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The Endocrinology of Reproduction in Lampreys and Applications for Male Lamprey Sterilization

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ABSTRACT. *This review briefly summarizes the latest findings on the reproductive endocrinology of sea lampreys* (Petromyzon marinus) *and the application of these findings to the control of sea lamprey populations by sterilization. Since the last Sea Lamprey International Symposium (SLIS) meeting in 1979, substantial new evidence has now clearly shown that lamprey reproduction is controlled by the neuroendocrine axis. This evidence includes the identification of reproductive hormones—five brain and six pituitary hormones of lampreys have been identified between 1986 and 2000. In addition, there have been extensive physiological and immunological studies on lamprey reproduction. It is concluded that lamprey reproduction is a highly synchronized process that is initiated and mediated by a complex neuroendocrine coordination and integration of environmental cues and hormonal mechanisms.*

This newly acquired information permits the pursuit of a new approach of sterilization for control of reproduction in male lampreys. It is proposed that lamprey gonadotropin-releasing hormone (GnRH) analogs can be developed for sterilizing sea lampreys in a sterile-male-release program in the Great Lakes region. This approach could complement other methods being used to control sea lampreys. The potential of using GnRH analogs (antagonist) is high because these compounds are proteins which are easily degraded within the organism, are non-toxic to humans, are easy to administer, can be administered in the field, are low in cost, and are relatively easy to synthesize. This report includes a summary of the experiments to identify putative GnRH agonists/antagonists that inhibit spermatogenesis without destroying the mating competitiveness of males.

INDEX WORDS: *Sea lampreys, GnRH, sterilization, GnRH analogs, reproduction, spermatogenesis.*

INTRODUCTION

There have been several extensive reviews on lamprey reproduction (Barannikova *et al*. 1995, Larsen 1980, Larsen and Dufour 1998, Sower and Gorbman 1999) and brain and pituitary hormones of lampreys (Sower 1990b, 1995, 1998; Sower and Kawauchi 2001) in recent years. The following sections summarize the most recent findings on reproductive hormones, especially gonadotropin-releasing hormone (GnRH). These findings have led to a focus of studies in examining methods in manipulating lamprey reproduction. Thus, this review in the second part also summarizes research on the application of hormonal control of reproduction, i.e., sterilization.

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Reproductive Cycle of the Lampreys

There are approximately 40 species of lampreys that are classified as parasitic or nonparasitic (Sower and Gorbman 1999). Lampreys spawn only once in their lifetime, after which they die. The parasitic lampreys are generally anadromous. The male and female reproductive cycles coinciding with the life cycle of the sea lamprey, *Petromyzon marinus*, are shown in Figure 1. In the parasitic sea lamprey, sexual maturation is a seasonal, synchronized process (Sower and Gorbman 1999). The sea lampreys begin their lives as freshwater ammocoetes (larval lampreys), which are blind filter feeding larvae. After approximately 5 to 7 years in freshwater streams, metamorphosis occurs and the ammocoetes become free-swimming, sexually-immature lampreys, which migrate to the sea or lakes. During the approximately 15-month-long parasitic sea

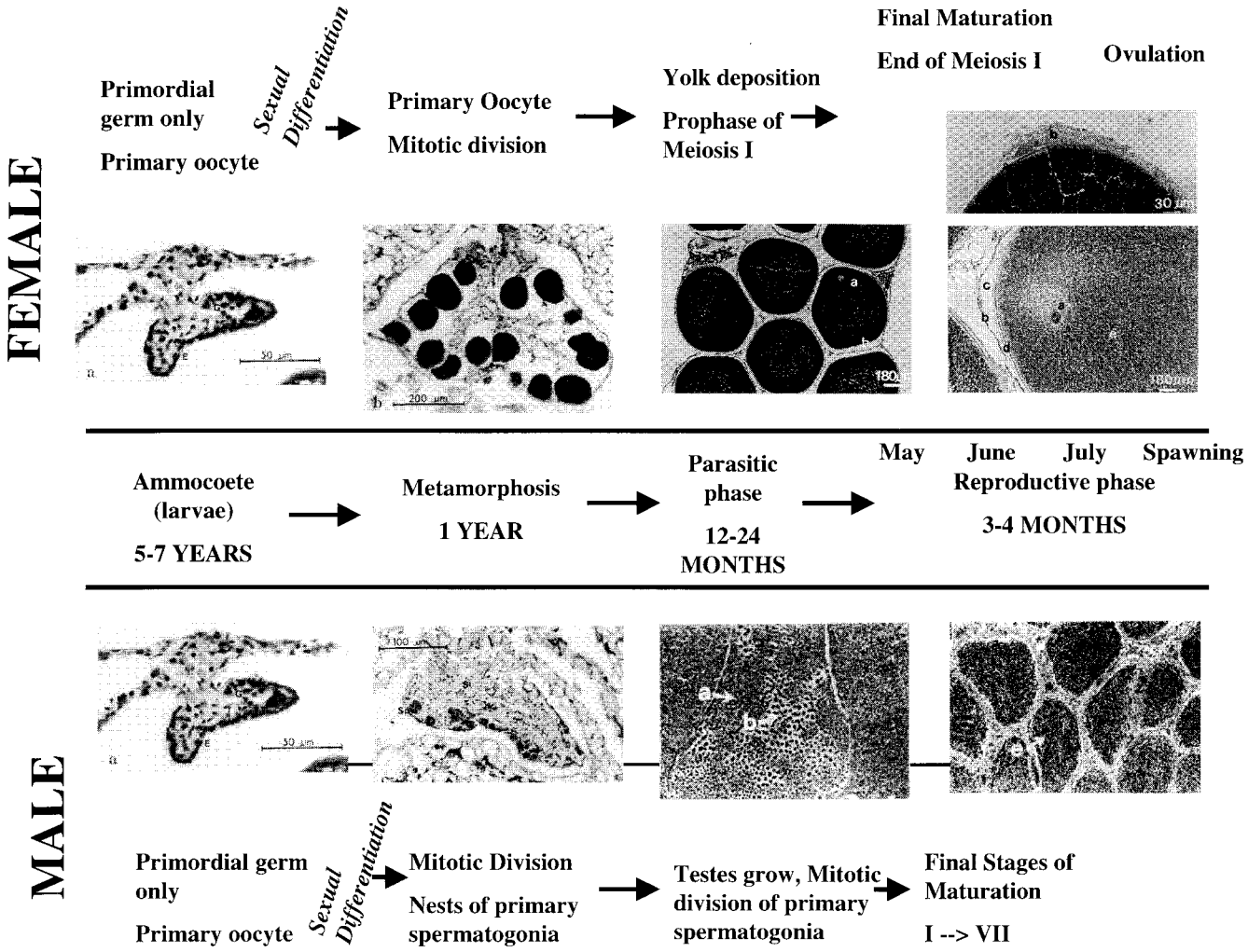

FIG. 1. Schematic diagram of female and male spermatogenesis during the life cycle of the sea lamprey. The histological pictures have previously been published in Fahien and Sower 1990, Bolduc and Sower 1992, and Sower and Gorbman 1999.

phase, gametogenesis progresses. In males, spermatogonia proliferate and develop into primary and secondary spermatocytes; in females, vitellogenesis occurs. After this period, lampreys return to freshwater streams and undergo the final maturational processes resulting in mature eggs and sperm, and finally spawning, after which the lampreys die.

