

THE ORIGIN AND EVOLUTION OF GONADOTROPIN-RELEASING HORMONE  
IN BONEY FISHES

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DOCTOR OF PHILOSOPHY

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### Abstract

Gonadotropin-releasing hormone (GnRH) is a decapeptide with a central role in vertebrate reproduction. Each of the GnRH peptides characterized by primary structure has a modified pyroglutamyl N-terminus and conserved amino acids in positions 1, 2, 4, 9 and 10. GnRH peptides belong to a family, but not a superfamily, of related peptides. The evolutionary changes in this neuropeptide family are examined in regard to structure and role in reproduction.

The primary structure of GnRH peptides from nervous tissue of boney fishes and a tunicate was determined by using high performance liquid chromatography (HPLC), radioimmunoassay (RIA) and sequence analysis. In addition, indirect investigations using HPLC and RIA were used to help delineate the evolution of GnRH.

In this thesis, mammalian GnRH (mGnRH) is characterized by primary structure from a living representative of an ancient boney fish, the Russian sturgeon *Acipenser gueldenstaedti*. As well, mGnRH is shown to be present in the brains of early-evolved teleosts such as the butterflyfish (*Pantodon buchholzi*), a boney tongued fish in the order Osteoglossiformes and the moray eel (*Muraena militaris*) in the order Anguilliformes. In the herring (*Clupea harengus pallasii*), three forms of GnRH are present in the brain: salmon GnRH (sGnRH), chicken GnRH-II

(cGnRH-II) and a novel form, herring GnRH (hGnRH). Herring represent the most phylogenetically ancient fish with sGnRH as confirmed by primary structure, although evidence is presented that the knifefish, *Xenomystus nigri*, (Osteoglossiformes) may also have sGnRH in their brains. As well, mGnRH disappears in early-evolved teleosts such as herring and knifefish. The identification of hGnRH by primary structure represents the first known appearance of three forms of GnRH within a species.

The presence of sGnRH and cGnRH-II in salmon was confirmed by primary structure. As well, the distribution of these two forms was shown to include eight other species of salmonids from three genera. The physiological role of GnRH was investigated in chinook salmon (*Oncorhynchus tshawytscha*) by measuring plasma gonadotropin levels in fish induced to ovulate by exogenous application of a sGnRH analogue. The sGnRH analogue induced ovulation by increasing plasma gonadotropin-II levels concomitant with decreasing plasma gonadotropin-I levels.

The primary structure of a novel form of GnRH, sea bream GnRH (sbGnRH), is identified in fishes of the order Perciformes: the sea bream (*Sparus aurata*), tilapia (*Oreochromis niloticus*) and another cichlid fish, *Haplochromis burtoni*. In addition, sGnRH for tilapia and cGnRH-II for sea bream and tilapia are characterized by primary structure. An abundance of sbGnRH in the pituitary

of percomorph fishes supports the role of sbGnRH as the physiological releaser of the gonadotropins. The phylogenetic origin of sbGnRH was traced using HPLC and RIA to the rockfish (*Sebastes rastelliger*; Scorpaeniformes).

The invertebrate origin of GnRH is shown by the identification of two novel forms of GnRH by primary structure in the tunicate, *Chelyosoma productum*. Tunicate GnRH-I is related to the ancestral lamprey GnRH-III by its structure and a putative salt bridge between residues 5 and 8. Tunicate GnRH-II contains a cysteine residue in position 6 and appears to be a homodimer. The identification of two GnRH peptides in tunicates represents a conservation of GnRH that spans 600 million years.

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**List of Abbreviations****Gonadotropin-releasing hormone (GnRH) Family**

cfGnRH: Catfish GnRH  
cGnRH-I: Chicken GnRH-I  
cGnRH-II: Chicken GnRH-II  
dfGnRH: Dogfish GnRH  
hGnRH: Herring GnRH  
lGnRH-I: Lamprey GnRH-I  
lGnRH-III: Lamprey GnRH-III  
mGnRH: Mammalian GnRH  
sbGnRH: Sea bream GnRH  
sGnRH<sub>a</sub>: Salmon GnRH analogue  
sGnRH: Salmon GnRH  
tGnRH-I: Tunicate GnRH-I  
tGnRH-II: Tunicate GnRH-II

