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Hormone injection experiments were conducted for 2 years during the spring when adult Petromyzon marinus returned to a freshwater stream to spawn. Successive injections of salmon gonadotropin and synthetic analogue of hypothalamic GnRH (gonadotropin-releasing hormone) advanced ovulation by 2 to 3 weeks in the tests of the 1st year. Such treatment in the doses used also elevated plasma estradiol levels almost threefold above those of untreated females prior to ovulation. A more extensive 2nd year experiment had essentially similar results, though the experiment was complicated by the delaying effects of an extended period of low ambient water temperatures. At temperatures which permitted eventual ovulation, either salmon gonadotropin or active GnRH in a dose-related manner advanced ovulation by at least several weeks. However, even in the extended period preceding ovulation, at low temperatures either salmon gonadotropin or GnRH injections elevated plasma estradiol levels in a dose-related and time-related manner. Injections of another analogue of GnRH, which in mammalian tests is a competitive inhibitor of GnRH, had little or no effect either on plasma estradiol levels or on incidence of ovulation. These results indicate that there may be hypothalamic control over pituitary and gonadal function in lampreys.


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Introduction

The lampreys comprise one of two surviving groups that are representatives of the most ancient class of agnathan vertebrates; they are unique and unlike other vertebrates in lacking a hypothalamic–hypophysial portal vascular or innervation system (Gorbman 1965). There is at this time no evidence that the agnathan hypothalamus exerts any regulatory influence on the

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tion is seasonal occurring in early summer and seems to be responsive to environmental cues. The sea lamprey, *Petromyzon marinus*, like Pacific salmon, develops in freshwater, migrates to the sea, and returns to freshwater to reproduce. They breed only once in their lifetime after which they die. The adenohypophysis has been shown to exert some influence on gonadal tissues at various reproductive phases (reviewed by Larsen 1980). Although there may be regulation of sexual development mediated through the hypothalamo–hypophysial–gonadal axis, such regulation is now only inferred and has yet to be established in the lampreys.

Our study was undertaken to evaluate the effects of exogenous administration of GnRH analogues and salmon gonadotropin on steroidogenesis and ovulation in an anadromous lamprey to determine if the completion of the reproductive processes is mediated by elements of the hypothalamo–hypophysial–gonadal axis as in other vertebrates. In this 1st study of the actions of two synthetic analogues of mammalian GnRH, the biological actions of GnRHa (gonadotropin-releasing hormone analogue; d-Ala<sup>6</sup>, des-Gly<sup>10</sup>-LH-RH ethylamide), GnRH antagonist (Ac<sup>-3</sup>Pro<sup>-4</sup> FD Phe<sup>2</sup>, d-Trp<sup>3,6</sup>LRF; GnRHant) or a partly purified coho salmon gonadotropin in the sea lamprey, *Petromyzon marinus* were tested. Following the administrations of these compounds singly or in combination at different dosages, we monitored the occurrence of ovulatory responses during the lamprey’s final spawning phase. In addition, plasma estradiol levels were measured as another possible indicator of pituitary responsiveness. Gonadotropin(s) have not been isolated from the lamprey pituitary glands, so that no radioimmunoassay of secreted gonadotropins is possible at this time.

**Methods and materials**

**Hormonal preparations**

Frozen coho salmon pituitaries collected in 1978 at the Fall Creek Hatchery, Oregon, were used to prepare the partly purified salmon gonadotropin (GTH) by ethanol extractions and gel filtration (Sower et al. 1982).

Gonadotropin-releasing hormone analogue, d-Ala<sup>6</sup>, des-Gly<sup>10</sup>-LH-RH ethylamide, GnRHa, a high potency analogue (reviewed by Donaldson et al. 1981), was obtained from Sigma Chemical Company. The GnRH antagonist used was Ac<sup>-3</sup>Pro<sup>-4</sup> FD Phe<sup>2</sup>, d-Trp<sup>3,6</sup>LRF-(GnRH ant) (Rivier, Rivier, Perrin et al. 1981). During the morning of the day when the fish were injected, all peptides were dissolved in 0.6% NaCl in distilled water. They were injected intraperitoneally into each fish. Blood samples (400–600 µL) were collected in heparinized syringes by cardiac puncture (1982) or from the caudal veins (1981). Plasma samples were kept frozen at −20°C until assayed.