Hypothalamus-Pituitary Axis in Lampreys

A key neuroendocrine function of the hypothalamus in the control of reproduction is the timed release of the decapeptide gonadotropin-releasing hormone (GnRH) in response to external and internal cues. GnRH acts on the pituitary to regulate the pituitary-gonadal axis for all vertebrates. Gonadotropins, secreted in response to GnRH, are released from the pituitary gland and are the major hormones influencing steroidogenesis and gametogenesis. Until about 17 years ago, there had been little evidence for brain control of reproduction in lampreys. However, substantial progress has been made in this area (Sower 1990b, 1997, 1998). Two molecular forms of GnRH have been identified and sequenced in the sea lamprey: lamprey GnRH-I and lamprey GnRH-III (Sherwood *et al.* 1986, Sower *et al.* 1993). In addition, the complementary DNAs (cDNA) of lamprey GnRH-I and -III have been identified (Suzuki *et al.* 2000, Silver *et al.* 2001). Lampreys are the most primitive vertebrates for which there are demonstrated functional roles for multiple GnRH neurohormones involved in pitu-

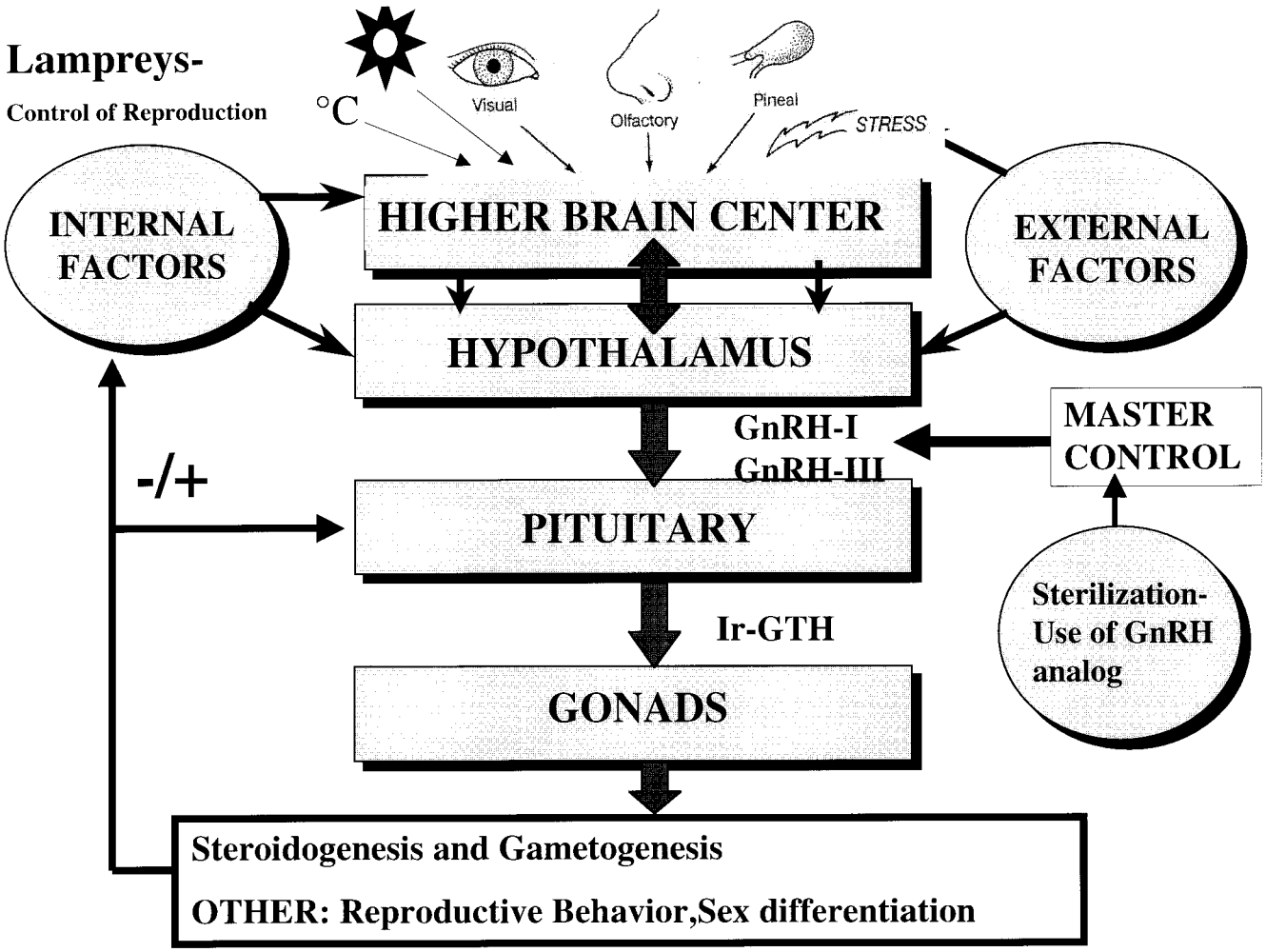

FIG. 2. Schematic diagram of the hypothalamic-pituitary-gonadal axis in the control of reproduction in the sea lamprey.

itary-reproductive activity (Fig. 2). Both lamprey GnRH-I and -III have been shown to induce steroidogenesis and spermiation/ovulation in adult sea lampreys (Deragon and Sower 1994, Gazourian *et al.* 1997, Sower 1990a, Sower *et al.* 1993). In lampreys undergoing metamorphosis, there is an increase of brain lamprey GnRH-I and -III that coincides with the acceleration of gonadal maturation (Youson and Sower 1991). In immunocytochemical studies, both immunoreactive (ir)-lamprey GnRH-I and -III can be found in the cell bodies of the rostral hypothalamus and preoptic area in larval and adult sea lamprey (King *et al.* 1988, Nozaki *et al.* 2000, Tobet *et al.* 1995, Wright *et al.* 1994). Most of the ir-GnRH in the brain of larval stage lampreys has been shown to be lamprey GnRH-III. Thus, lamprey GnRH-III may be the more active form during gonadal maturation. Such information suggests that the structure and function of the GnRHs in vertebrates are highly conserved throughout vertebrate evolution.