**Unidentified hormones observed during purification**

H-I, H-II, H-III: Herring  
S-I, S-II, S-III: Salmon  
sbGnRH-I, sbGnRH-II, sbGnRH-III: Sea bream  
T-I, T-II, T-III, T-IV: Tilapia

**Pituitary Hormones**

GH: Growth hormone

GTH-I, LH: Gonadotropin-I, Luteinizing hormone

GTH-II, FSH: Gonadotropin-II, Follicle Stimulating Hormone

PRL, PRL<sub>177</sub>, PRL<sub>188</sub>: Prolactin

**Analysis Techniques**

HPLC: High performance liquid chromatography

irGnRH: Immunoreactive GnRH

MALDI-MS: Matrix-assisted laser desorption/ionization mass  
spectroscopy

RIA: Radioimmunoassay

**Solvents**

ACN: CH<sub>3</sub>CN; acetonitrile

HFBA: heptofluorobutyric acid

TEAF: triethylammonium formate

TEAP: triethylammonium phosphate

TFA: trifluoroacetic acid

**Molecular Terms**

cDNA: Complementary DNA

cRNA: Complementary RNA

GAP: GnRH associated peptide

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## Common and taxonomic names of boney fishes used in this thesis

Common name	Order	Genus and species
Alligator gar	Lepisostiformes	<i>Lepisosteus spatula</i>
Bowfin	Amiiformes	<i>Amia calva</i>
Brook Trout	Salmoniformes	<i>Salvelinus fontinalis</i>
Butterflyfish	Elopiformes	<i>Pantodon buchholzi</i>
Catfish	Siluriformes	<i>Clarias spp.</i>
Cichlid	Perciformes	<i>Haplochromis burtoni</i>
Eels	Anguilliformes	<i>Muraena miliaris</i>
Goldfish	Cypriniformes	<i>Carassius auratus</i>
Grayling	Salmoniformes	<i>Thymallus arcticus</i>
Herring	Clupeiformes	<i>Clupea harengus pallasii</i>
Knifefish	Osteoglossiformes	<i>Xenomystus nigri</i>
Medaka	Cypridontiformes	<i>Oryzias latipes</i>
Pumpkinseed	Perciformes	<i>Lepomis gibbosus</i>
Reedfish	Polypteriformes	<i>Calamoichthys calabaricus</i>
Rockfish	Scorpaeniformes	<i>Sebastes rastrelliger</i>
Sabalo	Characiformes	<i>Prochilodus lineatus</i>
Salmon	Salmoniformes	<i>Oncorhynchus spp.</i>
Sea bream	Perciformes	<i>Sparus aurata</i>
Snook	Perciformes	<i>Centropomis undecimalis</i>
Sturgeon	Acipenseriformes	<i>Acipenser spp.</i>
Tilapia	Perciformes	<i>Oreochromis spp</i>
Whitefish	Salmoniformes	<i>Prosopium/Corigonium spp.</i>
Zebrafish	Cypriniformes	<i>Brachydanio rerio</i>

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## Chapter 1

### GENERAL INTRODUCTION

Present day fish species comprise nearly one half of all known vertebrate species (see Nelson, 1984). Of these species about 50 are jawless fishes and 800 are cartilaginous fishes (see Walker and Liem, 1994). The most numerous and varied among the fishes are the boney fishes, Osteichthyes, with 22,000 species to which about 100 new species are added each year (Bone and Marshall, 1992; Walker and Liem, 1994).

Fish have evolved many reproductive adaptations, which in part, reflects their habitation of freshwater, seawater and brackish water on every continent. Indeed, the reproductive strategies of fishes are as diverse as the fish themselves. Pivotal to the control of reproduction is a brain hormone, gonadotropin-releasing hormone (GnRH). The identification of the primary structures of GnRH in the lamprey (Sherwood et al., 1986a; Sower et al., 1993), shark (Lovejoy et al., 1992a), salmon (Sherwood et al., 1983) and catfish (Ngamvongchon et al., 1992a) provides initial evidence that GnRH is present in jawless, cartilaginous and boney fishes.

The structure of GnRH, however, varies within fish and in other vertebrates. One interesting feature of GnRH is that it is present in different forms among the classes, yet each form is conserved in length and key amino acids. Only five amino acid positions are known to change in vertebrate GnRH peptides. The variability of GnRH structure among species

is of special interest from an evolutionary perspective. It is possible to determine the emergence of each GnRH structure in extant species, the functional changes that result from structural changes and the tissue location in which each form of GnRH is expressed.

