S multiperifusion system. Indirect measurement of the pituitary response to GnRH was accomplished by measuring estradiol released from testes sections which were incubated in the perifusion effluent. In addition to native lamprey

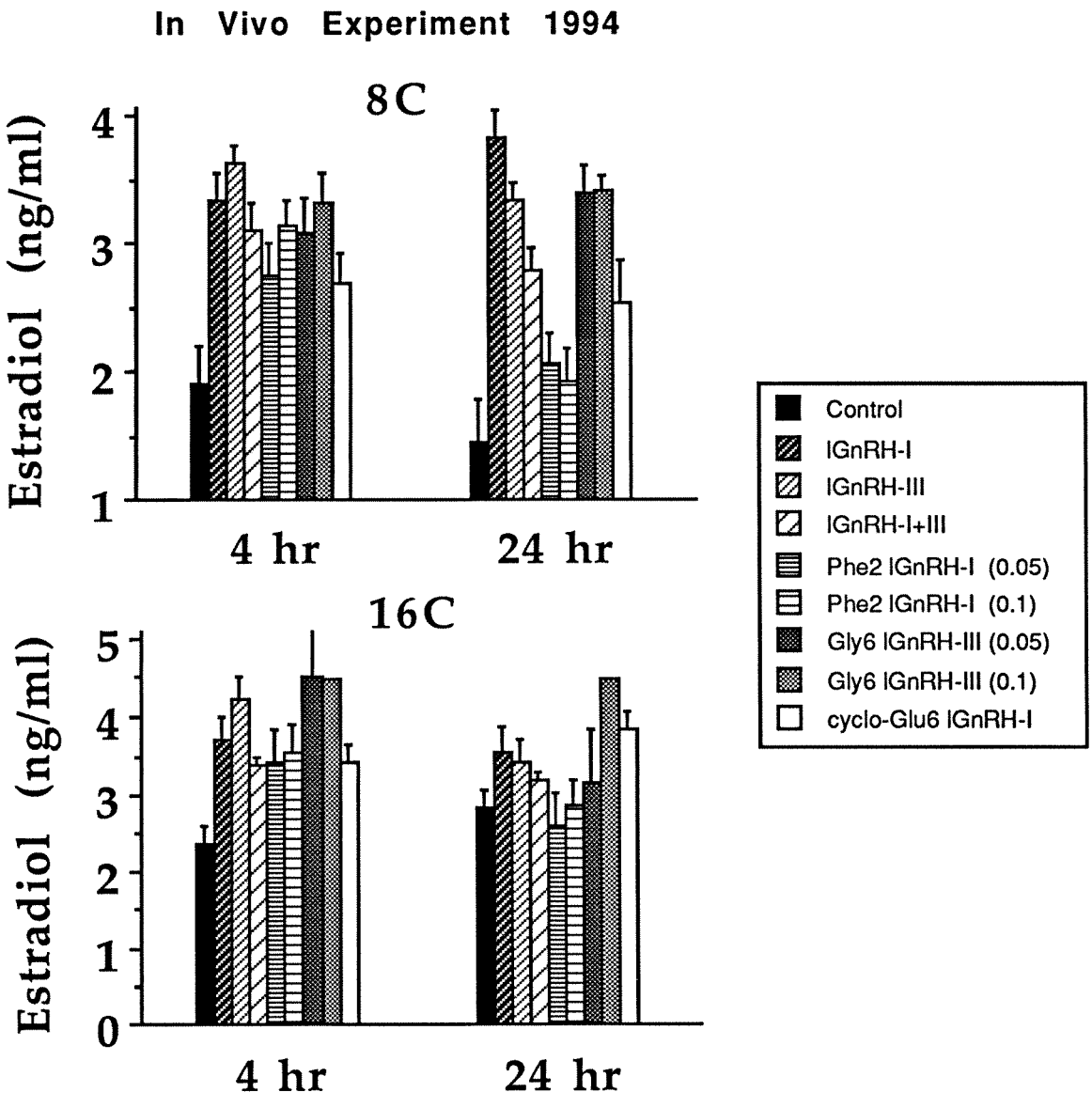
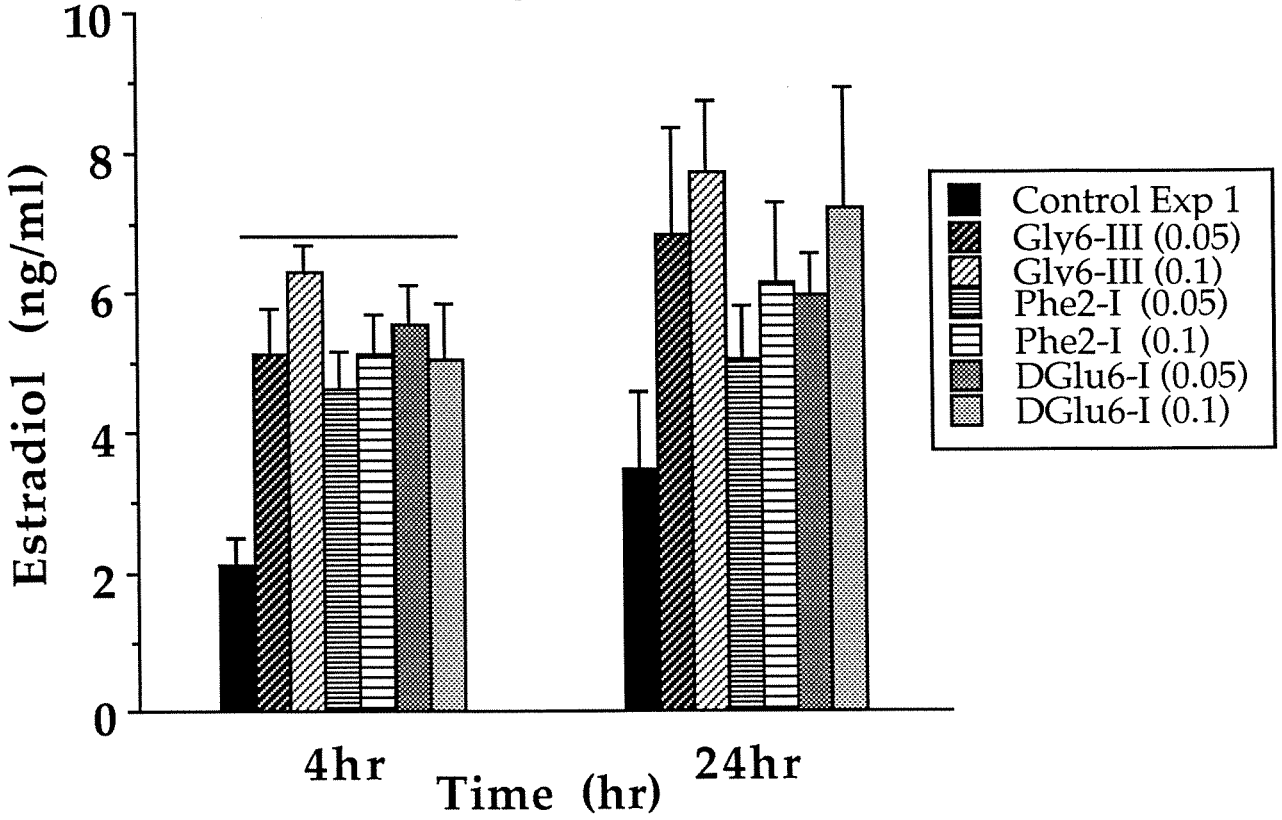

