General and Comparative Endocrinology 108, 327–339 (1997)
Article No. GC976981

Characteristics of GnRH Binding in the Gonads and Effects of Lamprey GnRH-I and -III on Reproduction in the Adult Sea Lamprey

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Accepted July 18, 1997

In the present study, both lamprey GnRH-I and -III stimulated steroidogenesis and induced ovulation in adult female sea lampreys during their final reproductive stage. One injection of lamprey GnRH-III at 0.1 or 0.2 μg/g lamprey stimulated plasma estradiol levels in lampreys held at each of three water temperatures, 13°, 17°, and 19°, corresponding to increasing stages of maturation. Four successive injections, 3 to 4 days apart, of lamprey GnRH-III at 0.1 or 0.2 μg/g body weight induced ovulation in 100 or 88% of lampreys, respectively, compared to 21% in controls by Day 31. Lamprey GnRH-III also had a direct stimulatory effect on estradiol production in the sea lamprey gonads *in vitro*. Lamprey GnRH-III at 100 or 1000 ng/ml stimulated estradiol levels in media incubated with either lamprey ovaries or testes. In contrast to a previous finding in which lamprey GnRH-III was more potent than lamprey GnRH-I in inducing spermiation in adult male sea lampreys (Deragon and Sower, 1994), the results from the present study indicate that lamprey GnRH-I and -III are equally potent in inducing ovulation and stimulating steroidogenesis in female sea lampreys. In addition, GnRH binding sites have been demonstrated for the first time in both the testis and the ovary of the adult sea lamprey using an analog of mammalian GnRH ([*D*-Lys^6] mammalian GnRH) as a labeled ligand. Scatchard analysis suggested the presence of a high affinity binding site in both the testis and the ovary. In summary, lamprey GnRH-III is biologically active in stimulating the pituitary–gonadal axis in adult female sea lampreys. This is the first report demonstrating the presence of a GnRH binding site in the gonads of an Agnathan. The evidence for a direct stimulatory effect of lamprey GnRH in the gonads, the presence of GnRH binding site, and the absence of GnRH in the plasma suggest that, like other vertebrates including rat, rabbit, teleost fish, and human, there may be a GnRH-like factor produced in the gonads of the lamprey and it may act as a paracrine/autocrine modulator of gonadal function. This study further strengthens the paracrine regulatory role of GnRH peptides in the gonads of vertebrates, which appear to be evolutionarily conserved.

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). The amino acid composition of a third form of lamprey GnRH, lamprey GnRH-II, is also known; however, the primary structure is yet to be determined (Sherwood *et al.*, 1986). The primary struc-

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0016-6480/97 $25.00

ture of lamprey GnRH-III differs in three amino acids compared with that of lamprey GnRH-I. The lamprey GnRH-III molecule is more similar to the other GnRH molecules than is lamprey GnRH-I: the lamprey GnRH-III molecule shares 80% sequence identity with chicken GnRH-II and dogfish GnRH; 70% sequence identity with catfish GnRH, lamprey GnRH-I, and salmon GnRH; and 60% homology with chicken GnRH-I. Although representative species of each vertebrate class possess at least two forms of GnRH, the significance of having multiple forms of GnRH within a species remains unclear.

Both lamprey GnRH-I and GnRH-III have been demonstrated to act as neurohormones stimulating 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 *et al.*, 1987; Sower, 1989, 1990). Lamprey GnRH-III has been shown to be biologically active in adult female and male sea lampreys, as determined by increased levels of plasma steroids (Sower *et al.*, 1993; Deragon and Sower, 1994). Lamprey GnRH-III has also been shown to induce spermiation in adult male sea lampreys (Deragon and Sower, 1994). However, further information is necessary on the actions of lamprey GnRH-I and -III to determine if, indeed, both are neurohormones mediating the pituitary–gonadal axis. Therefore, one objective of this study was to determine the effects of lamprey GnRH-I and -III on steroidogenesis and ovulation in the adult female sea lamprey.

Temperature has been considered to be an important environmental factor for the final maturational processes in adult sea lampreys. Fahien and Sower (1990) noted a positive correlation between both estradiol levels and concentration of brain GnRH just prior to spawning with increasing water temperature in adult male sea lampreys. Bolduc and Sower (1992) showed a similar positive correlation between both estradiol levels and concentrations of brain GnRH with water temperature in adult female sea lampreys. These data strongly suggested that there is a significant correlation between temperature and neuroendocrine events during reproduction in adult lampreys. Therefore, another objective of this study was to determine the effects of lamprey GnRH-III on steroidogenesis at different temperatures corresponding to increasing stages of maturation in the adult sea lamprey.

In lampreys, physiological and immunocytochemical data have clearly shown that lamprey GnRH-I and -III act at the pituitary to stimulate gonadal activity (see review Sower, 1990; Fahien and Sower, 1990; Sower and Larsen, 1991; Youson and Sower, 1991; Bolduc and Sower, 1992; Sower *et al.*, 1993). Even though gonadotropins have not been isolated from lamprey pituitaries, there is substantial indirect and direct 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 is not considered to travel via systemic circulation (Nozaki *et al.*, 1994). This is supported by studies in which lamprey GnRH-I and -III (Fahien and Sower, 1990; Millar and King, 1987; Sower, unpublished) have not been detected in plasma. However, the question remains whether there is a GnRH-like factor produced in gonads and whether GnRH administered interperitoneally has potentially any direct effects on the gonads.

There is compelling evidence that GnRH is produced and has a physiological role in the gonads of several teleost species (Pati, 1995; Pati and Habibi, 1993a,b). GnRH peptide has recently been isolated and biochemically characterized from the ovary of the goldfish (Pati, 1995; Pati and Habibi, in preparation). Lin and Peter (1996) showed that salmon GnRH transcript and low levels of chicken GnRH-II transcript are present in the goldfish ovary. GnRH receptors have been characterized in the ovary and testis of goldfish, *Carassius auratus* (Pati and Habibi, 1993a,b), and in the fully mature ovary of the African catfish, *Clarius gariepinus* (Habibi *et al.*, 1994), and seabream, *Spararus aurata* (Nabissi *et al.*, 1997). Fully mature goldfish ovaries contain two classes of GnRH binding sites, a high affinity/low capacity and a low affinity/high capacity site, while immature ovaries contain only a low affinity binding site (Pati and Habibi, 1993b). The goldfish testes also contain two classes of GnRH binding sites, a high affinity/low capacity and a low affinity/high capacity site (Pati and Habibi, 1993a).