Of further interest in the evolution of the GnRH neurohormone is the presence of two or more forms within the brain of a single species. Some species such as humans have a single form of GnRH in the brain, but other species appear to have two forms (see Sherwood et al., 1993a) or even three forms of GnRH (Sherwood et al., 1993b; Somoza et al., 1994). In addition, there is some evidence that distinct forms of GnRH are located in discrete areas of the brain (Kah et al., 1989; White et al., 1994). It is not only the multiple forms and functions of GnRH in the brains of fishes that is of interest, but also the process by which these forms arose. GnRH appears to be a good molecule to examine the process of peptide evolution through changes seen in its structure, location and function.

The suggestion of multiple GnRH forms in the brains of vertebrate species is based largely on indirect evidence garnered from the immunological profiles of fractions from high performance liquid chromatography (HPLC) of brain extracts. This method can be used to show that multiple forms are present, but not to identify definitively the GnRH forms. Therefore, I undertook to determine the primary structure of GnRH peptides from the brains and pituitaries

of extant fish that represent widely separated species in the evolution of boney fishes.

Fish arose from ancestors that appeared during the late Cambrian period 500 million years ago (see Carroll, 1988; Walker and Liem, 1994). The chordate characteristics observed in fish are hypothesised to have arisen from an ancestral protochordate, a group which is represented today by tunicates and amphioxus. Therefore, if GnRH can be shown to exist throughout the vertebrates, the origin of GnRH or GnRH-like peptides may lie within invertebrates. If GnRH is present in animals ancestral to fish, the lineage of GnRH would span 600 million years of evolution, and might lend insight into the evolution of neurohormonal control of reproduction.

#### **Discovery of GnRH**

GnRH was first identified by primary structure from porcine (Matsuo et al., 1971) and ovine (Burgess et al., 1972) hypothalami. This peptide was originally given the name luteinizing hormone-releasing hormone because it released luteinizing hormone (LH) from the pituitary. However, this peptide was also found to release another pituitary gonadotropin, follicle stimulating hormone (FSH) and hence the name was changed to gonadotropin-releasing hormone. The form originally identified in several mammalian species was named mammalian GnRH (mGnRH). Subsequently, GnRH was purified from the brains of other

vertebrate species and found to differ by 10-50% in amino acid sequence compared with mGnRH (Fig. 1.1). The GnRH peptides are named after the species in which they were first identified. All of the vertebrate GnRH forms have been found to cause the release of gonadotropins from pituitary cells of either the host or other species. Therefore, one primary role of GnRH in vertebrates is to elicit the release of pituitary gonadotropins from gonadotrophs.

#### **Multiple forms of GnRH in one species**

A large number of jawed vertebrates have two forms of GnRH, chicken GnRH-II (cGnRH-II) and another form. The other form of GnRH may vary among classes, but at least two forms are present in representatives of each class. The form that is generally accepted to be the releaser of the gonadotropins has an origin, location and function different from cGnRH-II (Murikami et al., 1991; Muske and Moore, 1994). Neurons that contain the GnRH form that releases gonadotropins are derived from the embryonic olfactory placode, then migrate to the forebrain (Muske and Moore, 1988). In contrast, neurons that contain cGnRH-II are primarily located in the caudal portions of the midbrain (for example, see Hayes et al., 1994).

The role of cGnRH-II appears to be neuromodulatory. In most species the location of cGnRH-II cell bodies and axons

Figure 1.1. Amino acid sequence of the GnRH peptides identified prior to the initiation of this thesis. Boxes indicate amino acids that differ compared with those in mGnRH.