In Vivo Experiment 1994
8C
Estradiol (ng/ml)
4
3
2
1
4 hr
24 hr
16C
Estradiol (ng/ml)
5
4
3
2
1
0
4 hr
24 hr
Control
lGnRH-I
lGnRH-III
lGnRH-I+III
Phe2 lGnRH-I (0.05)
Phe2 lGnRH-I (0.1)
Gly6 lGnRH-III (0.05)
Gly6 lGnRH-III (0.1)
cyclo-Glu6 lGnRH-I

Fig. 1

In Vivo Experiments 1995

8°C



16°C

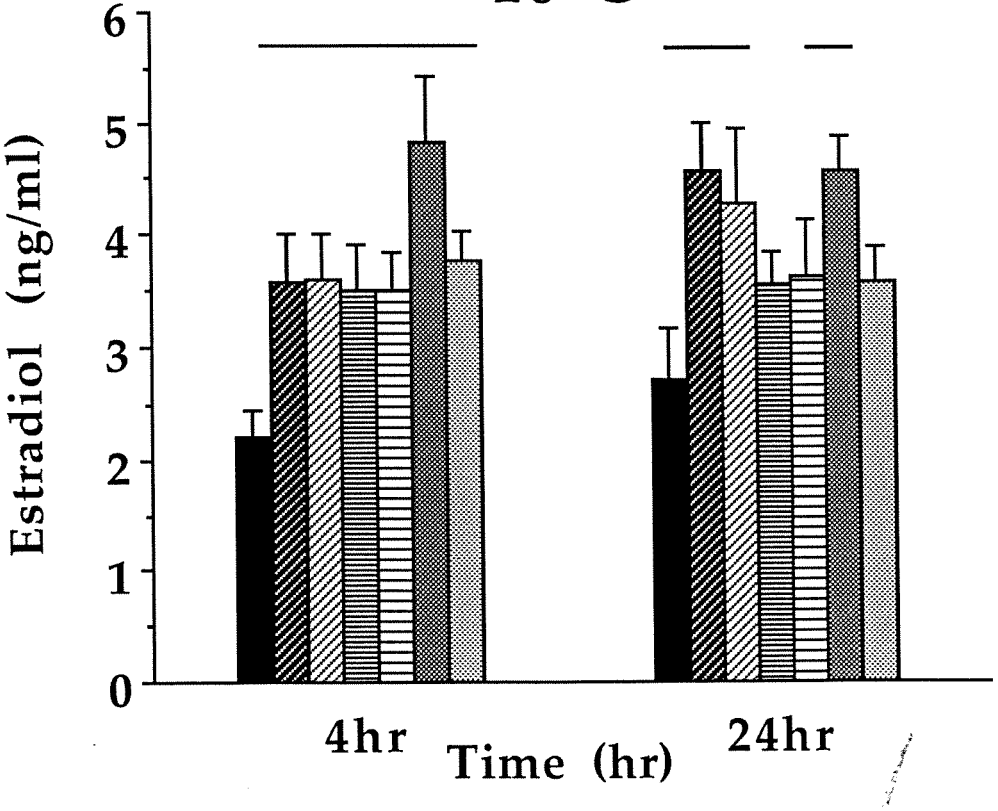


Fig 2

GnRH-I or -III, seven lamprey GnRH analogs were also examined: [D-Glu^6] lamprey GnRH-I, cycle [Glu^6-Trp^7-Lys^8] lamprey GnRH-I, cycle [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, and [Gly^6] lamprey GnRH-III.

The response of the pituitary, as measured by an increase of estradiol release by ovarian tissue incubated at 18°C, was significantly enhanced by GnRH-I at 1000 ng/ml (data not shown). Pituitary responsiveness was significantly enhanced by GnRH-III at 1000 ng/ml at 18°C. In addition, GnRH-III at 1000 ng/ml directly stimulated the ovaries incubated at 14°C. GnRH-I at 1000 ng/ml demonstrated a slight direct effect on the testis incubated at 14°C and 18°C. Lamprey GnRH-III demonstrated a more significant direct effect on the testis at 14° and 18°C (data not shown).

A significant decrease of estradiol release from testis incubated at 14°C as a measure of pituitary responsiveness was noted following perifusion with [D-Glu^6] GnRH-I at 10, 100, and 1000 ng/ml (Fig. 3, #1). There was no significant direct effect of [D-Glu^6] GnRH-I on the testis.Cycle [D-Glu^6-Trp^7-Lys^8] GnRH-I at 100 and 1000 ng/ml significantly diminished pituitary responsiveness of the testis incubated at 14°C (Fig. 3, #2). There was no significant direct effect of Cycle [D-Glu^6-Trp^7-Lys^8] GnRH-I on the testis at 14°C. [D-Glu^6-Trp^7-Lys^8] GnRH-I at 10 and 1000ng/ml directly stimulated the testis incubated at 14°C (Figs. 3,#3-6). [Gly^6] GnRH-I at 10, 100, and 1000 ng/ml directly stimulated the testis incubated at 14°C (Fig. 3,#7).

Summary of Experiments 1 and 2 -- These data indicate that there are significant differences in the response to the various analogs. In addition, there are significant differential responses among the analogs compared to controls.

Experiment 3: Behavior experiment

The objective of this study was to determine the effects of lamprey GnRH-I and -III analogs (antagonists) on the nesting and spawning behavior of adult males and on the development of the eggs fertilized by treated males. Lampreys used in this experiment were given fin clips and introduced to the spawning channel on June 6, 1994, to acclimate until the start of the experiment. Sixty male lampreys were left unclipped and used as controls. Another sixty male lampreys were given one of six fin clips, for the six treatment groups. Sixty female lampreys were also introduced to the spawning channel at this time. Water temperature was constantly monitored by a thermograph.