**1982 studies**

Twenty-two adult landlocked sea lampreys *Petromyzon marinus* were sent by air from the Hammond Bay Biological Station, Millersburg, Michigan to Seattle, on 2 June and 6 June, 1981. These animals had been captured by a trap in the Cheboygan River, Michigan, on their anadromous spawning migration after their parasitic phase in Lake Huron, about 13 months before their normal spawning time. The lampreys were maintained in two 0.6-m diameter cylindrical tanks (114 L) supplied with flowing lake water. Nineteen of the lampreys were males. The temperature ranged between 16.6 and 22°C. The specimens used averaged 165 g in body weight.

On 15 June 1981, the lampreys were anesthetized by immersion in 0.2 g/L of ethyl m-aminobenzoate methanesulfonate (MS-222), and injected in the morning either with saline (control) followed 3 days later by saline or with GnRHa at 0.01 mg/fish (day 3). Lampreys were reinfected on day 11 with either saline or GTH. Plasma samples were taken on day 4, 24 h after the GnRHa injection. The female lampreys were checked every other day to determine if they had ovulated as judged by external physical characteristics such as softness of the abdominal region and eggs free flowing from the cloaca on application of gentle pressure on anterior abdominal wall.

The lampreys were killed on the day they were determined to have ovulated. An ovulatory response was considered to have occurred if an individual female had more than 50% of her eggs loose in the body cavity.

**1982 studies**

Landlocked sea lampreys were captured by trap in the Cheboygan River, in May, 1982 on their anadromous spawning migration following completion of their parasitic lake phase. They were retained in raceways at Hammond Bay Biological Station, Michigan, supplied with flow-through lake water ranging in ambient temperature from 5.5 to 20°C. Two weeks prior to injection, the lampreys were divided into groups by transfer into five 1.5-m diameter fiberglass cylindrical tanks. The lampreys averaged 230 g in body weight and 41.9 cm in body length. One week before injections, all lampreys were individually identified with Floy tags, and in addition the dorsal fins were clipped to designate mode of treatment.

The treatment regimes and their scheduling are summarized in Table 1. Each treatment group contained at least 10 females and 2 males. Thus, 168 lampreys were subjected to the designated injection treatments three times over a 2-month period. Plasma samples were taken 24 h after the first injections as indicated. Plasma samples were also taken when individual fish ovulated or had died. Ovulatory responses were monitored in all groups twice per week by direct examination.

**Radioimmunoassay**

Plasma estradiol-17β level was measured by radioimmunoassay (RIA) as described by Sower and Schreck (1982). The radioimmunoassay was validated for adult sea lamprey plasma. Comparisons were made of plasma subjected to extraction and partition chromatography on Celite columns (Abraham 1973) and plasma extracted twice with diethyl ether (100 µL plasma : 1.5 mL diethyl ether). Since similar values were obtained from both procedures, ether extraction was used exclusively. Serial dilutions of extracted lamprey plasma were parallel with the curve of the estradiol-17β standards. Briefly, 100 µL of plasma samples were extracted twice with diethyl ether; the extract was evaporated to dryness with nitrogen gas.
and assayed directly for estradiol. Antiestradiol-17β antibody (S-244) was obtained from Dr. G. Niswender (Colorado State University, Fort Collins, CO) and diluted 1:8500 in phosphate-buffered saline–gelatin (PG). This assay is highly specific for estradiol-17β. The lower limit of detection was about 6 pg/mL. The antibody binding efficiency ranged between 47 and 57% in the assays. The intra- and inter-assay coefficients of variation for the estradiol RIA of lamprey plasma were 3.2% \((n = 9)\) and 13% \((n = 10)\), respectively.

**Statistics**

The percent ovulation data were analyzed by use of a \(2 \times 2\) contingency table followed by the Bonferroni approach (Neter and Wasserman 1974). Data for hormone concentrations were analyzed by a Student–Newman–Keuls test after preliminary analysis of variance. In all tests the level of significance for differing groups was \(P < 0.05\).

**Results**

1981

By day 11 after the day of the initial injection, 22% of treated lampreys had ovulated; at this time none of the controls had ovulated (Fig. 1). The first clear spontaneous ovulation of the controls was not until day 21. By day 28, 56% of hormone-treated lampreys had ovulated, compared with 22% of the controls. Mortality was about 30%, possibly a consequence of shipment and frequent handling.

