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

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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-Glu⁶] lamprey GnRH-I, cyclo [Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I, cyclo [D-Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I, [Gly⁶] lamprey GnRH-I, [D-Phe^{2,6}, Pro³] lamprey GnRH-I, [Phe²] lamprey GnRH-I, [Trp³] lamprey GnRH-I, and [Gly⁶] lamprey GnRH-III. All peptides tested in vivo, except [Trp³] 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-Glu⁶] lamprey GnRH-I at all doses suppressed the putative pituitary response on the testis at 14°C, whereas cyclo [Glu⁶-Trp⁷-Lys⁸] 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.

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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. © 2000 Elsevier Science B.V. All rights reserved.

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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 perfusion 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 perfusion 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-Ala⁶, Pro⁹] 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 Pro¹, 4-FD-Phe², 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}, Pro³] 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 [Gly⁶] lamprey GnRH-III were purchased from American Peptide (Sunnyvale, CA). The lamprey GnRH-I analogs [D-Glu⁶] lamprey GnRH-I, cyclo [Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I, cyclo [D-Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I, [Gly⁶] lamprey GnRH-I were obtained from Dr. Goodman (UC San Diego) (Sower et al., 1995). [D-Phe^{2,6}, Pro³] lamprey GnRH-I, [Phe²] lamprey GnRH-I and [Trp³] 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), [Phe²] lamprey GnRH-I (0.05 or 0.1 µg/g lamprey), [Gly⁶] lamprey

GnRH-III (0.05 or 0.1 $\mu\text{g/g}$ lamprey) and cyclo-[Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I (0.1 $\mu\text{g/g}$ lamprey). Two more analogs, [Gly⁶] lamprey GnRH-I (0.05 or 0.1 $\mu\text{g/g}$ lamprey) and [Trp³] lamprey GnRH-I (0.05 or 0.1 $\mu\text{g/g}$ lamprey), were also tested. The following lamprey GnRH analogs were tested in 1995: [Gly⁶] lamprey GnRH-III (0.05 or 0.1 $\mu\text{g/g}$ lamprey), [Phe²] lamprey GnRH-I (0.05 or 0.1 $\mu\text{g/g}$ lamprey) and [D-Glu⁶] lamprey GnRH-I (0.05 or 0.1 $\mu\text{g/g}$ 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 perfusion 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 perfusate collected from the perfusion 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 morphology as described by Fahien and Sower (1990).

2.5. *In vitro* perfusion system

An Acusyst-S multiperfusion system was used to deliver medium at the same rate and temperature to six chambers of constant volume (400 μ l). 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