On June 16, 20, 24 and 29, 1994, clipped males were injected with 0.1 ml of one of the following treatments: Trp^3 Gly^6 lamprey GnRH-I (0.05μg/g BW); Trp^3 Gly^6 lamprey GnRH-I (0.1μg/g BW); D-Phe^{2,6} Pro^3 lamprey GnRH-I (0.05μg/g BW); D-Phe^{2,6} Pro^3 lamprey GnRH-I (0.1μg/g BW); Gly^6 lamprey GnRH-III (0.05 μg/g BW); or Gly^6 lamprey GnRH-III (0.1 μg/g BW).

Sixty male sea lampreys were weighed and introduced to the spawning channel on June 7, 1995, to acclimate until the start of the experiment. Fifty female lampreys were added to the spawning channel at this time. On June 15, 45 males were given fin clips and injected with 75 μg of microencapsulated Gly^6 lamprey GnRH-I (15 lampreys) or control microspheres (30 lampreys). Fifteen male lampreys were injected on June 20 with 75 μg of microencapsulated Gly^6 lamprey

Figure #1

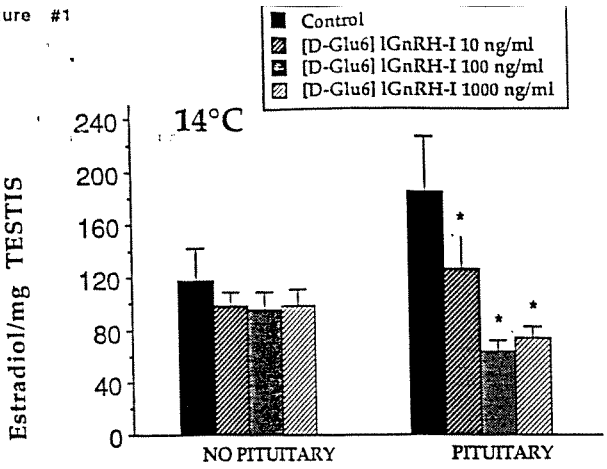


Figure #2

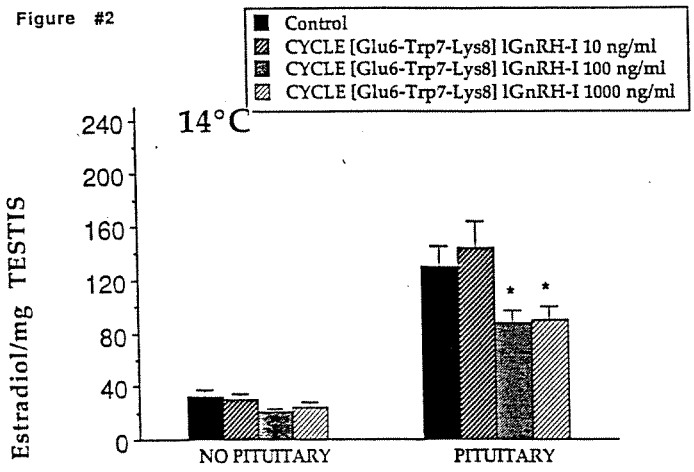


Figure #3

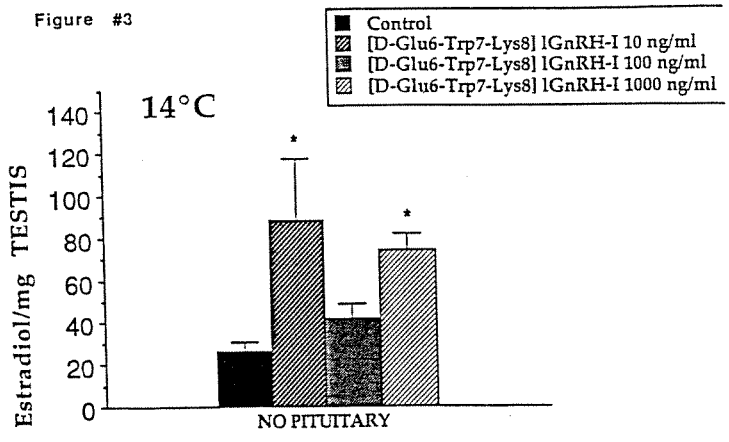


Figure #4

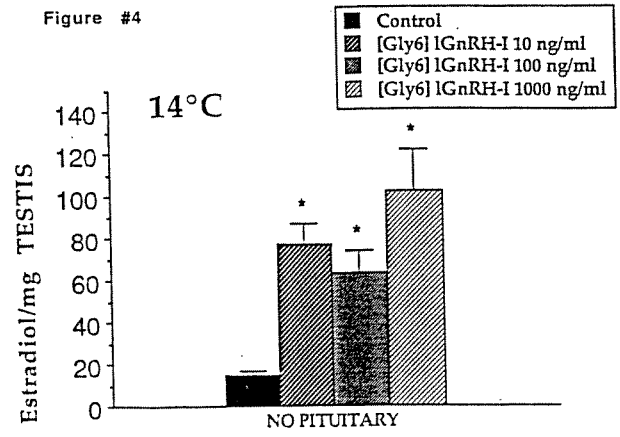


Figure #5

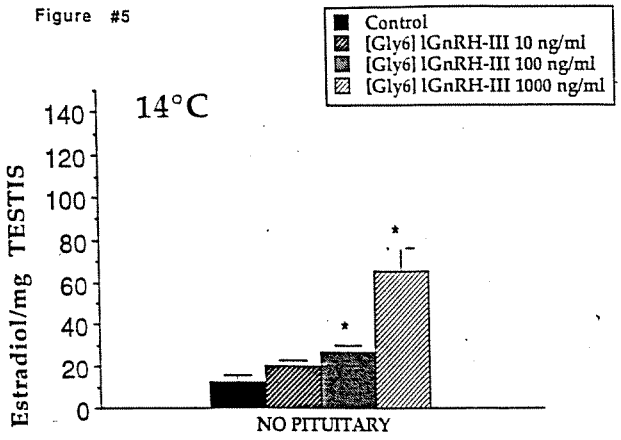


Figure #6

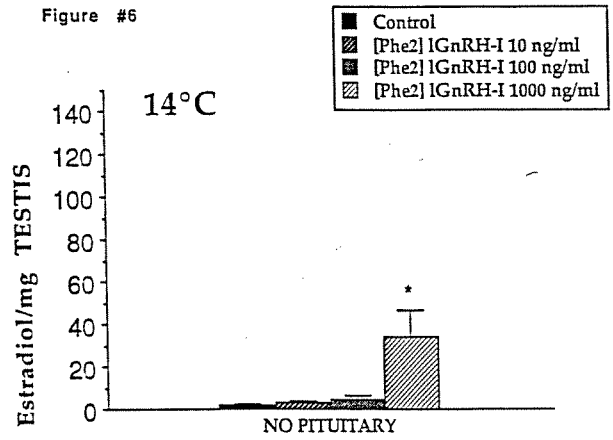
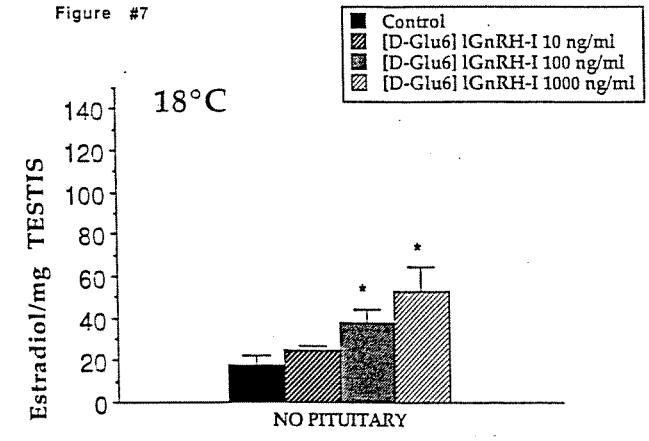


Figure #7



GnRH-I. Water temperature was continually monitored by a thermograph.

Following introduction to the spawning channel, the lampreys in the spawning channel were observed daily for any signs of spawning behavior. When a pair of lampreys was observed spawning, they were removed from the spawning channel and artificially spawned. One portion of eggs stripped from a female was fertilized with sperm from an untreated male to serve as a control and another portion of eggs from the same female was fertilized with the sperm from a treated male. Eggs were placed in 10-L glass battery jars (250 mm in diameter) containing 6 L of Lake Huron water and immersed in a constant temperature water bath at 18.3°C, the optimum temperature for development of sea lamprey embryos (Piavis, 1961). After 18 days of incubation, each batch of embryos was terminated to determine survival and assess the degree of development of the prolarvae according to the method of Piavis (1961).

The results of the incubation of eggs are shown in the following two figures (Fig. 4 and 5) as mortality data (% mean±SE). As noted, there were no significant differences of the survival of the eggs fertilized by treated males compared to controls. A total of 58 batches of eggs were incubated in this part of the study at Hammond Bay Biological Station.