Mean plasma estradiol was significantly elevated in the treated fish (11.54 ± 1.70 ng/mL) compared with the controls (4.76 ± 0.29 ng/mL) on day 4 (Fig. 1). Plasma estradiol significantly decreased in the controls and treated lampreys but did not differ between groups on the day of ovulation.

**1982 steroid responses**

Plasma estradiol during the first phase of treatment was significantly elevated 24 h (day 1) after lampreys were treated twice with salmon gonadotropin compared with controls (Fig. 2). Plasma estradiol remained elevated after a second injection of gonadotropin (4.69 ± 1.08 ng/mL). With no second injection of salmon...
gonadotropin, plasma estradiol diminished (1.36 ± 0.16 ng/mL) and was not significantly different from controls (1.06 ± 0.12 ng/mL). High plasma estradiol levels were found at 24 h (day 3) after an injection of GnRHa which followed a gonadotropin injection on day 0 (Fig. 2). Plasma estradiol responded in a significant dose-related manner (4.38 ± 0.50, 1.83 ± 0.23, or 1.28 ± 0.11 ng/mL) 24 h after injections of GnRHa (50, 5, or 0.5 μg/kg) which followed a gonadotropin injection on day 0.

Following two injections of GnRHa (50 μg/kg), plasma estradiol was significantly elevated compared with controls (Fig. 2). Such treatment yielded higher values of estradiol (3.82 ± 0.61 ng/mL) 24 h following 50 μg/kg of GnRHa compared with controls (1.07 ± 0.15 ng/mL); however, with no second hormone injection, plasma estradiol diminished by day 4 but was still significantly higher (2.30 ± 0.40 ng/mL) compared with controls (1.06 ± 0.12 ng/mL).

In most cases, plasma estradiol was not significantly different from controls after injections of GnRHant at different dosages (Fig. 2). On day 4 plasma estradiol was higher in lampreys treated with two injections of GnRHant (50 μg/kg), two injections of GnRHant
1982 SEA LAMPREY

FIG. 3. Accumulative percent ovulations at day 12 after the third sequence of injections of female sea lamprey injected in 1982 with saline on day 0 and 3, GTH (100 µg/kg lamprey) on day 0 and 3, GTH on day 0, GTH followed at 3 days by GnRHa (50), GTH followed at 3 days by GnRHa (0.5), GnRHa followed at 3 days by GnRHa, GnRHa on day 0; GnRHa (50) followed at 3 days by GnRHa (50), GnRHa (5) followed at 3 days by GnRHa (5), GnRHa (0.5) followed at 3 days by GnRHa (0.5), GnRHa (50) on day 0, or GnRHa (5) on day 0.

(0.5 µg/kg), or one injection of GnRHa (5 µg/kg) on day 0, but only significantly higher in lampreys treated with two injections of GnRHa (0.5 µg/kg), 1.5 ± 0.18 ng/mL, compared with controls, 1.06 ± 0.12 ng/mL.

1982 ovulation responses

There were no ovulatory responses following the first two phases of injections. It is probably significant that the ambient water temperature (13°C) was unusually low for this date at this place. Petromyzon marinus have not been known to spawn when the temperature is less than 15.5°C (L. King personal communication). Heaters were introduced into the tanks and the water was warmed to 21°C. At this temperature, the same animals were injected a third time with the same combinations of hormones. The observed ovulations all followed the third set of injections.

Thus, at day 12 after the third sequence of injections was begun, ovulation had occurred in 78% of the lampreys treated with GTH followed in 2 days by GnRHa (50 µg/kg), in 64% of those treated with a single injection of GnRHa (50 µg/kg), in 57% of those treated with GTH followed 2 days later by GTH, and in 56% of those treated with GnRHa (50) followed after 2 days by GnRHa (50). These incidences may be contrasted with 18% in control lampreys (Fig. 3). In lampreys treated with GnRHa at different dosages and combinations little or no ovulation was observed (18%). A single injection of GTH, or GTH followed after 2 days by a low dose of GnRHa (5) or by GnRHa (0.5), was ineffective in accelerating ovulation. The experiment was terminated on day 18 because of high mortalities.

Discussion

The principal question addressed by the experiments described here is whether there is hypothalamic control over reproduction in lampreys. This question in lampreys has special significance since these animals, with the hagfishes, are modern descendants of the most primitive vertebrates available for study. Proof that there is hypothalamic regulation of adenohipophysial function in this group would imply that evolution of this mechanism most likely antedated the origin of all known vertebrates.