Reproductive Steroids

The physiological role of gonadal sex steroids and the identity of other potentially important sex hormones need to be clarified in the lamprey. Plasma estradiol and progesterone have been measured as indicators of gonadal activity in the sea lampreys under various physiological studies. Estradiol (Barannikova *et al.* 1995, Fukayama and Takahashi1985, Katz *et al.* 1982, Sower *et al.* 1985b) and progesterone (Barannikova *et al.* 1995,

Linville *et al*. 1987, Sower 1989) are two steroids that have been associated with reproductive activity in both female and male lampreys. In an earlier study, plasma estradiol but not testosterone was elevated in response to mammal GnRH analog in male and female sea lampreys (Sower *et al*. 1985a). These studies and the demonstrated absence of androgen receptors in the lamprey testis suggest that testosterone may not have a role during the final spermatogenic phases in adult male lampreys. However, Kime and Rafter (1981) and Kime and Callard (1982) found 15 hydroxylated compounds to be produced in the gonads of river and sea lampreys. Based on effects of partial hypophysectomy and gonadectomy on plasma levels of various steroids in river lampreys, *Lampetra fluviatilis* (Kime and Larsen 1987) suggested that the true sex hormones responsible for development of secondary sex characteristics may be 15-hydroxylated derivatives of estradiol and testosterone. However, both the ovary in brook lamprey, *Lampetra aznandreai Vladykov*, (Belvedere and Colombo 1983) and the testis in sea lamprey (Callard *et al*. 1980) have been shown to be capable of synthesizing estradiol. In addition, estradiol and progesterone have been extracted from the ovary (Botticelli *et al*. 1963). Estradiol from these and other studies is certainly a major player in mediating reproduction in lampreys.

Estradiol's role in reproduction is further supported by recent information on the cloning of steroid receptors. The first steroid receptors, lamprey progesterone-like receptor, the estrogen-like receptor, and a corticoid-like receptor have been identified (Thornton 2001). Thornton (2001) proposed that the first steroid receptor in vertebrates was an estrogen receptor, followed by a progesterone receptor. This was based on identification and phylogenetic analysis of steroid receptors in basal vertebrates and reconstruction of the sequences and functional attributes of ancestral proteins. The androgen receptor was not identified and is proposed to have been created by gene duplication after the lamprey lineage diverged from other vertebrates (Thornton 2001). Specific regulation of physiological processes by androgens and corticoids are unique to gnathostomes and are proposed in this study to be relatively recent innovations that emerged after these duplications. Thus from this study and information stated above, estrogen regulation of reproductive maturation and function appears to be one of the major steroid controls in both male and female lamprey reproduction.

Circulating Hormones During Reproductive Cycles

Lampreys are clearly seasonal and temperature-responsive in the timing of their anadromous migrations and reproduction. Many of these processes are coordinated by the neuroendocrine axis through a number of different hormones. For the hormones identified to date, the relative pattern of circulating hormone concentrations during lamprey reproductive cycles are shown in Figure 3. As indicated, there are progressive changes in hormonal levels over time that occur in a changing endocrine environment. It has now been well documented that an essential environmental cue to initiate metamorphosis in sea lampreys is the increase in water temperature in the spring (Holmes *et al*. 1994, Holmes and Youson 1994). As described by Youson (1997), circulating concentrations of thyroxine (T4) and triiodothyronine (T3) drop dramatically at the onset of metamorphosis and do not appear to regulate lamprey metamorphosis as observed in other vertebrate metamorphoses, but these hormones are important in the developmental process. As an indication of the complex simultaneous processes in lampreys undergoing metamorphosis, there is an increase of brain lamprey GnRH-I and -III that coincides with the acceleration of gonadal maturation (Youson and Sower 1991). There is relatively little known of the circulating reproductive hormones coinciding with the reproductive processes during the parasitic phase. However, in adult lampreys, there are seasonal correlations between changes in brain GnRH and gametogenic and steroidogenic activity of the gonads in adult male and female sea lampreys (Bolduc and Sower 1992, Fahien and Sower 1990). In sea lamprey females, it has been demonstrated that lamprey GnRH-III is present in higher concentrations than lamprey GnRH-I during the final stages of the reproductive season (MacIntyre *et al*. 1997). Lamprey GnRH-I concentrations do not change significantly during the reproductive season, whereas lamprey GnRH-III undergoes significant increases during the same period. These results suggest that lamprey GnRH-III may be the major form regulating reproductive processes in the female sea lamprey during the period of final reproductive maturation.

In reproduction, numerous activities are organized in some related manner with respect to maturation, migration, and mobilization of fat stores. As reviewed by Larsen and Dufour (1998), the adult lampreys are not feeding during the final reproduc-