Summary of Experiment 3 --In contrast to experiments completed a few years ago, in which an analog of lamprey GnRH was effective in inducing higher mortality of fertilized eggs compared to controls, there were no differences noted in this experiment. In the previous experiments, the lampreys had been injected more often. Thus, the treatment regimes seem to a critical factor. Thus, in 1995, we used an encapsulated GnRH analog which allowed a slow continuous time-release of GnRH in the lamprey during the entire spawning season. This ensured the full effects of GnRH in the lamprey and subsequently on behavior and development of the eggs. We were only able to test one GnRH analog in 1995. However, we propose that further testing of GnRH analogs is necessary and will likely yield a method of sterilizing male lampreys for use in this program in the Great Lakes.

Experiment 4: Microencapsulation experiment

The objective of this study was to test the release rates of GnRH within the lamprey from treatment with microcapsules of GnRH or GnRH implants. The microcapsules and implants for this experiment were obtained from Aquapharm Inc, Bethesda, Maryland. All doses contained 75μg of DAla^6 Pro^9 Net mammal GnRH. Microcapsules were injected in 0.1 ml of emulsion fluid using an 18 gauge needle. Implants were inserted using an 11 gauge cancer implantation needle.

Male lampreys were given injections of microcapsules or implants on June 17, 1994. The three treatment groups consisted of the following:

1) forty-three male lampreys were given intraperitoneal injections (IP) of the DAla^6 Pro^9 Net mammal GnRH microcapsules,
2) thirty male lampreys were given intramuscular injections (IM) of the DAla^6 Pro^9 Net mammal GnRH microcapsules, and,
3) thirty male lampreys were given intraperitoneal implants, containing DAla^6 Pro^9 Net mammal GnRH.

Lampreys were then maintained in 4' diameter circular tanks during the duration of the experiments. Lampreys were sampled at 1, 2, 3 and 4 weeks post-injection, when a 0.5ml blood