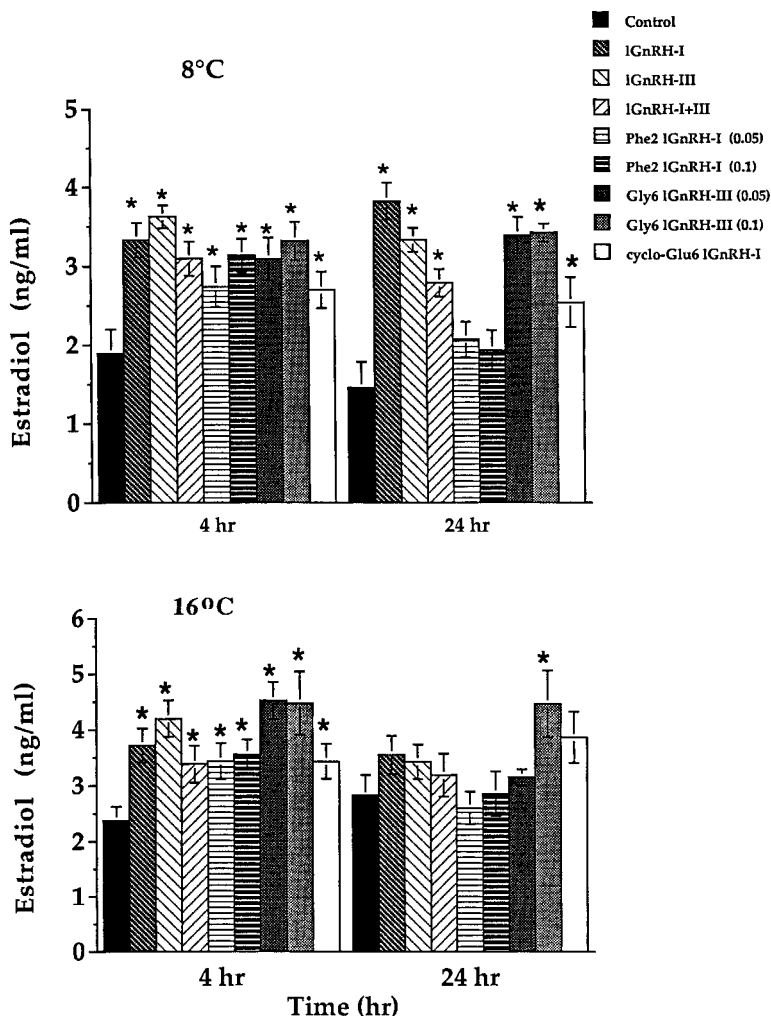


Fig. 1. Plasma oestradiol levels (ng/ml) of male lampreys injected during the 1994 season with 0.6% saline (control), lamprey GnRH-I, lamprey GnRH-III, lamprey GnRH-I + GnRH-III, [Phe²] lamprey GnRH-I, [Gly⁶] lamprey GnRH-III or cyclo [Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I. Individual lampreys received a dose of 0.05 or 0.1 μ g 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 \pm SEM. * Denotes significance at $P < 0.05$.

analogs. Peptides tested included lamprey GnRH-I and -III at concentrations of 1000 ng/ml; and analogs, [D-Glu⁶] lamprey GnRH-I, cyclo [Glu⁶, Trp⁷, Lys⁸] lamprey GnRH-I, cyclo [D-Glu⁶, Trp⁷, Lys⁸] lamprey GnRH-I, [Gly⁶] lamprey GnRH-I, [Gly⁶] lamprey GnRH-III, [D-Phe^{2,6}, Pro³] lamprey GnRH-I, and [Phe²] lamprey GnRH-I at concentrations of 10, 100 and 1000 ng/ml. Each injection of GnRH or analogs into the perfusion system was followed by a rinse with 50 μ l 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- μ l fractions were collected every 6 min.