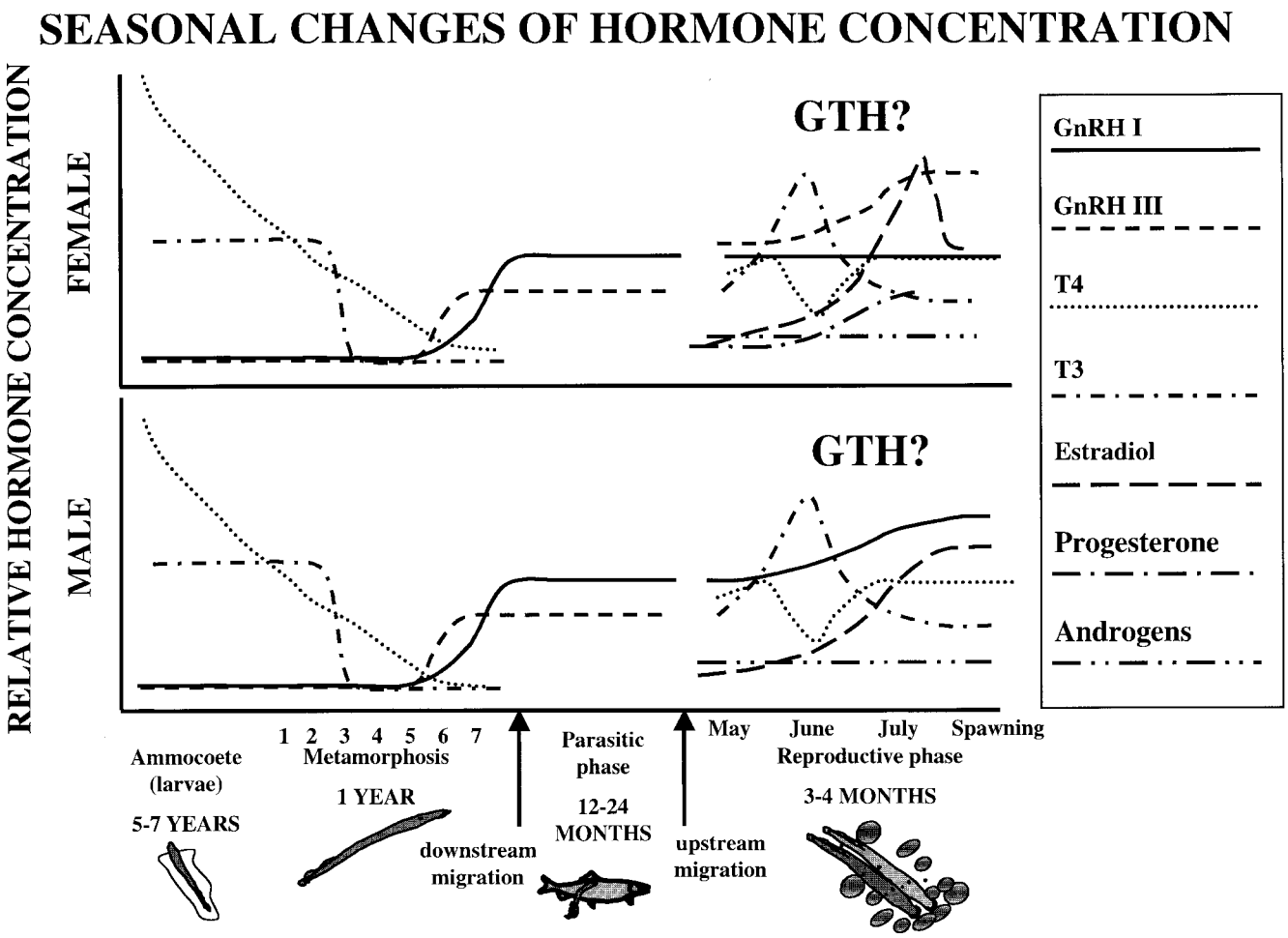

FIG. 3. Schematic diagram of the relative circulating hormone concentration during the life cycle of the female sea lamprey. T4 (thyroxine), T3 (triiodothyronine), GnRH-I (lamprey gonadotropin-releasing hormone-I) GnRH-III ((lamprey gonadotropin-releasing hormone-III) E2 (estradiol) and prog (progesterone) and androgens.

tive period and there is significant mobilization of body tissue used to cover energy requirements used for gonadal growth. It is proposed that a number of different endocrine systems could be involved in these various activities including thyroid and reproductive hormones. Annual cycles in the gonads and thyroid gland and their interactions have been described for numerous species of vertebrates. Several studies of other fish species have indicated that sex steroids can influence thyroid activity (Sower *et al.* 1984). In earlier studies, plasma thyroxine was significantly elevated in female sea lampreys following administration of a GnRH analog (Sower *et al.* 1985b). Not surprisingly, there are also coordinated changes in plasma T3 and T4 in males and females during the final maturational period (Fig. 3). Plasma T3 shows a significant peak coinciding with a significant drop of T4 early in the final maturational period. Whether these thyroid hormones are coordinated or independently associated with lamprey reproduction is not yet known. However, it is evident that there are associated changes of hormones with the final maturational period and migration.

Isolation of cDNA Encoding the Precursor to Lamprey GnRH-I

The cDNA encoding lamprey prepro- GnRH-I was isolated and sequenced in an agnathan, the sea lamprey (Gamble *et al.* 1997, Suzuki *et al.* 2000). The lamprey GnRH-I precursor was the first identified in an ancient lineage of vertebrates and has the

same overall tripartite structure as other vertebrate GnRH precursors. The amino acid sequence of lamprey GnRH-I and the processing site (Gly-Lys-Arg) have been highly conserved during 500 million years of evolution with 60 to 70% identity compared to those of tetrapod and teleost GnRH precursors. In contrast, the GnRH associated peptide regions were markedly divergent with less than 20% identity compared to all identified vertebrate precursors. Unlike all other known vertebrate GnRH precursors, which typically have one and in a single case two transcripts, three distinct transcripts were isolated and sequenced in lampreys. The lamprey GnRH-I transcripts, termed GAP49, GAP50, and GAP58, differed in the length of the GAP coding sequence and were demonstrated to be the products of a single gene. Analysis of the lamprey GnRH-I gene intron-2 splice junction demonstrated that alternate splicing produces the different lamprey GnRH-I transcripts. Lamprey GnRH-I is the first GnRH gene demonstrated to utilize splice sequence variants to produce multiple transcripts.

The Interrelationship of NPY, PMY, GABA and GnRH in the Sea Lamprey

Many factors have been identified in vertebrates which are able to modulate reproductive events through the influence on the hypothalamic-pituitary-gonadal axis. Two such modulators have been found to establish relationships with GnRH neurons from the earliest stages of their development. Thus, neuropeptide Y(NPY) and γ-aminobutyric acid (GABA) have been shown to influence reproductive processes in vertebrates, and have been found in and around GnRH neurons during their migration in several different species. In sea lampreys, both peptide methionine-tyrosine (PMY, a neuropeptide y-like hormone) and GABA were determined to interact with the reproductive neuroendocrine axis (MacIntyre *et al*. 1997, Reed *et al*. 2002). NPY is a 36 amino acid peptide that has been shown to act at the level of the hypothalamus and pituitary to alter GnRH and gonadotropin (GTH) release, respectively (Larhammar 1996). Immunocytochemical studies have determined that, in both teleosts and mammals, NPY-containing cells can be identified in close proximity to GnRH containing cells (Larhammar 1996). Whether NPY exerts a stimulatory or inhibitory effect at either of these levels has proven to be highly dependent on the hormonal milieu. In teleosts, NPY is able to stimulate GnRH and GTH release from the hypothalamus and pituitary and potentiate GnRH induced GTH release when conducive steroidal conditions exist (Larhammar 1996).

PMY, a neuropeptide y-like hormone, was isolated first from the intestine and then from the brain of the sea lamprey (Conlon *et al*. 1994, Conlon *et al*. 1991). PMY is structurally more similar to NPY than other NPY-family members as it has the same amino acid residues at key positions identified in all other vertebrate forms of NPY (Conlon *et al*. 1991). Studies showed that PMY suppressed estradiol levels in female sea lamprey (MacIntyre *et al*. 1997). It was further demonstrated that PMY elevated brain lamprey GnRH-I and -III content, which is consistent with the function of NPY observed in other vertebrates (MacIntyre *et al*. 1997). At this time, it is undetermined whether PMY altered estradiol concentration through direct action at the ovaries or if PMY affected pituitary function.

The inhibitory neurotransmitter, GABA, is distributed somewhat ubiquitously throughout the central nervous system. GABA and agents which alter GABAergic function significantly affect reproductive processes through their actions at the hypothalamus and pituitary. In mammals, GABA containing neurons have been visualized along the GnRH neuronal migratory pathway, and in some cells GnRH and GABA were co-expressed (Tobet *et al*. 1996a). The development of GnRH containing neurons was delineated in the prolarval and larval sea lamprey (Tobet *et al*. 1996b). The results from Tobet *et al.* (1996a, 1996b) and Reed *et al.* (2002) provide supporting evidence for the hypothesis of a regulatory role of GABA on GnRH neurons in the sea lamprey.

Pituitary Hormones in Lampreys

Until recently, the only pituitary hormone that had been structurally identified in the lamprey was arginine vasotocin (Lane *et al*. 1988). From 1995 to 2000 research identified the first anterior pituitary hormones and cDNA/genes in lampreys (Table 1; Sower and Kawauchi 2001).