Unlike the goldfish, the mature ovary of the common carp (*Cyprinus carpio*), African catfish, and seabream contain only one class of high affinity binding site for GnRH (Pati and Habibi, 1991; Habibi *et al.*, 1994; Nabissi *et al.*, 1997). HPLC analysis of catfish and seabream ovarian extract revealed the presence of two fractions that bind specifically to the catfish ovary and demonstrate the ability to release gonadotropin from cultured goldfish pituitary. One of the fractions does not correspond with any other known form of GnRH while the second coelutes with mammalian GnRH (Habibi *et al.*, 1994). Habibi *et al.* (1988) demonstrated an inhibitory role of GnRH agonist on the process of progesterone-induced oocyte meiosis in the goldfish. These studies provide firm evidence that GnRH or GnRH-like substances are produced in the gonads of teleost fish. Therefore, the objectives of this part of the study were to determine the effects of lamprey GnRH-III on sea lamprey ovaries and testes *in vitro* and to demonstrate GnRH binding sites in the gonads of the sea lamprey.

MATERIALS AND METHODS

Lampreys

Adult sea-run sea 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, New Hampshire, and the Lamprey River in Newmarket, New Hampshire, in May and June of 1994 and 1996 during their upstream spawning migration from the ocean. The lampreys were transported to the freshwater fish hatchery at the University of New Hampshire (Durham, NH) and maintained in an artificial spawning channel supplied with flowthrough reservoir water at an ambient temperature range 13–20° under natural photoperiod.

Adult female land-locked sea lampreys, which averaged 200 g in body weight, were sent from Hammond Bay Biological Station, Michigan, to Logan Airport and then transported to the freshwater fish hatchery at the University of New Hampshire and maintained in an artificial spawning channel supplied with flowthrough reservoir water at an ambient temperature range 13–20° under natural photoperiod.

Peptides

Lamprey GnRH-I was obtained from Peninsula Laboratories, Inc. (CA) and American Peptide Co. (CA). Lamprey GnRH-III was generously donated by Dr. Russell Doolittle, University of California at San Diego, and was also obtained from American Peptide Co. For the receptor studies, D-Lys^6 mGnRH was purchased from Peninsula Laboratories Inc. D-Lys^6 mammalian GnRH was iodinated using a modification of the chloramine-T method and purified as described in Stopa *et al.* (1988). The specific activity (80 Ci/mmol) was determined by a self-displacement assay as previously described by Knox *et al.* (1994).

Effects of Lamprey GnRH-I and -III on Steroidogenesis

A total of 180 sea-run female sea lampreys were used in this experiment. Adult female sea lampreys were tested with a single injection of saline, lamprey GnRH-I, lamprey GnRH-III, or a combination of lamprey GnRH-I and -III. The experiment was performed at three ambient temperatures of reservoir water during the course of the final reproductive stage of the lampreys: 13° (June 1), 17° (June 13), and 19° (July 1, 1994). Each group of 10 lampreys was tested with saline (control), lamprey GnRH-I (0.1 or 0.2 μg/g body weight), lamprey GnRH-III (0.1 or 0.2 μg/g body weight), or a combination of 0.1 μg/g each lamprey GnRH-I and -III. Within 30 min of injections, all peptides were dissolved in 0.6% NaCl in distilled water and were injected intraperitoneally. The lampreys were anesthetized with 0.2 g/liter ethyl *m*-amino benzoate methanesulfonate (MS222). Blood samples were collected 4 and 24 hr after injections by cardiac puncture with heparinized syringes. After centrifugation of the blood samples, plasma was collected and stored at −20° until assay for estradiol and progesterone. Plasma estradiol and progesterone levels were measured by radioimmunoassay as described previously (Sower *et al.*, 1983; Sower and Schreck, 1982). At the 24-hr blood sampling, gonads were removed and placed in Bouin's solution for later histological examination, as described previously by Sower *et al.* (1985). Briefly, gonadal tissue was removed and placed in

Bouin's solution overnight, and then transferred to 70% ethanol with lithium carbonate ($LiCO_3$). Samples were embedded in paraplast, sectioned at 8–10 μm with a microtome, and stained with hematoxylin and eosin. The ovaries were examined for morphological changes within the ovary during final maturation period, as described by Bolduc and Sower (1992).

Effects of Lamprey GnRH-I and -III on Ovulation

A total of 120 land-locked sea lampreys were used in the experiment. Adult female sea lampreys were tested with four successive injections of saline (control), lamprey GnRH-I, lamprey GnRH-III, or a combination of lamprey GnRH-I and -III on June 5, 8, 12, and 15. Six groups of 10 lampreys each were injected with saline, lamprey GnRH-I (0.1 or 0.2 μg/g body weight), lamprey GnRH-III (0.1 or 0.2 μg/g body weight), or a combination of lamprey GnRH-I and -III (0.1 μg/g body weight each). The female lampreys were checked every other day to determine if they had ovulated. An ovulatory response was judged by external characteristics, such as the softness of the abdominal region and eggs free flowing from the cloaca on application of gentle pressure on the anterior abdominal wall (Sower *et al.*, 1983).

In Vitro Effects of Lamprey GnRH-III on Sea Lamprey Gonads

Gonads were collected from adult male and female sea-run sea lampreys and placed in Hanks' Solution with 25 m*M* Hepes (pH 7.0). The gonads were incubated for 4 hr with a saline control, or lamprey GnRH-III in a dose of 10, 100, or 1000 ng/ml in triplicate. After incubation, the media was removed and stored at −20° until assay for estradiol and progesterone. Steroid levels were measured by radioimmunoassay, as described previously (Sower *et al.*, 1983; Sower and Schreck, 1982). A section of ovary was also removed and placed in Bouin's solution for later histological examination, as described previously by Sower *et al.* (1985) and Bolduc and Sower (1992).

Statistics

Data for hormone concentrations were analyzed by a Fisher PLSD test after a preliminary analysis of variance. In all tests, the level of significance for differing groups was $P < 0.05$.