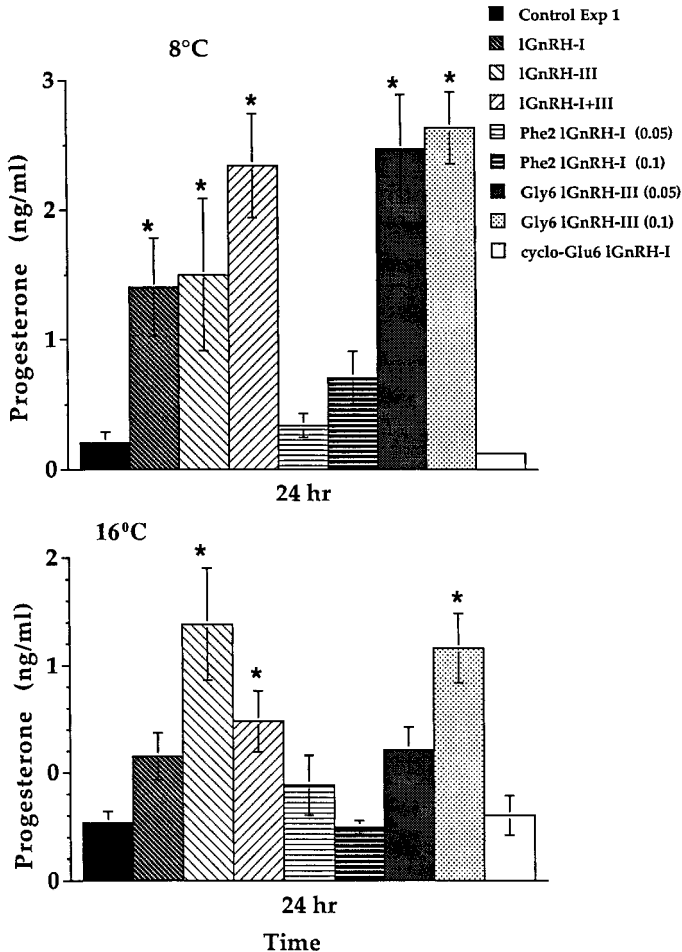


Fig. 2. Plasma progesterone levels (ng/ml) at 24 h of male lampreys injected during the 1994 season with 0.6% saline (control), lamprey GnRH-I, lamprey GnRH-III, lamprey GnRH-I+GnRH-III, [Phe²] lamprey GnRH-I, [Gly⁶] lamprey GnRH-III or cyclo [Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I. Individual lampreys received a dose of 0.05 or 0.1 μ g peptide/g lamprey. Lampreys were maintained in holding tanks at 8°C (top) and 16°C (bottom). Bars depict \pm 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- μ l 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- μ l 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 $P < 0.05$.

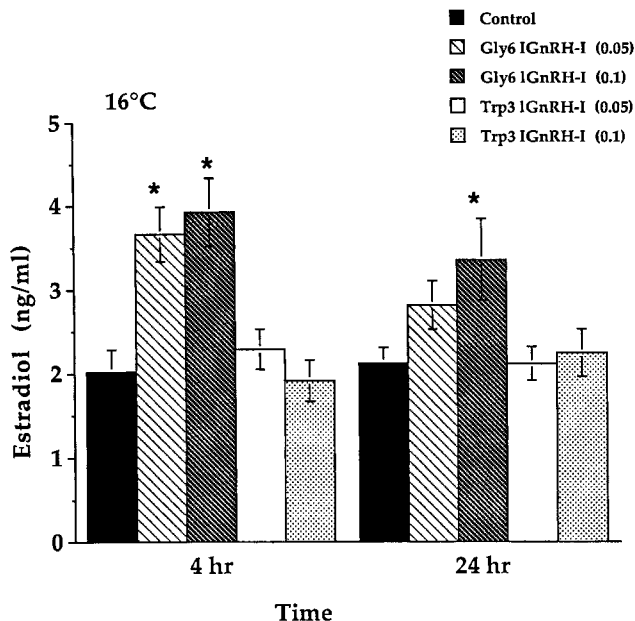


Fig. 3. Plasma oestradiol levels (ng/ml) of male lampreys injected during the 1994 season with 0.6% saline (control), [Gly⁶] lamprey GnRH-I or [Trp³] lamprey GnRH-I. Individual lampreys received a dose of 0.05 or 0.1 μ g peptide/g lamprey. Lampreys were maintained in holding tanks at the AFAIR laboratory at 16°C. Plasma samples were taken 4 and 24 h after injection. Bars depict \pm 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 [Phe²] 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 [Phe²] lamprey GnRH-I at both concentrations and cyclo [Glu⁶-Trp⁷-Lys⁸] 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 [Trp³]