The complete primary amino acid sequences for the pituitary hormones have been determined except for GTH and thyrotropin (TSH). Evidence from physiological and immunocytochemical studies strongly supports the presence of a gonadotropin-like molecule in lampreys, indicating that a reasonably typical pituitary-gonadal relationship exists in this group (Hardisty and Baker 1982, Larsen and Rothwell 1972, Sower 1990b, Sower

TABLE 1. Summary of the identified hormones by primary amino acid sequencing and identified cDNAs or genes from the brain and pituitary in lampreys. GnRH, gonadotropin releasing hormone; PMY, NPY, neuropeptide Y-related peptide; ACTH, adrenocorticotropin; MSH-A, MSH-B, melanotropins; NHF, naso-hypophysial factor; AVT, arginine vasotocin; POM, proopiomelanotropin, and POC, proopiocortin.

Tissue	Hormone	Gene/cDNA	Function	References
Brain				
	lamprey GnRH-I		Neurohormone	(Sherwood *et al.* 1986)
	lamprey GnRH-III		Neurohormone	(Sower *et al*. 1993)
	somatostatin-14		Unknown	(Sower *et al*.1994)
	PMY-like (NPY)		Reproduction	(Conlon *et al*. 1994)
	lamprey tachykinin		Unknown	(Waugh *et al*. 1994)
		NPY		(Soderberg *et al*.1994)
		lamprey GnRH-I		(Gamble *et al*. 1997, Suzuki *et al*. 2000)
		lamprey GnRH-III		(Silver *et al.* 2001)
Pituitary				
	ACTH		steroidogenesis	(Takahashi *et al.* 1995a)
	MSH-A		melanotropic	(Takahashi *et al*. 1995a)
	MSH-B		melanotropic	(Takahashi *et al*. 1995a)
	NHF		unknown	(Sower *et al*. 1995)
	AVT		unknown	(Lane *et a*l. 1988)
		POM		(Takahashi *et al*. 1995b)
		POC		(Heinig *et al*. 1995)
		AVT		(Suzuki *et al*. 1995)
		GH		(Kawauchi *et al.* 2002)

1998). In river lampreys (*L. fluviatilis*), hypophysectomy and substitution therapy with pituitary extracts or mammalian GTHs indicated pituitary regulation of the gonads (Larsen 1980). Moreover, injection of salmon gonadotropin preparation into adult spawning sea lamprey advanced ovulation by several weeks and elevated plasma estradiol levels (Sower *et al*. 1983). In addition, previous studies demonstrated that there are two distinct high affinity binding sites in the pituitary for lamprey GnRH-I and -III and that these hormones differentially regulate lamprey pituitary function (Knox *et al*. 1994, Materne *et al*. 1997, Sower 1997, Sower 1998). A mammalian-like immunoreactive luteinizing hormone was shown to be present by immunocytochemistry in the pituitary of the sea lamprey (Wright 1983).

Based on this evidence of a GTH-like molecule in lampreys, a concerted effort has been made to identify GTH in sea lampreys. Recently, using immunocytochemistry, Nozaki *et al*. (1999) detected immunoreactive (ir) GTH in the sea lamprey pituitary using two different cytochemical approaches: 1) lectin histochemistry and 2) immunohistochemistry**.** Based on these recent results, molecular and protein isolation procedures for lamprey GTH are now focused on using probes and antibodies to ovine luteinizing hormone (oLH).

Characterization of GnRH Binding Sites in the Pituitary and Gonads

To date, the only direct action of GnRH on the lamprey pituitary has been the characterization of two high affinity GnRH-binding sites in the adult female sea lamprey. In recent studies, *in vitro* binding analysis was performed on pituitary sections in an effort to better understand the differential roles of the two GnRH binding sites throughout the development and sexual maturation (stage I, II, III, and ovulation) of the female sea lamprey, and to characterize the affinity for GnRH binding sites of four potential lamprey GnRH antagonists in the sexually mature male land-locked lamprey. Two high-affinity GnRH binding sites were observed throughout the development and final sexual maturation of female sea lamprey (Materne *et al*. 1997). Concentration of sites increased in correlation with increased gonadal maturation and brain GnRH concentration, peaking near and at ovulation. All four lamprey GnRH analogs demonstrated two specific binding compartments of high and low affinity with

inhibition constants comparable to those of the native lamprey GnRH-I & III.

GnRH Analogs in Mammals and Fish

Since 1971, when the primary structure of mammal GnRH (or LHRH) was determined, over 7,000 analogs to GnRH have been made and tested in hundreds of various studies in mammals. In mammals, there has been great success using various mammalian GnRH analogs for sterilization, conception, and other therapeutic and clinical applications. As an example, Lupron Depot, a GnRH analog, is now one of the leading chemical treatments for advanced prostate cancer and endometriosis. In humans, it is typically given as a monthly injection because it has been microencapsulated. Continuous treatment of Lupron Depot results in decreased levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH). In males, testosterone is reduced to castrate levels. In pre-menopausal females, estrogens are reduced to post-menopausal levels. The most active synthetic agonists are found to be those with D-amino acid substitution in position 6. The most effective GnRH antagonists are those that also have substitutions in position 6 as well as substitution of amino acids in positions 1, 2, and 3.

A considerable amount of research has been devoted to the effects of GnRH and analogs on reproduction in fish during the past 15 years. Almost all of the research to date has been focused on GnRH-based spawning induction therapy in a number of commercially important species (Zohar *et al*. 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* (Crim and Glebe 1984, Sower *et al*. 1982), 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.

Applications for Male Lamprey Sterilization

During the past few years, the Great Lakes Fisheries Commission has been searching for alternative methods to control sea lamprey populations in the Great Lakes region. In its 1992 Strategic Plan, the Great Lakes Fishery Commission stated that one of its major objectives was to suppress sea lamprey populations to target levels by reduction of the use of lampricides and by development of new control methods by 2010. Lee Hanson, USFWS, Hammond Bay Biological Station, Michigan, pioneered work on the use of bisazir for sterilization of sea lamprey (Hanson and Manion 1978, 1980; Hanson 1981). Bisazir is currently being used in a sterile-male release program, but is extremely hazardous to humans and required a special facility to be constructed at the Hammond Bay Biological Station in 1991 for its use. Other chemosterilants that are non-hazardous need to be considered. In March 1992, the Great Lakes Fisheries Commission sponsored a workshop on sex determination/ differentiation that recommended development of new methods for sterilization as a high priority for future investigations. Therefore, a goal of my laboratory has been to develop a method of sterilizing male sea lampreys using a lamprey GnRH antagonist. This approach has been possible because of recent work on the identification and function of GnRH in lampreys.

Use of GnRH Analogs for Sterilization in Male Sea Lampreys

There is potential for using GnRH analogs to sterilize male sea lampreys (Fig. 4). Putative lamprey GnRH analogs have been tested to determine those that are reproductively active in the sea lamprey. Reproductive activity was evaluated by measuring the GnRH analogs ability to stimulate or inhibit plasma steroid levels *in vivo*. In addition, a pituitary perifusion method has been used to evaluate pituitary response to various GnRH analogs. Even though lamprey gonadotropins have yet to be isolated, pituitary responsiveness can be determined by the analogs ability to bind to GnRH pituitary receptors as has been previously demonstrated.