Membrane Preparation

On the morning of sampling, one male lamprey was removed from the tank and decapitated, and a testis sample was taken from the region just posterior to the liver and was immediately placed in Bouin's solution for histological preparation and examination as described by Sower *et al.* (1985). The testes were examined and stages identified based on morphology as described by Fahien and Sower (1990). The remaining testis was immediately dissected out and transferred into ice-cold assay buffer [10 m*M* Tris(hydroxymethyl)-aminomethane (Tris–HCl), containing 1 m*M* dithiothreitol and 0.5% bovine serum albumin (BSA) (Fraction-V) (pH 7.4)]. Approximately 1–2 g of testis from one fish was used in each experiment. Membrane preparation was carried out using a protocol described previously (Pati and Habibi, 1993b). In brief, the cleaned fresh testis was homogenized in 25 ml of freshly prepared ice-cold 25 m*M* sucrose in assay buffer containing 1 m*M* phenylmethanesulfonyl fluoride (PMSF) and 5 m*M* ethylenediaminetetraacetic acid (EDTA). Initially, a polytron was used for a total of 1 min (at 10-sec intervals, placing the samples on ice between intervals). The tissue was then homogenized using a glass Teflon homogenizer, followed by centrifugation at 500*g* for 5 min (4°). The supernatant was then further centrifuged at 17,000*g* for 30 min at 4°, and the pellet (crude membrane preparation) was separated, rinsed gently, and resuspended in the assay buffer (approximately 400 μg protein per 100 μl), unless otherwise stated. In all experiments, the testicular membrane preparation was used within 60 min in a radioreceptor assay. For protein determination, aliquots of crude membrane preparation were washed twice in distilled water by centrifugation (25,000*g*) and assayed for protein content using a Lowry kit (Sigma, St. Louis, MO). For the ovarian membrane studies, ovaries were obtained from adult female sea lampreys (*Petromyzon marinus*) collected from the Cocheco Rivery in Dover, New Hampshire, in the summer of 1993. The ovaries were immediately frozen on dry ice and sent to the University of Calgary, Canada, where subsequent ovarian membrane preparation and GnRH

binding were conducted using a protocol previously described (Pati and Habibi, 1991).

Binding Procedure

The binding assay was based on techniques described previously (Pati and Habibi, 1993b); incubations (in triplicate) were carried out in polypropylene microcentrifuge tubes (500 µl), which were precoated overnight with 2.5% BSA. The incubations were terminated after the appropriate time (as determined by the equilibrium binding study) by centrifugation (23,000*g*) for 5 min at 4°, followed by aspiration of the supernatant. The bottom part of the centrifuge tube containing the pellet was cut off and transferred into a clean scintillation tube for determination of radioactivity using a gamma counter. Parallel incubations were performed in all experiments for determination of nonspecific binding in the absence of gonadal membrane to determine binding to the tubes (<1.0% of added radioactivity), and also in the presence of excess (10^{-6} *M*) unlabeled D-Lys^6 mammalian GnRH for the determination of nonspecific binding to the tissue.

Data Analysis

A computerized nonlinear least-square curve-fitting program (LIGAND) was used for Scatchard analysis of the displacement data (Munson and Rodbard, 1980).

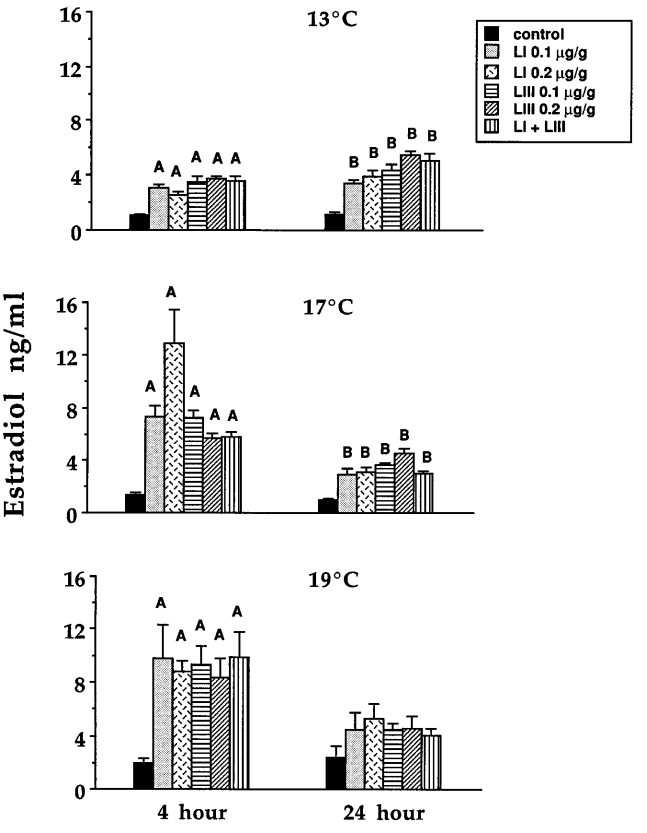

FIG. 1. Mean plasma estradiol levels (ng/ml) in female sea lampreys injected with saline (control), lamprey GnRH-I (0.1 or 0.2 µg/g), lamprey GnRH-III (0.1 or 0.2 µg/g), or a combination of 0.1 µg/g lamprey GnRH-I and -III at 13° (upper graph), 17° (middle graph), and 19° (lower graph) at 4 and 24 hr after injection. Bars depict SEM. A indicates significance between control at 4 hr, B indicates significance between control at 24 hr, and C indicates significance between 4- and 24-hr groups at $P < 0.05$.

RESULTS

Effects of Lamprey GnRH-I and -III on Steroidogenesis: Estradiol Response

In the group of lampreys treated at 13°, plasma estradiol increased significantly ($P < 0.05$) at 4 hr in response to lamprey GnRH-I at 0.1 µg/g and 0.2 µg/g, lamprey GnRH-III at 0.1 and 0.2 µg/g, and a combination of 0.1 µg/g lamprey GnRH-I and -III compared to controls (Fig. 1). Similarly, at 24 hr, plasma estradiol increased significantly ($P < 0.05$) in response to lamprey GnRH-I at 0.1 and 0.2 µg/g, lamprey GnRH-III at 0.1 and 0.2 µg/g, and a combination of 0.1 µg/g lamprey GnRH-I and -III compared to controls. There was a significant difference ($P < 0.05$) in estradiol levels between lamprey GnRH-III at 0.1 and 0.2 µg/g.

In the group of lampreys treated at 17°, plasma estradiol increased significantly ($P < 0.05$) at 4 hr in response to lamprey GnRH-I at 0.1 and 0.2 µg/g, lamprey GnRH-III at 0.1 and 0.2 µg/g, and a combination of 0.1 µg/g lamprey GnRH-I and -III compared to controls (Fig. 1). In addition, there was a significant difference in estradiol levels between lamprey GnRH-I at 0.1 and 0.2 µg/g. At 24 hr, plasma estradiol increased significantly ($P < 0.05$) in response to both 0.1 and 0.2 µg/g lamprey GnRH-I, 0.1 and 0.2 µg/g lamprey GnRH-III, and a combination of lamprey GnRH-I and -III compared to controls.