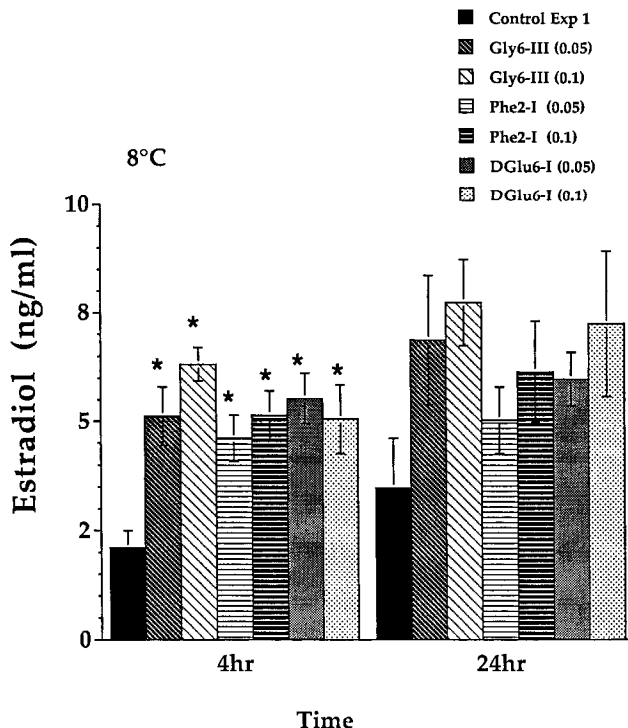


Fig. 4. Plasma oestradiol levels (ng/ml) of male lampreys injected during the 1995 season with 0.6% saline (control), [Gly⁶] lamprey GnRH-I, [Phe²] lamprey GnRH-III or [D-Glu⁶] lamprey GnRH-I. Individual lampreys received a dose of 0.05 or 0.1 µg peptide/g lamprey. Lampreys were maintained in holding 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 [Gly⁶] 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 [Gly⁶] 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

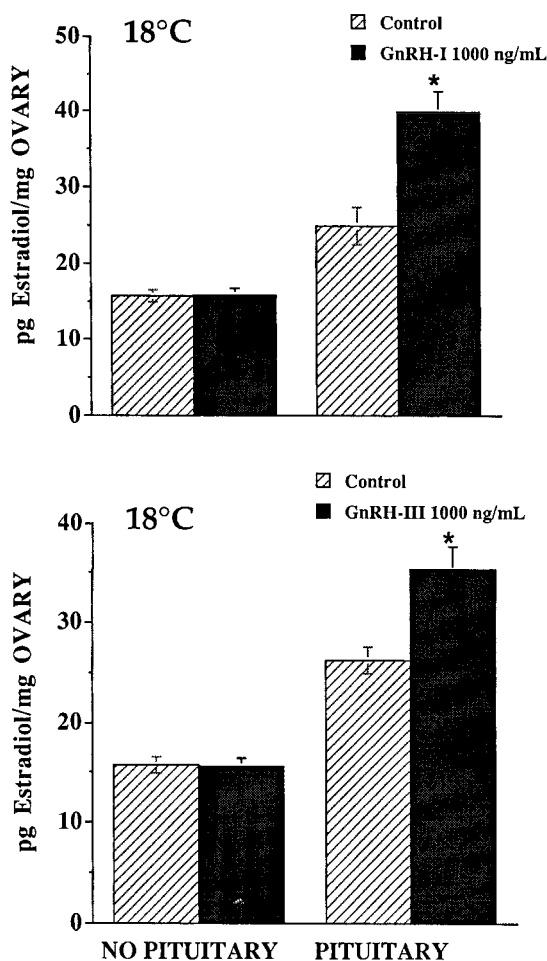


Fig. 5. Ovarian responsiveness to pituitary perfusion effluent at 18°C following injections of 1000 ng/ml lamprey GnRH-I (top) and 1000 ng/ml lamprey GnRH-III (bottom). No pituitary represents the direct effects of GnRH on the ovary and pituitary represents the effects of GNRH on the pituitary. Bars depict \pm 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 [Gly⁶] lamprey GnRH-III (0.05 or 0.1 µg/g lamprey) and [D-Glu⁶] 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