	1	2	3	4	5	6	7	8	9	10												
MAMMAL	p	GLU	-	HIS	-	TRP	-	SER	-	TYR	-	GLY	-	LEU	-	ARG	-	PRO	-	GLY	-	NH <sub>2</sub>
CHICKEN-I	p	GLU	-	HIS	-	TRP	-	SER	-	TYR	-	GLY	-	LEU	GLN	PRO	-	GLY	-	NH <sub>2</sub>		
CATFISH	p	GLU	-	HIS	-	TRP	-	SER	HIS	GLY	-	LEU	ASN	PRO	-	GLY	-	NH <sub>2</sub>				
SALMON	p	GLU	-	HIS	-	TRP	-	SER	-	TYR	-	GLY	TRP	LEU	PRO	-	GLY	-	NH <sub>2</sub>			
CHICKEN-II	p	GLU	-	HIS	-	TRP	-	SER	HIS	GLY	TRP	TYR	PRO	-	GLY	-	NH <sub>2</sub>					
DOGFISH	p	GLU	-	HIS	-	TRP	-	SER	HIS	GLY	TRP	LEU	PRO	-	GLY	-	NH <sub>2</sub>					
LAMPREY-III	p	GLU	-	HIS	-	TRP	-	SER	HIS	ASP	TRP	LYS	PRO	-	GLY	-	NH <sub>2</sub>					
LAMPREY-I	p	GLU	-	HIS	TYR	SER	LEU	GLU	TRP	LYS	PRO	-	GLY	-	NH <sub>2</sub>							

relative to the pituitary indicate that it is not the primary releaser of gonadotropins (Sharp et al., 1990; Muske and Moore, 1994; Northcutt and Muske, 1994). Moreover, cGnRH-II has a different tissue of origin and pattern of embryonic development than forebrain GnRH (Millam et al., 1993a; Muske and Moore, 1994). In some species, the axons of cGnRH-II cells differ in ultrastructure when compared to those associated with gonadotropin release (Northcutt and Muske, 1994). Despite the observation that cGnRH-II may not be delivered to the pituitary gonadotrophs, the peptide, given *in vitro* or *in vivo*, is one of the most potent forms of GnRH for release of gonadotropins (Habibi et al., 1992; Ngamvongchon et al., 1992b).

#### **Delivery of GnRH to the pituitary**

The role of GnRH in releasing gonadotropins has been most extensively studied in mammals. In this class, GnRH is produced mainly by cells with axons terminating adjacent to the hypophyseal portal system. GnRH is released from the axon terminals and is borne by the blood to the pituitary. In the pituitary GnRH acts upon specific receptors on the surface of gonadotrophs to elicit the release of gonadotropins. In contrast to mammals, most fishes do not have a hypophyseal portal system, but rather have GnRH secretory axons that pass through the infundibular stalk of the pituitary and terminate in close proximity to pituitary gonadotrophs.

The direct system of GnRH delivery from the brain to the pituitary is characteristic of the boney fishes and, in particular, the teleost fishes. The presence of axon terminals that store GnRH in the pituitary makes teleosts ideal fishes to use for the identification of the GnRH peptide that elicits the release of the gonadotropins. The GnRH peptide identified in the isolated pituitary is interpreted to have the function of releasing gonadotropin because the pituitary is the final destination of the peptide. As well, the high concentration of GnRH in the pituitary makes purification easier.

#### **A family of GnRH peptides in fishes**

Multiple forms of GnRH in the brains of fish have been identified by immunohistochemistry or high performance liquid chromatography (HPLC). In particular, HPLC has been used to investigate the forms of GnRH for comparison among species. To date, the fishes have the widest diversity of GnRH forms among the vertebrates. However, much of the available information on the forms of GnRH in fish brains comes from the indirect evidence of immunohistochemistry and HPLC elution position rather than from determination of primary structure.

Of the eight known forms of GnRH, seven of these forms are present in the fishes. In all cases, GnRH is a decapeptide that is modified after translation, resulting in a pyroglutamic N-terminus. Amino acids 1, 2, 4, 9 and 10

are always conserved. Amidation of the C-terminus is through the donation of an amide group after cleavage of Gly from the precursor molecule. The other amino acids from least to most variable are 3, 5, 6, 7 and 8. The eighth amino acid is the most variable; there are five known variants in this position. The receptor binding ability of the hormone is attributed to amino acids 4-10 and hormone action is attributed to amino acids 1-3 (see Naor, 1990). Analogues with a modified C-terminus are more resistant to degradation as are analogues with a D-amino acid substitution in position 6. These are the two principle areas of the molecule that are prone to degradation (Goren et al., 1990; Zohar et al., 1990a) and, hence, modifications enhance potency by decreased degradation. In addition, salt bridges between amino acids 5 and 8 tend to stabilize the U-shaped configuration of the molecule around a beta-turn loop at Gly<sup>6</sup> (Karten and Rivier, 1978).