In the group of lampreys treated at 19°, plasma estradiol increased significantly ($P < 0.05$) at 4 hr in response to lamprey GnRH-I at 0.1 and 0.2 μg/g, lamprey GnRH-III at 0.1 and 0.2 μg/g, and a combination of 0.1 μg/g lamprey GnRH-I and -III compared to controls. At 24 hr, there were no significant increases in plasma estradiol levels in response to lamprey GnRH-I at either 0.1 or 0.2 μg/g, lamprey GnRH-III at either 0.1 or 0.2 μg/g, or a combination of 0.1 μg/g lamprey GnRH-I and -III compared to controls.

Effects of Lamprey GnRH-I and -III on Steroidogenesis: Progesterone Response

In the group of lampreys treated at 13°, there was a significant increase ($P < 0.05$) at 4 hr in progesterone levels in lampreys treated with 0.1 μg/g lamprey GnRH-I compared to controls (Fig. 2), but there was no significant increase in progesterone levels in lampreys treated with 0.2 μg/g lamprey GnRH-I, either 0.1 or 0.2 μg/g lamprey GnRH-III, or a combination of 0.1 μg/g lamprey GnRH-I and -III compared to controls. At 24 hr, there was a significant increase ($P < 0.05$) in progesterone levels in lampreys treated with 0.2 μg/g lamprey GnRH-I and 0.1 μg/g lamprey GnRH-III compared to controls. There was not a significant increase in progesterone levels in lampreys treated with 0.1 μg/g lamprey GnRH-I, 0.2 μg/g lamprey GnRH-III, or a combination of 0.1 μg/g lamprey GnRH-I and -III compared to controls. In general, at 13°, there was a decrease in progesterone levels from 4 to 24 hr.

In the group of lampreys treated at 17°, there was a significant increase ($P < 0.05$) at 4 hr in response to 0.1 and 0.2 μg/g lamprey GnRH-I, 0.1 and 0.2 μg/g lamprey GnRH-III, and a combination of 0.1 μg/g lamprey GnRH-I and -III compared to controls (Fig. 2). At 24 hr, levels of progesterone were nondetectable in lampreys treated with 0.2 μg/g lamprey GnRH-I, 0.1 μg/g lamprey GnRH-III, and a combination of 0.1 μg/g lamprey GnRH-I and -III. In the lampreys treated with 0.2 μg/g lamprey GnRH-III, only one of nine samples had a detectable level of progesterone. In the 0.1 μg/g lamprey GnRH-I group, three of six samples had detectable progesterone levels. There were no significant differences in progesterone levels between 0.1 μg/g lamprey GnRH-I compared to controls. In general, at 17° there was a decrease in progesterone levels from 4 to 24 hr.

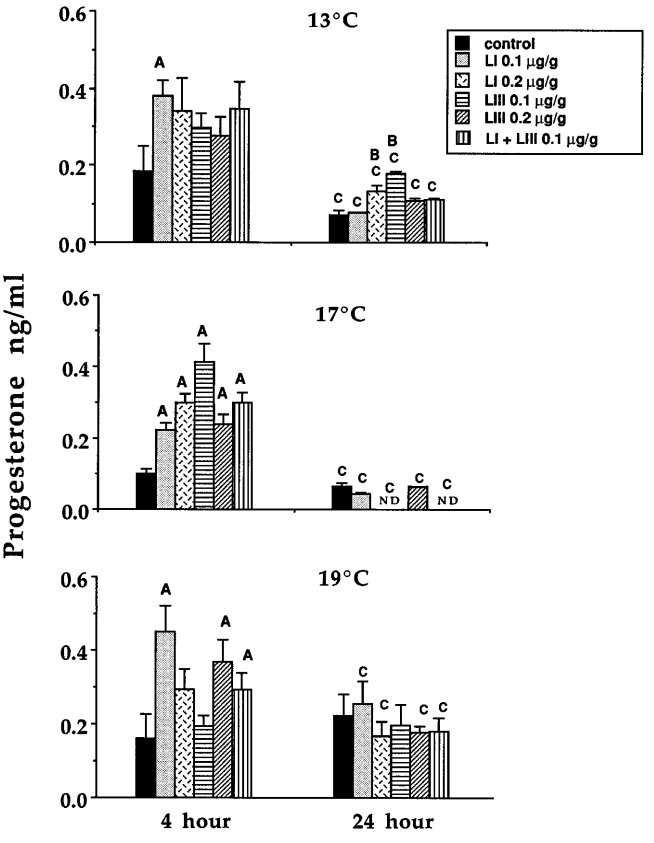

FIG. 2. Mean plasma progesterone levels (ng/ml) in female sea lampreys injected with saline (control), lamprey GnRH-I (0.1 or 0.2 μg/g), lamprey GnRH-III (0.1 or 0.2 μg/g), or a combination of 0.1 μg/g lamprey GnRH-I and -III at 13° (upper graph), 17° (middle graph), and 19° (lower graph) at 4 and 24 hr after injection. Bars depict SEM. A indicates significance between control at 4 hr, B indicates significance between control at 24 hr, and C indicates significance between 4- and 24-hr groups at $P < 0.05$.

In the group of lampreys treated at 19°, there was a significant increase ($P < 0.05$) at 4 hr in progesterone levels in lampreys treated with 0.1 μg/g lamprey GnRH-I, 0.2 μg/g lamprey GnRH-III, and a combination of lamprey GnRH-I and -III compared to controls (Fig. 2). However, there was not a significant difference between 0.2 μg/g lamprey GnRH-I or 0.1 μg/g lamprey GnRH-III compared to controls. At 24 hr, there was no significant change in progesterone levels between either 0.1 or 0.2 μg/g lamprey GnRH-I, 0.1 or 0.2 μg/g lamprey GnRH-III, or a combination of 0.1 μg/g lamprey GnRH-I and -III compared to controls.

Effects of Temperature on Reproduction: Estradiol and Progesterone Responses

In the control group of lampreys, there was a significant elevation ($P < 0.05$) of estradiol levels at 4 hr at 19° compared to 13° and 17°. There were no significant differences in progesterone levels between 13°, 17°, and 19°.