The effects of mammalian and lamprey GnRH analogs are summarized in the following paragraphs. In early studies, injections of a synthetic agonist of mammalian GnRH ([D-Ala^6, Pro^9] NEt mammalian GnRH) significantly elevated plasma estradiol and advanced ovulation by at least several weeks in adult female lampreys (Sower *et al*. 1983).

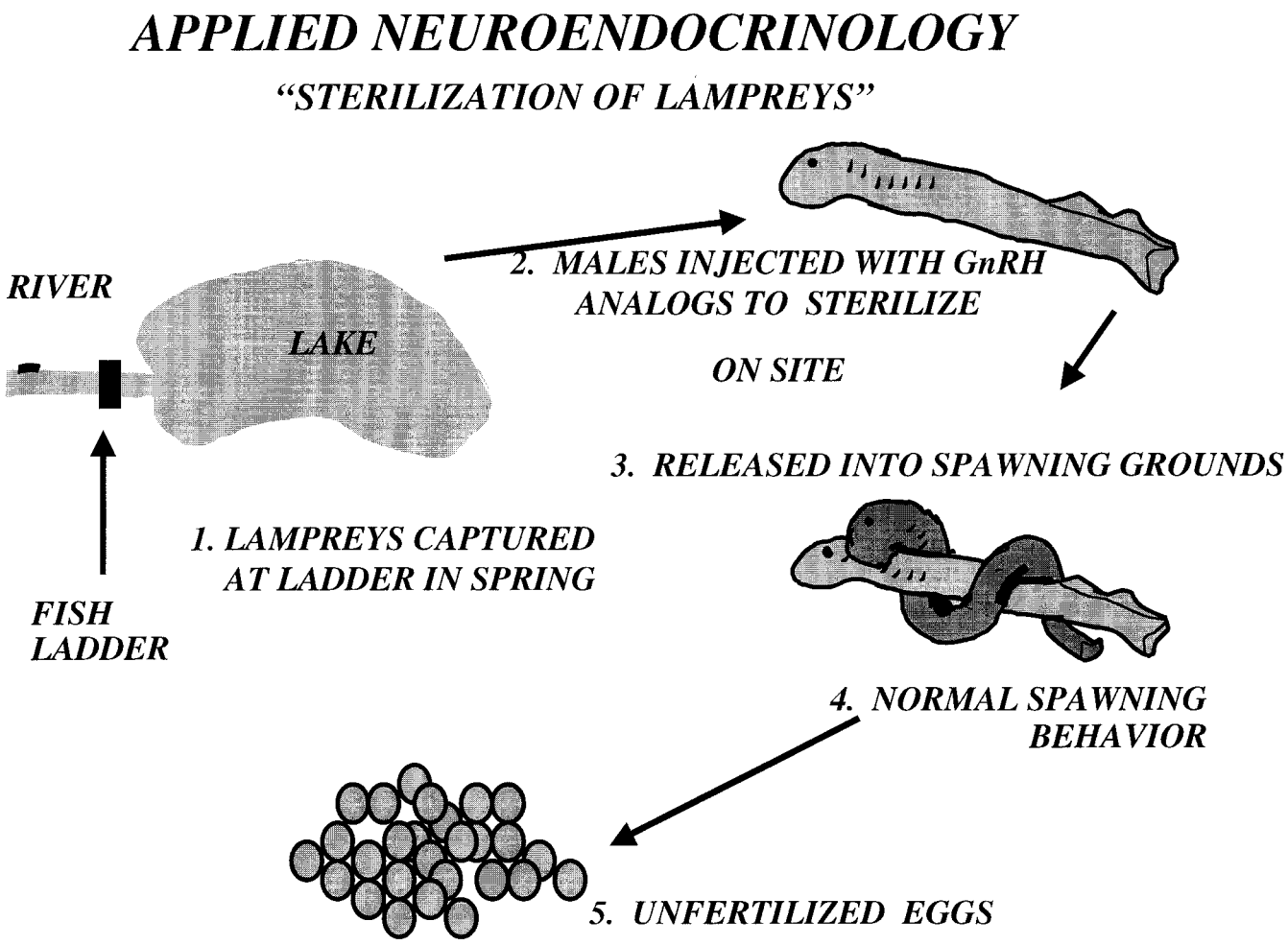

FIG. 4. Schematic diagram of Applied Neuroendocrinology. Application of the potential use of GnRH analogs in inducing sterilization in male sea lampreys.

In this same study, a mammalian GnRH antagonist ([Ac-3 Pro^1, 4-FD-Phe^2, D-$Trp^{3,6}$] mammalian GnRH), which is a competitive inhibitor of GnRH in mammalian systems, had no apparent effect on plasma estradiol concentrations or on timing of ovulation. These data confirmed that the receptors for GnRH in the sea lamprey are specific and can distinguish between variants in this molecule. [D-$Phe^{2,6}$,Pro^3] lamprey GnRH was one of the first GnRH analogs tested in lamprey and found to be a putative antagonist. It inhibited ovulation in mature female lampreys, and inhibited spermiation and reduced plasma progesterone levels in the male sea lampreys (Sower 1989, Sower *et al.* 1987).

Some GnRH-I analogs (but not GnRH-III) have been shown that they can influence the spawning behavior in lampreys—actually enhancing the spawning act rather than decreasing it. Earlier studies investigated the effects of GnRH and analogs on spawning behavior in adult male and female sea lamprey during three successive spawning seasons (Sower and Hanson 1992). In each of these experiments, three or four groups of 12 sea lampreys each were injected two times with saline, lamprey GnRH-I, lamprey GnRH agonist [D-Ala^6,Pro^9 NEt lamprey GnRH], or a GnRH antagonist [D-$Phe^{2,3}$,Pro^3 lamprey GnRH]. After the second injection, the lampreys were introduced into an artificial stream channel and behaviors of spawning activity, resting, nest building, swimming, and fanning were monitored. The lampreys were observed four times daily for 10 minutes every 1/2 hr during 2-hr periods (experiments 1 and 2) or were observed for 6 hrs on a continuous basis (experiment 3). In experi-