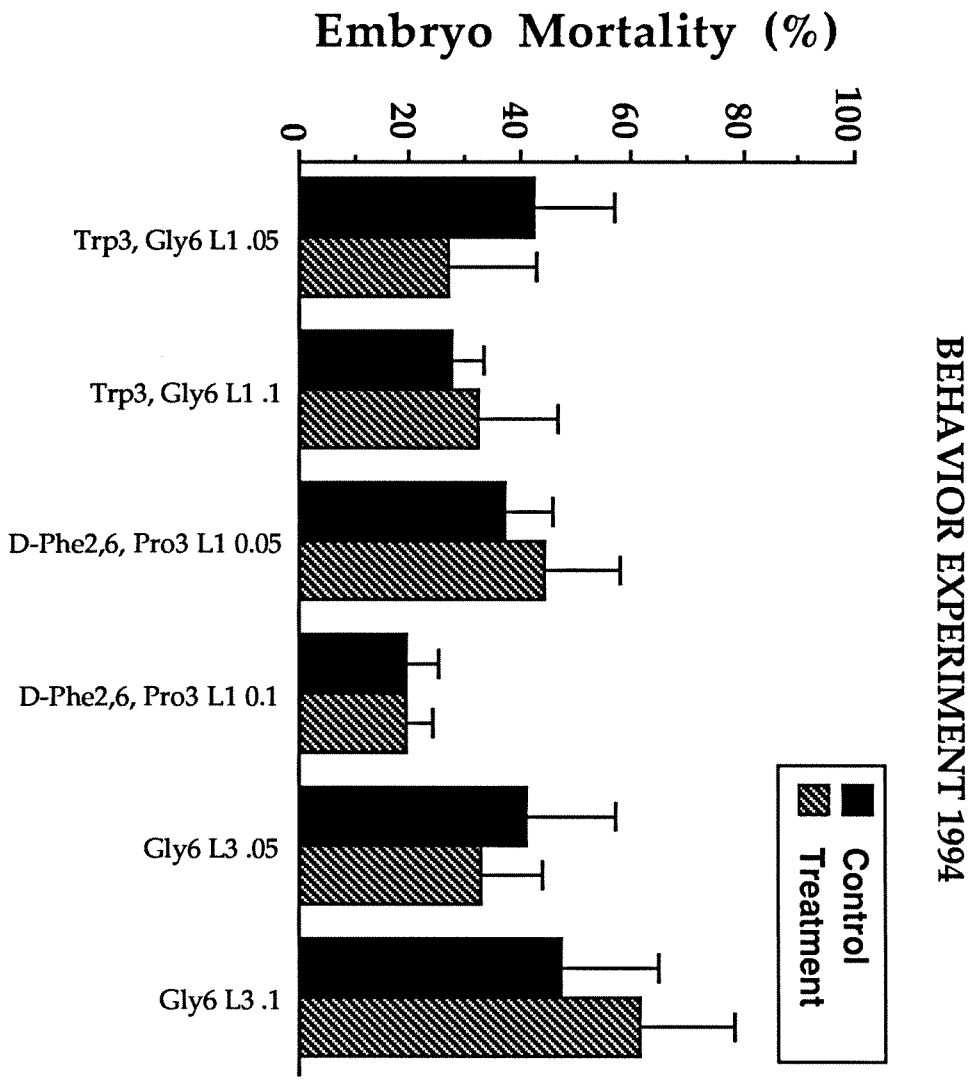

Embryo Mortality (%)
0
20
40
60
80
100
Trp3, Gly6 L1 .05
Trp3, Gly6 L1 .1
D-Phe2,6, Pro3 L1 0.05
D-Phe2,6, Pro3 L1 0.1
Gly6 L3 .05
Gly6 L3 .1
Control
Treatment
BEHAVIOR EXPERIMENT 1994

Fig 4

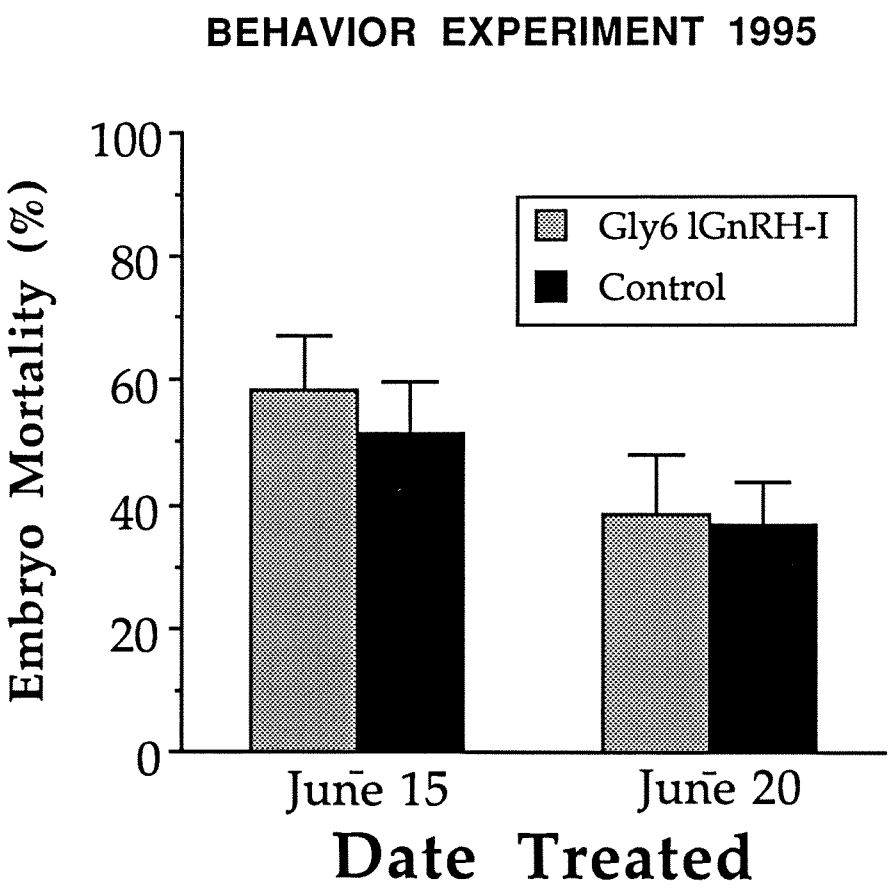

BEHAVIOR EXPERIMENT 1995
Embryo Mortality (%)
100
80
60
40
20
0
Gly6 lGnRH-I
Control
June 15
June 20
Date Treated

Fig 5

sample was taken as previously described (Fahien and Sower, 1990). The blood was then centrifuged and the plasma was removed and stored at -80°C until it could be assayed for DAla^6 Pro^9 Net mammal GnRH by HPLC and specific GnRH radioimmunoassay. Data are shown in Fig 6.

Intraperitoneal injections of the DAla^6 Pro^9 Net mGnRH microcapsules: DAla^6 Pro^9 Net mGnRH was detected in 28 of 43 lampreys (63.4%) sampled after one week and 9 of 31 lampreys (29.0%) after two weeks. DAla^6 Pro^9 Net mGnRH was not detected in any lampreys after 2 weeks. Plasma DAla^6 Pro^9 Net mGnRH levels decreased from week 1 (5.0 ± 1.3 ng/ml) to week 2 (1.4 ± 0.5 ng/ml) (as shown in the following figure).