Histological Examination of the Ovary

At all three temperatures, the germinal vesicle was located at the periphery of the egg, the vitelline membrane was double, and the majority of space within the oocyte was filled with yolk platelets. At both 13° and 17°, the ovaries were in Stage II of development, indicated by an elevation of the thecal layer from the double vitelline membrane. At 19°, the ovaries were in Stage III of development, indicated by a complete separation of the thecal layer from the vitelline membrane.

Effects of Lamprey GnRH-I and -III on Ovulation

On Day 24, 44% of the lampreys treated with 0.1 μg/g lamprey GnRH-I had ovulated (Fig. 3), 16% with 0.2 μg/g lamprey GnRH-I, 27 and 31% in those lampreys treated with 0.1 and 0.2% lamprey GnRH-III, respectively, and 43% of the lampreys treated with a combination of lamprey GnRH-I and -III had ovulated compared to no ovulating females in the control group of lampreys.

By Day 31, all of the lampreys treated with either 0.1 μg/g lamprey GnRH-I or -III had ovulated (Fig. 3), compared to 74 accumulative percentage ovulation in the lamprey GnRH-I at 0.2 μg/g group, 88% of lampreys treated with lamprey GnRH-III (0.2 μg/g), 79% of lampreys treated with a combination of 0.1 μg/g lamprey GnRH-I and -III, and only 21 accumulative percentage ovulation in the control group of lampreys.

On Days 24 and 31 there was a higher accumulative percentage ovulation in lampreys treated with lamprey GnRH-I and -III compared to controls. Lamprey GnRH-I and -III appeared to be equally potent neurohormones accelerating the onset of ovulation in the female sea lamprey.

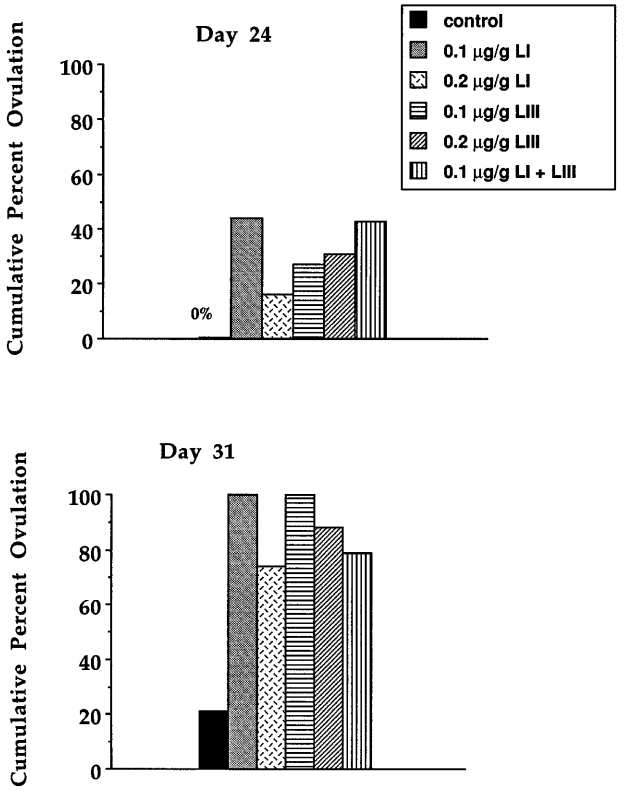

FIG. 3. Percentage ovulation on Day 24 (upper graph) and Day 31 (lower graph) after four successive injections of female sea lampreys with saline (control), lamprey GnRH-I (0.1 or 0.2 μg/g), lamprey GnRH-III (0.1 or 0.2 μg/g), or a combination of 0.1 μg/g lamprey GnRH-I and -III.

In Vitro Effects of Lamprey GnRH-III on Sea Lamprey Gonads

In the ovaries, there was a significant increase in estradiol levels (Fig. 4) in response to 10, 100, and 1000 ng/ml lamprey GnRH-III compared to controls. Progesterone levels were not detected in the media of incubated ovaries.

In the testes, there was a significant increase ($P < 0.05$) in estradiol levels (Fig. 4) in response to 100 and 1000 ng/ml lamprey GnRH-III compared to controls. There was a significant increase ($P < 0.05$) in progesterone levels in response to 1000 ng/ml lamprey GnRH-III compared to controls. Progesterone levels were nonde-

tectable in testes treated with 10 ng/ml lamprey GnRH-III.

Equilibrium Binding

The time course of ^{125}I-labeled D-Lys^6 mammalian GnRH binding to testicular membrane preparations was determined over a period of 100 min. Equilibrium was achieved after 10 min of incubation at 4° and the ligand binding was stable for the following 45 min (Fig. 5); thereafter, the binding decreased abruptly. Increasing the incubation temperature to 22° resulted in unstable specific binding (results not shown). In general, optimal binding was observed after 20 min of incubation at 4° and therefore this binding condition was used for subsequent studies.

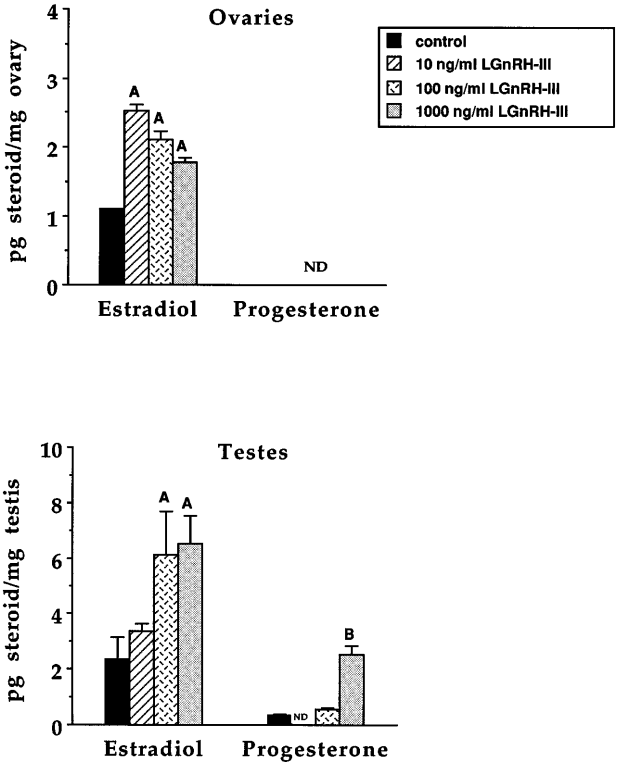

FIG. 4. Mean plasma estradiol and progesterone levels (pg/mg gonad) of sea lamprey ovaries (upper graph) and testes (lower graph) treated with saline (control) or 10, 100, or 1000 ng/ml lamprey GnRH-III *in vitro*. Bars depict SEM. A indicates significance between estradiol groups and B indicates significance between progesterone groups at $P < 0.05$.