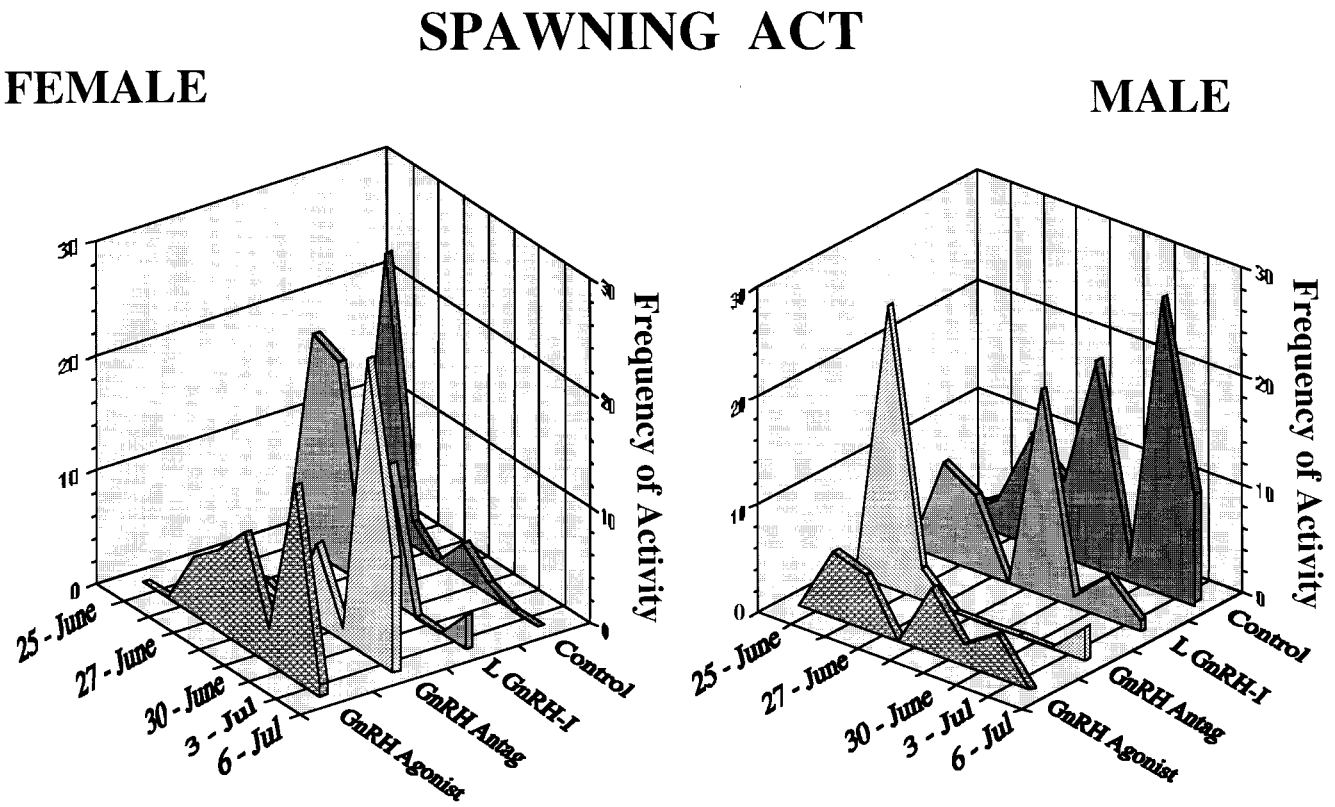

FIG. 5. The effect of injecting saline (control); lamprey GnRH-I; lamprey GnRH agonist [D-Ala^6,Pro^9 Net lamprey GnRH] or a GnRH antagonist [D-Phe^{2,3},Pro^3 lamprey GnRH] on sea lamprey spawning behavior activity.

ment 2, spawning behavior was inhibited in females treated with lamprey GnRH agonist or antagonist compared to controls (Fig. 5). However, in the males, lamprey GnRH agonist or antagonist stimulated earlier spawning activity compared to the controls. In experiment 3, lamprey GnRH antagonist induced earlier spawning activity in males while lamprey GnRH agonist inhibited spawning activity and lamprey GnRH delayed spawning activity compared to the controls (Fig. 5). These data suggested that lamprey GnRH-I influences spawning behavior in sea lampreys. Furthermore, the responses to GnRH and analogs were different in males compared to females, suggesting that different neuroendocrine mechanisms may be involved. Other studies (Sower, unpublished data) have indicated that lamprey GnRH-III does not influence the lamprey spawning behaviors. Therefore, because lampreys have two GnRHs that act as neurohormones controlling the pituitary-gonadal axis and act in a differential manner, it is proposed that an analog to lamprey GnRH-III can be developed in which the spawning behavior would not be affected, yet the lampreys would be sterilized.

Temperature has been considered an important environmental factor for the final maturational processes in adult sea lampreys (Bolduc and Sower 1992, Fahien and Sower 1990). Therefore, the effects of different temperatures were examined 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 an *in vivo* study, the effects of lamprey GnRH-I, -III, and analogs on plasma estradiol concentrations in the male sea lamprey were examined at 8 and 16°C (Gazourian *et al*. 2000). All peptides tested *in vivo*, except [Trp^3] lamprey GnRH-I, effectively stimulated plasma estradiol after 4 hours in lampreys held at 8 or 16°C. In the *in vitro* studies, lamprey GnRH-I and -III significantly stimulated the pituitary-gonadal axis estradiol when incubated at 18°C. [D-Glu^6] lamprey GnRH-I at all doses suppressed the putative pituitary response on the testis at 14°C,

TABLE 2. Inhibition constants (K_I) of lamprey GnRH-putative antagonists in the pituitary of adult male land-locked sea lamprey* (Petromyzon marinus). *Affinity at the type-I GnRH-binding site was significantly higher than affinity at the type-II ($P > 0.0001$). Self displacement assay with [DAla^6, Pro^{9}Net]-mammalian GnRH was used as a control and demonstrated high affinity for both GnRH binding sites. The data were plotted on a logit-log plot and the K_I determined from the IC_{50} using the Prism software.

	K_I (type-I) (M)	K_I (type-II) (M)
[DAla^6, Pro^{9}Net]-m GnRH	3.11×10^{-12}	8.9×10^{-8}
[D-Phe^2,Gly^6]-lGnRH-I	1.37×10^{-9}	5.65×10^{-6}
[D-Phe^2,Gly^6]-lGnRH-III	3.83×10^{-12}	6.55×10^{-7}
[D-Phe^{2,6},Pro^3]-lGnRH-III	3.0×10^{-11}	5.22×10^{-5}
[Ac-Delta-3Pro,4FDPhe^2-D-Trp^{3,6}]-lGnRH-III	7.1×10^{-9}	8.94×10^{-6}

whereas cyclo [Glu^6-Trp^7-Lys^8] lamprey GnRH-I only suppressed the pituitary at a dose of 100 and 1,000 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, -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.

GnRH analogs were tested which had modifications in the second and third positions of the native molecule (Gazourian *et al*. 2000). 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). Similar to the studies in mammals, replacement of the native Tyr^3 of lamprey GnRH-I with tryptophan rendered the analog completely inactive, suggesting that the third position of lamprey GnRH-I is critical for binding and/or activation of the receptor (Gazourian *et al*. 2000).