There has been surprisingly little prior research on control over reproduction in Agnatha despite its significance. The earlier experiments of Evenet and Dodd (1963) and Larsen (1973) showed by hypophysectomy and mammalian gonadotropin injections that there is a relationship between the lamprey adenohypophysis and gonadal function. In hagfishes there seems to be no such relationship (Matty et al. 1976; Gorbman 1983). However, the hypophysial gonadotropic activity of lampreys appears to be directed more toward regulation of gonadal steroidogenesis than gametogenesis (see review by Gorbman 1983). A further difference between petromyzontids and myxinoïds is in the detectability of immunoreactive GnRH in the hypothalamus. In lampreys, Crim et al. (1979) found such immunoreactivity in discrete preoptic neurones and in axonal projections toward the
neurohypophysis. They found no such immunoreactivity in the brains of hagfishes (*Eptatretus stouti*).

Thus, there is evidence of normal occurrence of immunoreactive GnRH in a part of the lamprey brain homologous with that brain region in higher vertebrates in which GnRH localization forms part of a neuroendocrine mechanism for gonadotropic regulation. If we add to this evidence the findings reported here in which exogenous GnRH stimulates directly or indirectly (through the adenohypophysis) two phases of ovarian function in *Petromyzon*, the case for hypothalamic control of lamprey reproduction is strengthened. Even without such direct experimental evidence there has been circumstantial evidence of hypothalamic control in that breeding activity and completion of gonadal development in lampreys is synchronized with season, and all of a population of lampreys will mature and breed simultaneously.

It remains unclear what physical environmental clue(s) (photoperiod, temperature) normally can trigger gonadal maturation and function since appropriate experiments have not been done. Our own experiments have shown that endocrine stimulation alone by injection of doses of fish gonadotropin or GnRH sufficient to elevate plasma estradiol will not evoke ovulation at lower environmental temperatures (mean: 13°C). When the temperature was increased to 21°C by use of electric heaters ovulation followed hormone injection. Unfortunately, this experiment was not adequately designed to critically test the effect of temperature since no control animals were retained at 13°C. Practical experience at the Hammond Bay Biological Station is that adult *P. marinus* kept at temperatures below 15.5°C will not ovulate for up to 8 months and will generally die before that time without ovulation (L. King, personal communication).

It is clear from our experiments that receptors in the lamprey adenohypophysis and (or) ovary recognize and respond to the nonapeptide GnRHa analogue that is active in mammals and teleost fish. It is also clear that the GnRHant analogue, which in mammalian tests (Rivier, Rivier, Perrin et al. 1981; Rivier, Rivier, and Vale 1981) is a competitive inhibitor of GnRH, has little or no activity in *Petromyzon*. Thus, receptors for GnRH in *Petromyzon* are apparently specific and can distinguish between molecular variants of this peptide.

The fact that the lampreys gonad responds specifically by steroid secretion and ovulation to injections of exogenous heterospecific gonadotropic preparations indicates that a reasonably typical pituitary—gonadal relationship exists in this group. The possibility that the gonad may respond directly to some degree to GnRH cannot be ruled out by our experimental design. If this evidence now can be accepted as reasonably well established then it is clear that in this agnathan, and presumably in its extinct ancestors nearer the primitive vertebrate evolutionary line, there already was an evolved brain—pituitary—gonadal regulatory mechanism for control of reproduction. Thus, the origin of this mechanism appears lost in the unknown prevertebrate forms about which we have no clear information. The apparent absence of such a regulatory mechanism for gonadal function in hagfishes must be a secondary degenerative evolutionary phenomenon. If this were not so, it would be difficult to explain why in hagfishes the adenohypophysis separates off from the adjacent pharyngeal epithelium and becomes opposed to the neurohypophysis.

In neither the hagfishes nor lampreys is there a vascular portal system which could carry GnRH from the brain to the adenohypophysis (Gorbman 1965; Jasinski 1969). If this is a primitive pattern, then in the lampreys we must assume that GnRH must reach the adenohypophysis by diffusion from the adjacent and coextensive neurohypophysis. If these several presumptions concerning the functional route for GnRH in lampreys is correct, then evolution of the portal vascular connection between neurohypophysis and adenohypophysis is the only major anatomical feature of the hypothalamo—hypophysial system that evolves within the vertebrates (Gorbman 1980).

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**References**