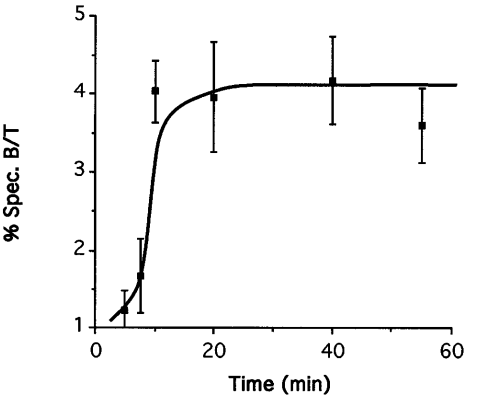

FIG. 5. Time course of ^{125}I-labeled D-Lys^6 mGnRH binding to the lamprey testicular membrane preparations at 4°. The results are expressed as fraction bound (bound/total (B/T), expressed as percentage of total counts) corrected for nonspecific binding measured in the presence of unlabeled D-Lys^6 mGnRH (10^{-6} *M*). Values are mean ± SEM of six observations (two experiments, each carried out in triplicate).

Tissue Concentration

Experiments were conducted to determine the correlation between testicular membrane concentration and D-Lys^6 mammalian GnRH binding. ^{125}I labeled D-Lys^6 mGnRH (30,000–40,000 cpm) was incubated in the presence or absence of 10^{-6} *M* unlabeled D-Lys^6 mGnRH with increasing concentrations of testicular membrane for a period of 20 min. Specific binding was determined by subtracting the binding in the presence of 10^{-6} *M* unlabeled D-Lys^6 mGnRH. The binding of D-Lys^6 mGnRH was found to be a function of tissue concentration with a positive linear correlation ($R^2 = 0.939$) over the range 125 to 500 µg protein per tube (Fig. 6). Based on these results, a membrane protein equivalent of approximately 400 µg was used per tube as standard experimental condition.

Competition Studies

Displacement of bound ^{125}I-labeled D-Lys^6 mGnRH (30,000–40,000 cpm per testis and 60,000 cpm per ovary) from the lamprey testicular and ovarian membrane preparations was studied by the addition of increasing concentrations of unlabeled D-Lys^6 mGnRH to the incubation tubes containing membrane prepared from testes at stage VI incubated at 4° for 20 min

and ovaries at a preovulatory stage incubated at 23° for 60 min. Addition of unlabeled D-Lys^6 mGnRH resulted in a dose-related displacement of ^{125}I-labeled D-Lys^6 mGnRH from lamprey testicular and ovarian membrane preparations (Figs. 7, 8). Testis data were analyzed on the pooled data from three separate assays, using a computerized curve fitting program (LIGAND). Scatchard analysis (Fig. 7, inset) of the displacement of D-Lys^6 mGnRH using mature lamprey testis indicated the presence of a single class of high affinity binding sites with an equilibrium dissociation constant (K_d) of 0.187 n*M* and binding capacity of 1.55 pmol/mg protein. Scatchard analysis (Fig. 8, inset) of the homologous displacement using mature lamprey ovarian membrane preparations indicated the presence of a single class of high affinity binding sites with an equilibrium dissociation constant (K_d) of 0.286 n*M* and binding capacity of 2.08 pmol/mg protein.

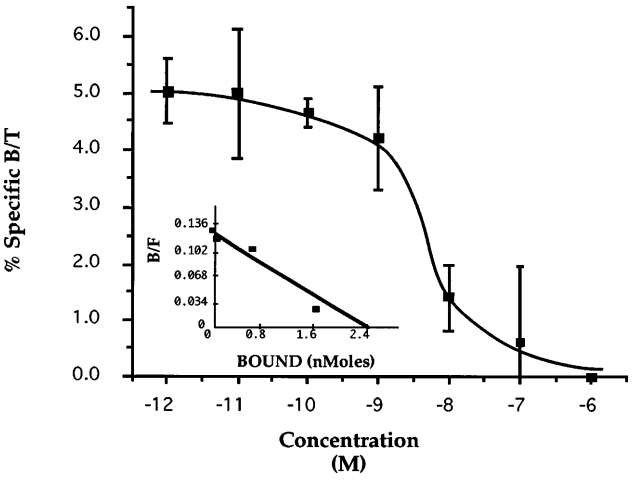

FIG. 7. Homologous displacement of ^{125}I-labeled D-Lys^6 mGnRH from the membrane prepared using lamprey testis at stage IV of maturity. The results were obtained from three separate experiments. Incubations were carried out in triplicate for a period of 20 min at 4°. Values (mean ± SEM) represents specific binding (bound/total in %) determined by subtraction of nonsaturable binding in the presence of excess unlabeled D-Lys^6 mGnRH (10^{-6} *M*). Inset: Scatchard plot of the displacement data, using a computerized curve-fitting program (LIGAND). B/F, bound/free.

DISCUSSION

In the present study, both lamprey GnRH-I and -III stimulated steroidogenesis and induced ovulation in adult female sea lampreys during their final reproductive stage. These data provide further evidence that lamprey GnRH-I and -III are both neurohormones involved in reproduction in the sea lamprey. The lamprey is one of the first vertebrates that has been shown to possess two forms of GnRH acting as hypothalamic neurohormones (Deragon and Sower, 1994). In contrast to a previous finding in which lamprey GnRH-III was more potent than lamprey GnRH-I in inducing spermiation in adult male sea lampreys (Deragon and Sower, 1994), the results from the present study indicate that lamprey GnRH-I and -III are equally potent in inducing ovulation and stimulating steroidogenesis in female sea lampreys. In addition, data from the present study showed that there is GnRH binding in the gonads of the adult sea lamprey.