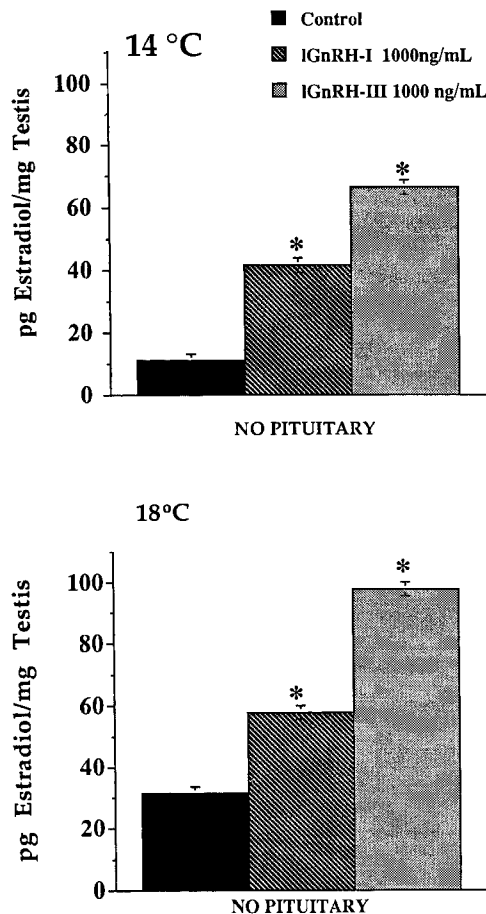


Fig. 6. Testis responsiveness to perfusion effluent at 14°C (A) and 18°C (B) following injections of 1000 ng/ml lamprey GnRH-I and 1000 ng/ml lamprey GnRH-III in the absence of pituitary, which represents the direct effect on the testis. Bars depict \pm SEM. * Denotes significance at $P < 0.05$.

-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 perfusion with [D-Glu⁶] lamprey GnRH-I at 10, 100, and 1000 ng/ml ($P < 0.05$). Cyclo [Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I at 100 ng/ml and 1000

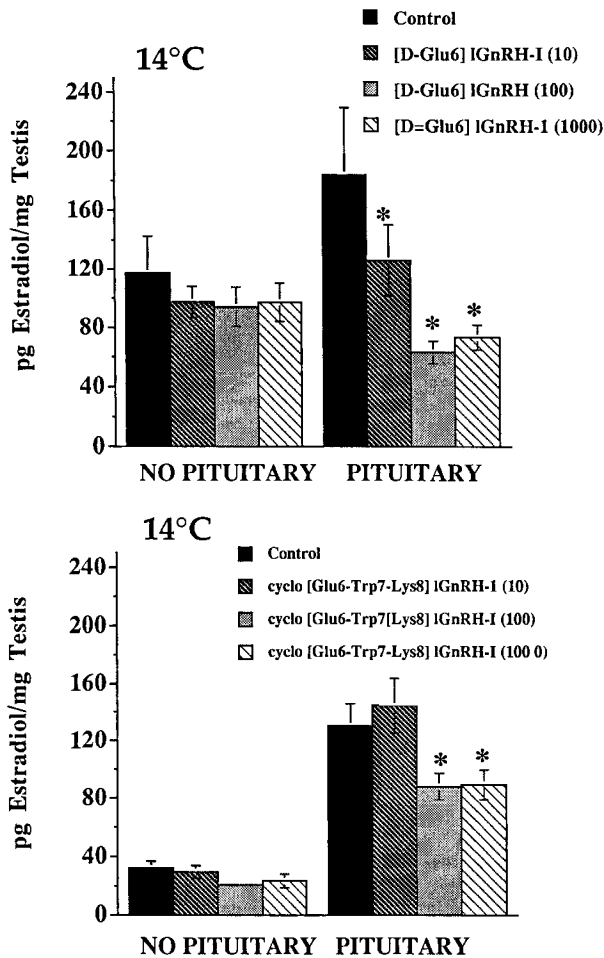


Fig. 7. Testis responsiveness to pituitary perfusion effluent at 14°C following injections of Control (0.6% saline) or 10, 100 or 1000 ng/ml [D-Glu⁶] lamprey GnRH-I (top) or cyclo [Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I (bottom). No pituitary represents the direct effects of GnRH on the testis and pituitary represents the effects of GnRH on the pituitary. Bars depict \pm SEM. * Denotes significance at $P < 0.05$.