In an unpublished study (O. Materne and Sower, unpublished data) the binding affinity in the pituitary of male sea lampreys was determined with lamprey GnRH-I and III analogs in an effort to better understand the structure-activity relations of the lamprey GnRH. Competition studies were performed using the iodinated [DAla^6, Pro^{9}NEt]-mammalian GnRH as the tracer with [D-Phe^2,Gly^6]-lGnRH-I (Peninsula labs, CA.), and the following lGnRH-III analogs: [D-Phe^{2,6},Pro^3], [D-Phe^2,Gly^6], and [Ac-Delta-3Pro,4FDPhe^2-D-Trp^{3,6}] from American Peptide, CA. All lamprey GnRH analogs tested demonstrated a high affinity binding for the type-I GnRH binding site while demonstrating a significantly lower affinity for the type-II site, the level of significance was $P < 0.0001$ (Table 2). Self-displacement with [DAla^6,Pro^9] NEt mammalian GnRH was used as a control and demonstrated high affinity for type-I and II binding sites. The studies testing these analogs showed a dose-dependent displacement by the lamprey GnRH analogs demonstrating a high affinity binding site and a significantly lower affinity binding site suggesting that both binding sites can discriminate between GnRH molecule variants. These inhibition data, taken together with *in vivo* studies showing a decrease in sperm motility when compared to controls (Sower, unpublished data), were found to correlate with previous studies where [Gly^6] and [D-Phe^{2,6},Pro^3]-lamprey GnRH-I as well as [Ac-Delta-3Pro,4FDPhe^2-D-Trp^{3,6}]-mammalian GnRH antagonists were found to decrease gametogenesis in male and female sea lampreys (Sower 1987, Sower 1989, Sower *et al*. 1983). These *in vivo* and *in vitro* studies suggest that both lamprey GnRH-I and lamprey GnRH-III molecules can be used in the design of antagonists.

In summary of the studies on GnRH analogs, proposed putative agonists/antagonists have been identified that may be used to enhance reproduction in lampreys. This would be a valuable tool in a sterile-male-release program in the Great Lakes.

Microencapsulation of GnRH

Lamprey GnRH analogs may prove to be safer, less expensive chemosterilants for use in the sea lamprey control program in the Great Lakes. However, this will require a method of controlled release of the GnRH analog which means one injection that can be done in the field. Experiments were conducted to determine the injection site and delivery agent that would best ensure a slow constant release of D-Ala^6 Pro^9 NEt mammal GnRH (GnRHa) over a period of 4 weeks (Hanson, Evans and Sower, unpublished data). In the plasma, GnRH is easily degraded by proteolytic enzymes. Therefore, a simple injection will not elevate plasma GnRH levels for the duration of time required to ensure the appropriate reproductive effect. If a GnRH analog is used to sterilize lampreys in the Great Lakes, a method will have to be used to maintain high levels of GnRH in the plasma for the length of the spawning season, about 6 weeks. It is believed that controlled release of GnRH could be attained by injection of biodegradable microspheres or implants containing a GnRH analog.

The effect of route of administration of GnRH analogs has been examined in a few fish species, but published information is quite scarce. Lam *et al*. (1976) evaluated the ovulation of goldfish (*Carassius auratus*) after intracranial or intraperitoneal injections of synthetic mammalian GnRH. Intracranial injections were more effective than intraperitoneal injections; however, the differences in effectiveness were not as significant as expected, probably because of the short half-life of GnRH in the bloodstream (Breton *et al*. 1990). It may be that the interval between successive injections (24 hours) was too long for full utilization of the advantage of the intracranial injections (Lam *et al*. 1976). Donaldson *et al*. (1981) examined the effects of intraperitoneal and intramuscular injections of mammal GnRH (1.0 mg/kg body weight) on ovulation in the coho salmon. No significant differences were found between these two treatment groups.

In experiments in lampreys, microspheres (dose of 75 µg GnRHa/ lamprey) and 2 mm implants (Aquapharm Technology Inc.) were injected either intramuscularly (IM) or intraperitoneally (IP) (S.A. Sower, University of New Hampshire, L. H. Hanson, Hammond Bay Biological Station, and E. Evans, University of New Hampshire, unpublished data). At week 1, plasma GnRHa was detected in two-thirds of the lampreys injected IP, either with microspheres (5.0 ± 1.3 ng/mL) or implants (1.6 ± 0.3 ng/mL). Ninety percent of the lampreys injected IM with microspheres had detectable levels of GnRHa (4.2 ± 1.1 ng/mL) after 1 week. At week 2, 92% of the lampreys injected IM with microspheres still had detectable levels of GnRHa (3.1 ± 1.2 ng/mL), while less than 30% of the other two treatment groups had detectable levels of GnRHa. Only the lampreys injected IM with microspheres still had detectable levels of GnRHa after 3 weeks (2.9 ± 1.5 ng/mL). Based on the results of this experiment, the best method of controlled release of GnRHa was determined to be via intramuscular injections using microspheres.

Summary of Application of GnRH Analogs in Male Lamprey Sterilization

As described above, there is excellent promise that a lamprey GnRH analog may present a viable alternative and/or complement to bisazir for use in the lamprey sterile-male-release program. A proposal that further testing of GnRH analogs will likely yield a method of sterilizing male lampreys for use in this program in the Great Lakes is summarized by the following:

1. The potential of using GnRH analogs (antagonist) is high because these compounds are proteins which are easily degraded within the organism, are non-toxic to humans, are easy to administer, are low in cost, and are relatively easy to synthesize. These compounds can easily be injected into the lampreys in the field by a single injection using a microencapsulation delivery system that allows the GnRH analog to be released in the lamprey during the spawning season. This system has been shown to be effective. However, because Aquapharm, the vendor of the microsphere preparation, no longer exists, new procedures for making these microencapsulated GnRH analogs will have to be developed.
2. GnRH analogs have been examined because they are the most likely compound to be approved by the FDA. As examples, the use of GnRH analogs has already been approved for use in enhancing fish reproduction in aquaculture. An analog of GnRH is one of the leading chemical treatments for advanced prostate cancer in men and endometriosis in women.
3. New methods in molecular biology and in

structural modeling of proteins will allow the screening of potential GnRH analogs that previously could not be done. Once the primary structure of GTH in lampreys has been determined, the development of a GTH radioimmunoassay can be done. This would be followed by extensive perifusion experiments of the pituitary, in which potential GnRH analogs can be screened.

4. Lampreys are among the few vertebrates to clearly demonstrate roles for multiple GnRH molecules as neurohormones involved in pituitary-gonadal function. In mammals, the research to date has only shown one GnRH involved in pituitary-gonadal function. Studies have shown that GnRH can affect reproductive behavior in vertebrates. Some GnRH-I analogs (but not GnRH-III) have been shown to influence the spawning behavior in lampreys—actually enhancing the spawning act rather than decreasing it. Thus, because lampreys have two GnRHs that act as neurohormones controlling the pituitary-gonadal axis and act in a differential manner, it is proposed that an analog to lamprey GnRH-III can be developed in which the spawning behavior would not be affected, yet the lampreys would be sterilized. However, the current limitations of determining the use of a GnRH analog include that there is only one time to test the analogs during the year and the current inability of measuring gonadotropin directly.

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