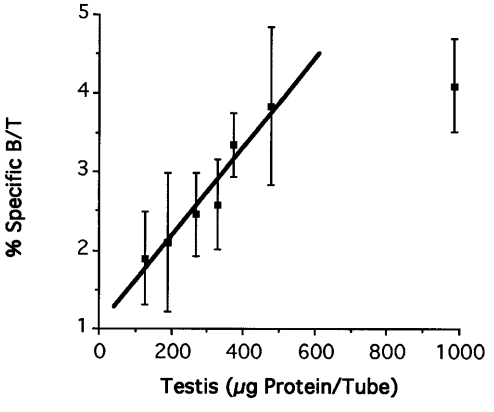

FIG. 6. Binding of ^{125}I-labeled D-Lys^6 mGnRH as a function of lamprey testicular membrane concentration (125.0 to 500.0 μg protein/tube). Specific binding of ^{125}I-labeled D-Lys^6 mGnRH was determined by subtracting the nonspecific binding in the presence of 10^{-6} *M* unlabeled D-Lys^6 mGnRH. Values are mean ± SEM of three observations.

Temperature has been considered an important environmental factor for the final maturational processes in adult sea lampreys. In the present study, there was a significant increase in estradiol levels at 19°, corresponding to later stages of final egg maturation, compared to levels at 13° and 17°, corresponding to earlier stages of egg maturation, in the control group of lampreys, providing further evidence for a relation-

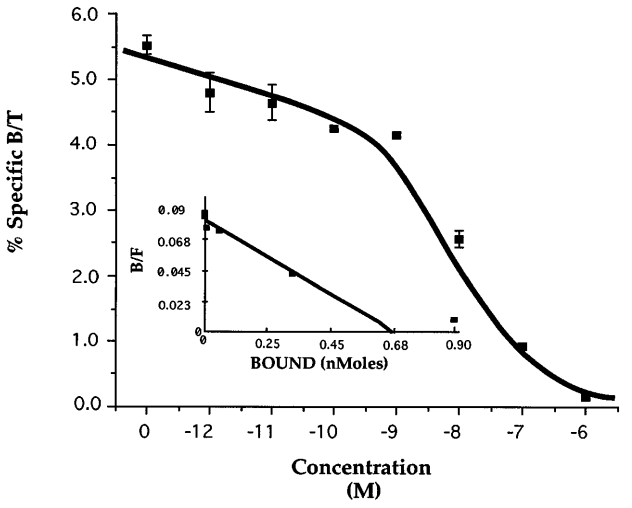

FIG. 8. Homologous displacement of ^{125}I-labeled D-Lys^6 mGnRH from the membrane prepared using lamprey ovaries at a preovulatory stage of maturity. The results were obtained from one experiment. Incubations were carried out in triplicate for a period of 60 min at 23°. Values (mean ± SEM) represents specific binding (bound / total) determined by subtraction of nonsaturable binding in the presence of excess unlabeled D-Lys^6 mGnRH (10^{-6} *M*). Inset: Scatchard plot of the displacement data, using a computerized curve-fitting program (LIGAND). B / F, bound / free.

ship between increased water temperatures and / or advance in time with the final reproductive processes in the lamprey. The morphology of the sea lamprey egg was similar to that observed by Bolduc and Sower (1992) for female sea lampreys undergoing final maturation. The germinal vesicle was located at the periphery of the egg and the vitelline membrane was double. There was a partial elevation of thecal layer from the double vitelline membrane at the two lower temperatures, and a complete separation of the theca when the water temperature reached 19°. Bolduc and Sower (1992) also noted the complete separation of the theca from the vitelline membrane around the entire oocyte. This complete separation was noted about 10 days before the first sign of ovulated eggs. Fahien and Sower (1990) noted a significant positive correlation between plasma estradiol levels and water temperature (13–19°) in adult male sea lampreys during their final spawning migration. In addition, there was an increase in brain GnRH concentration coincident with an increase in temperature just prior to spawning. In the present study, there were no significant differences in progesterone levels between any of the three temperatures. Fahien and Sower (1990) also found no correlation between water temperature and progesterone levels. Bolduc and Sower (1992) measured plasma estradiol levels and concentration of brain GnRH in adult female sea lampreys during their spawning migration for two different seasons, 1988 and 1989. In 1988, there were no significant changes in brain GnRH concentration or plasma estradiol levels, while in 1989 brain GnRH concentrations and plasma estradiol levels gradually increased through time until just prior to spawning, when levels decreased. One proposed explanation for these differences was the temperature differences between the two seasons: between June 19 and July 9 in 1988, water temperatures remained below 20°, while during this same time period in 1989, the water temperature remained above 20°. Therefore, the lack of elevated temperature in 1988 may have effected brain GnRH concentrations and plasma estradiol levels.

Under natural conditions, ovulation occurs mainly in a defined range (optimal temperature for ovulation) of temperatures that are characteristic of a given species (Epler *et al.,* 1985). The optimal temperature for spawning in the sea lamprey is considered to be about 21°, and the sea lamprey will not spawn at temperatures below 15.5° (Sower, 1990). In the present study, an ovulatory response was first observed in control lampreys on Day 24, when the water temperature first reached 21°, in agreement with the previously reported optimal temperature for spawning. Ovulatory responses were induced in GnRH-treated lampreys at water temperatures as low as 16°. Applegate (1950) suggested that the spawning of the sea lamprey is strongly influenced by water temperature. Rapid drops in water temperature up to and during the peak of spawning activity caused a noticeable decline in both nest building and spawning activities. Sea lampreys do not usually start to build their nests until the water warms to about 15° (Manion and Hanson, 1980). Bertmar (1985) also noted the importance of temperature in the timing of the spawning migrations and that cold water delays spawning. In a study by Linville *et al.* (1987) adult sea lampreys were transferred from the Ocqueoc River in Michigan, with a water temperature of 22°, to an artificial channel that had a water temperature of 12° over a 2-hr acclimation period. This sudden drop in temperature was accompanied by a decreased level of spawning activity. In addition, there

were large fluctuations in water temperature (between 8 and 17°), which seemed to suppress the level of activity of reproductive behaviors, compared to lampreys having high levels of activity in water temperatures remaining around 20–22° in the Ocqueoc River. Injections of partly purified salmon gonadotropin or a synthetic GnRH analog at 13° were sufficient to elevate plasma estradiol levels in adult female sea lampreys, but not ovulation at similar temperatures (Sower *et al.*, 1983). When water temperature was raised to 21°, and after a third injection of salmon gonadotropin or synthetic GnRH analog, early ovulatory responses were observed. Contrary to these findings, Sower *et al.* (1985) demonstrated a decrease in plasma estradiol levels between adult female sea lampreys at 5–6° (4–6 ng / ml) and female lampreys at 11° (2–3 ng / ml) and therefore suggested that higher temperature and / or advance in time of maturation may be inversely related to plasma estradiol levels.