Intramuscular injections of the DAla^6 Pro^9 Net mGnRH microcapsules: DAla^6 Pro^9 Net mGnRH was detected in 27 of 30 lampreys (90.0%) sampled after one week, 23 of 25 lampreys (92.0%) sampled after two weeks and 6 of 12 lampreys (50.0%) sampled after three weeks. Plasma DAla^6 Pro^9 Net mGnRH levels decreased from week 1 (4.2 ± 1.1 ng/ml) to week 2 (3.1 ± 1.2 ng/ml) to week 3 (2.9 ± 1.5 ng/ml).

DAla^6 Pro^9 Net mGnRH implants: DAla^6 Pro^9 Net mGnRH was detected in 18 of 29 lampreys (62.1%) sampled after one week, 5 of 21 (23.8%) lampreys sampled after two weeks and only 1 of 10 (10%) lampreys sampled after three weeks. Plasma DAla^6 Pro^9 Net mGnRH levels decreased from week 1 (1.6 ± 0.3 ng/ml) to week 2 (0.7 ± 0.2 ng/ml). After three weeks, only one lamprey still had a detectable level of DAla^6 Pro^9 Net mGnRH (0.4 ng/ml).

Summary of Experiment 4 -- Based on the results of this experiment in which the GnRH analog stayed elevated during the three week period following IM injection, in contrast to the two IP type injections, we then decided to use intramuscular injections of encapsulated GnRH analog as the most effective delivery system for the 1995 behavior experiment.

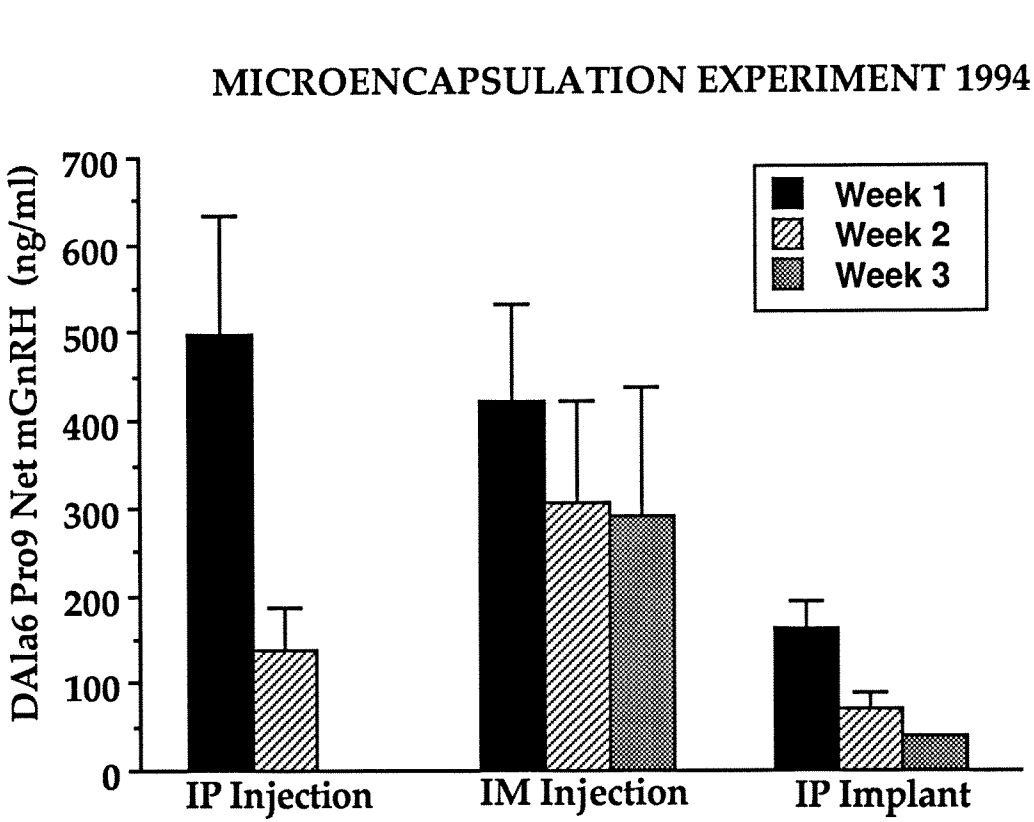

MICROENCAPSULATION EXPERIMENT 1994
DAla6 Pro9 Net mGnRH (ng/ml)
700
600
500
400
300
200
100
0
Week 1
Week 2
Week 3
IP Injection
IM Injection
IP Implant

Fig 6

IV. REFERENCES CITED

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