Table 1

Direct effects of GnRH analogs at 14°C

Testis responsiveness to perfusion effluent in the absence of pituitary at 14°C following injections of Control (0.6% saline) or 10, 100 or 1000 ng/ml [D-Glu⁶] lamprey GnRH-I, cyclo [Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I, cyclo [D-Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I, [Gly⁶] lamprey GnRH-I, [Gly⁶] lamprey GnRH-III, [D-Phe^{2,6}, Pro³] lamprey GnRH-I and [Phe²] 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-Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I at 10 and 1000 ng/ml, [Gly⁶] lamprey GnRH-I at 10, 100, and 1000 ng/ml, [Gly⁶] lamprey GnRH-III at 100 and 1000 ng/ml, and [Phe²] lamprey GnRH-I at 1000 ng/ml directly stimulated the testis incubated at 14°C ($P < 0.05$) (Table 1). [D-Glu⁶] lamprey GnRH-I at 100 and 1000 ng/ml, [Gly⁶] lamprey GnRH-I at 10, 100, and 1000 ng/ml, [Gly⁶] lamprey GnRH-III at 1000 ng/ml, [D-Phe^{2,6}, Pro³] lamprey GnRH-I at 10 and 1000 ng/ml and [Phe²] 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 perfusion effluent in the absence of pituitary at 18°C following injections of Control (0.6% saline) or 10, 100 or 1000 ng/ml [D-Glu⁶] lamprey GnRH-I, cyclo [Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I, cyclo [D-Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I, [Gly⁶] lamprey GnRH-I, [Gly⁶] lamprey GnRH-III, [D-Phe^{2,6}, Pro³] lamprey GnRH-I and [Phe²] 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-Glu⁶] lamprey GnRH-I at all doses suppressed pituitary response on the testis at 14°C, whereas cyclo [Glu⁶-Trp⁷-Lys⁸] 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-Glu⁶] lamprey GnRH-I and [Gly⁶] lamprey GnRH-I, respectively. Two additional analogs, cyclo-[Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I and cyclo-[D-Glu⁶-Trp⁷-Lys⁸] 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, [Gly⁶] lamprey GnRH-I acted antagonistically by delaying ovulation by 3 weeks as compared to controls, while [D-Glu⁶] 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 II β -bend at the level of Gly⁶-Leu⁷ 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 [Phe²] lamprey GnRH-I, [Trp³] lamprey GnRH-I and others was examined. [Phe²] 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, [Phe²] 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 His²-Trp³ 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 Tyr³ 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 [Gly⁶] lamprey GnRH-I, [Gly⁶] lamprey GnRH-III, [D-Glu⁶] lamprey GnRH-I and cyclo-[Glu⁶-Trp⁷-Lys⁸] 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-[Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I or [D-Glu⁶] lamprey GnRH-I also stimulated plasma oestradiol in vivo. In the in vitro studies, [Gly⁶] lamprey GnRH-I at all doses and [Gly⁶] 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 [Gly⁶] lamprey GnRH-I and -III directly elevated oestradiol production. These data support the in vivo data with both [Gly⁶] lamprey GnRH-I and -III elevating oestradiol levels. In addition, cyclo [D-Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I at 10 and 1000 ng/ml directly stimulated the testis at 14°C, whereas [D-Glu⁶] 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-Glu⁶-Trp⁷-Lys⁸] lamprey GnRH-I and [D-Glu⁶] lamprey GnRH-I elevated oestradiol levels in vivo. The lower activity of [Gly⁶] 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 Gly⁶ may have modified the structure of the molecule, possibly promoting the conformation required for receptor interaction, or that the substitution of Gly⁶ augmented the resistance of the molecule to enzymatic degradation. Enzymatic degradation of both mammalian GnRH and salmon GnRH primarily results in cleavage of the Tyr⁵–Gly⁶ or Gly⁶–Leu⁷ 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 Gly⁶ 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.

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