The results of the *in vitro* experiment indicate that lamprey GnRH-III has a direct steroidogenic effect on sea lamprey gonads, as evidenced by an increase in estradiol levels. Abrams (1991) was unable to detect a direct effect of lamprey GnRH-I at 1, 10, or 1000 ng / ml on perifused sea lamprey ovaries. However, using these same methods, Gazourian and Sower (1994) demonstrated a direct effect of lamprey GnRH-III (1000 ng / ml) on sea lamprey ovaries, as indicated by a significant increase in estradiol levels. In this same study, both lamprey GnRH-I and -III (1000 ng / ml) had significant direct effects on sea lamprey testes. However, neither lamprey GnRH-I (Fahien and Sower, 1990; Millar and King, 1987) nor lamprey GnRH-III (Sower, unpublished) has been detected in the plasma of the sea lamprey and therefore it has been proposed that GnRH does not exert direct effects on the gonads via systemic circulation in the lamprey. In addition, studies by Knox *et al.* (1994) demonstrated the presence of two high affinity binding sites in the proximal pars distalis of the sea lamprey pituitary gland, providing further evidence that hypothalamic GnRH acts on the pituitary–gonadal axis. The direct gonadal effects of GnRH demonstrated in this study suggest that there may be some GnRH-like factor produced locally in sea lamprey gonads that may modulate gonadal function. An alternate possibility is that the gonads may be directly innervated by GnRH-containing tracts from the brain. This possibility has been suggested from results of studies in amphioxus, rats, and teleosts (reviewed in Schreibman and Margolis-Nunno, 1987), although in lampreys there is no evidence to date of direct innervation of the gonads (Sower, unpublished). Either possibility of a GnRH-like factor or direct innervation of the gonads is supported by the characterization of GnRH binding in the gonads in the present study. In this study, a single class of high affinity / high capacity binding sites has been characterized in the testes with an equilibrium dissociation constant (K_d) of 0.187 n*M* and binding capacity of 1.55 pmol / mg protein. Ovarian data also demonstrated the presence of a single class of high affinity / high capacity binding sites with an equilibrium dissociation constant (K_d) of 0.286 n*M* and binding capacity of 2.08 pmol / mg protein, respectively.

Knox *et al.* (1994) characterized and localized GnRH receptors in the pituitary of the adult female sea lamprey. The study revealed two high affinity binding sites with K_d values of 1.5×10^{-12} *M* and 5.0×10^{-9} *M*, located primarily in the proximal pars distalis with slight binding in the rostral pars distalis, demonstrating for the first time direct evidence of GnRH activity on the lamprey pituitary. In addition the goldfish pituitary also demonstrated two classes of GnRH binding sites; a high affinity / low capacity site, and a low affinity / high capacity site (Habibi *et al.*, 1987). Fully mature goldfish ovaries contain two classes of GnRH binding sites, a high affinity / low capacity and a low affinity / high capacity site, while immature ovaries contain only a low affinity binding site (Pati and Habibi, 1993b). The goldfish testis also contain two classes of GnRH binding sites, a high affinity / low capacity and a low affinity / high capacity site (Pati and Habibi, 1993a). In comparison, the present study demonstrated that both mature lamprey ovarian and testicular tissue contain only one high affinity GnRH binding site, unlike that of mature goldfish gonadal tissue.

In mammals, GnRH or GnRH-like peptides have been determined to be present in extrahypothalamic regions of the central nervous system and in nonneural tissues such as gonads, the placenta, mammary glands, and the pancreas (King and Millar, 1991). High affinity binding sites for GnRH analogs have been found in testicular Leydig cells and ovarian granulosa cells (King and Millar; 1991, for review see Tsafriri, 1987;

Chieffi *et al.,* 1991). GnRH and GnRH agonists have been demonstrated to cause a decrease in testicular LH receptors and inhibit testicular steroidogenesis in hypophysectomized male rats (Hsueh and Erickson, 1979a) and inhibit ovarian steroidogenesis in hypophysectomized female rats (Hsueh and Erickson, 1979b). More recent studies have indicated that GnRH or a GnRH-like factor may modulate gonadal function in teleosts (for review see Habibi and Pati, 1993; Pati, 1995). Two classes of GnRH binding sites have been characterized in the ovary of the goldfish, *C. auratus*, (Pati and Habibi, 1993a) and GnRH peptide has been isolated and biochemically characterized from the goldfish ovary (Pati, 1995; Pati and Habibi, in preparation). In small ovarian follicles, there is a single class of low affinity binding sites, while in larger follicles, there are high affinity / low capacity binding sites and low affinity / high capacity binding sites. Two classes of GnRH binding sites have also been characterized in the male goldfish (Pati and Habibi, 1993b). However, fully mature ovary of the common carp, African catfish, and seabream contains one class of high affinity GnRH binding sites. GnRH-like substances have also been isolated from the ovary of the catfish and seabream. *In vitro* studies have demonstrated direct inhibitory effects of GnRH agonists in the goldfish ovary. GnRH agonists had an inhibitory effect on oocyte meiosis (Habibi *et al.*, 1988, 1989). GnRH agonists and salmon and lamprey GnRH-III also reduced gonadotropin-induced steroidogenesis in goldfish follicles (Habibi *et al.*, 1989; Pati, 1995). These studies suggest that GnRH peptides may play a paracrine role in the regulation of gonadal function in teleosts. Likewise, the data from the present study also suggest that there may be a local GnRH-like factor regulating gonadal function in lampreys.

In summary, lamprey GnRH-I and lamprey GnRH-III stimulated steroidogenesis and induced ovulation in the adult female sea lamprey. These data provide further evidence that lamprey GnRH-I and -III are both neurohormones involved in the reproduction of the lamprey. However, it is likely that some of the noted increased steroidogenic responses are due to direct effects of GnRH on the gonads. The evidence for a direct stimulatory effect of lamprey GnRH in the gonads in terms of steroidogenesis, the presence of GnRH binding site, and the absence of GnRH in the plasma of the lamprey indicate that there may be a GnRH-like factor produced in the gonads of the lamprey, which may modulate gonadal function in this early evolved vertebrate species.

ACKNOWLEDGMENTS

This research was supported by NSF (IBN-94-07767 and IBN-9022834) to S.A.S. and D.P. was supported by an Alberta Minister of Advanced Education International Education Award. We thank Rebekah Gamble, Janet MacIntyre, Olivier Materne, and Kunimasa Suzuki for technical assistance.

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