#### **Organization of the GnRH gene**

The gene has been identified for mGnRH in human, rat, and mouse (see Seeburg et al. 1987). In domestic chicken, the gene for chicken GnRH-I (cGnRH-I) has been identified (Dunn et al, 1993). In fishes, the salmon GnRH (sGnRH) gene has been identified in two species, Atlantic salmon, *Salmo salar* (Klungland et al., 1992) and sockeye salmon, *Oncorhynchus nerka* (Coe et al., 1995). Also, the cDNA has been identified for sGnRH in three other species of teleosts:

masu salmon *O. masou* (Suzuki et al., 1992), midshipman, *Porichthys notatus* (Grober et al., 1995) and African cichlid, *Haplochromis burtoni* (Bond et al., 1991). The cDNA encoding cGnRH-II has been characterized for two teleost fish, the African catfish, *Clarias gariepinus*, (Bogerd et al., 1994) and the African cichlid, *H. burtoni*, (White et al., 1994). Additionally, the cDNA encoding catfish GnRH (cfGnRH) has been characterized from the African catfish, *C. gariepinus*, (Bogerd et al., 1994). The cDNA encoding mGnRH in the frog *Xenopus laevis* has also been described (Hayes et al., 1994).

In every case for GnRH, the organization of the gene is the same in that only four exons are present. The exons encode, as shown by cDNA analysis, a 5' untranslated region followed by a signal peptide and the hormone. A cleavage site encoding Gly-Lys-Arg follows the hormone and precedes the coding region for a GnRH associated peptide (GAP) of approximately 50-60 amino acids in length, depending on the species. Each cDNA is terminated by a 3' untranslated region that includes a polyadenylation region.

The least conserved portions of the precursors are the 5' and 3' regions followed by the signal peptide. However, the signal peptide retains a hydrophobic core essential to all signal peptides. The GAP portion of the GnRH precursor is moderately conserved within precursors of a given form of GnRH. For example, the amino acid sequence identity of the sGnRH GAP in salmonids versus cichlid is 66% conserved as is

the cGnRH-II GAP between catfish and cichlid. However, the GAP sequence is not conserved between forms of GnRH such as mGnRH GAP in humans and the cGnRH-II GAP sequences of fish. In contrast, the nucleotide sequence encoding the hormone is well conserved. Therefore, the evolution of GnRH can be studied at one level (orders of fish) by the protein structure of the hormone, but at a finer level (species of fish) by the more rapidly changing GAP sequence rather than the slower changing hormone sequence.

#### **Duplication of the GnRH gene**

Of considerable interest in the evolution of GnRH forms is the appearance of multiple forms of GnRH within a single species. The derivation of new forms from existing forms is more likely to occur by gene duplication or exon duplication than by duplication of the genome. The forms present in a single species must have arisen from preexisting ancestral forms that were modified. Analysis of the GAP among GnRH precursors may indicate the origin of a GnRH form. However, it is necessary to determine the protein structure and identify novel forms before attempting to isolate the cDNA or gene. In the precursor, low conservation of the signal peptide and GAP in addition to the short coding region of the hormone (30 nucleotides) has foiled attempts to find novel forms of GnRH by using DNA probes or primers. Only cDNA precursors of identified GnRH peptides have been isolated to date.

One observation about the GnRH cDNA architecture is that each cDNA encodes only one hormone; two hormones are not encoded within one cDNA. Specific coding of each GnRH form in a separate cDNA provides evidence that gene duplication rather than exon duplication was the probable mechanism involved in an increase in the number of GnRH forms. As noted above, it also implies that each GnRH form has a distinct GAP. GAP appears to change too rapidly for use as a conserved probe to detect the cDNA that encodes the precursor. Therefore, it is imperative to determine the primary structure of novel GnRH peptides, which can be the basis of designing accurate DNA probes to the hormone region encoded in the cDNA.

#### **Evolution of GnRH in vertebrates**

The most ancient forms of GnRH that are known to govern reproduction in vertebrates are lamprey GnRH-I & III (Sherwood et al., 1986a; Sower et al., 1993). These two forms have been shown to be related to sexual maturation and to indirectly increase steroid levels in mature fish (Sower et al., 1993; Wright et al., 1994). At least two forms of GnRH are present in the brains of representatives from each class of vertebrates. In the cartilaginous fishes, dogfish GnRH (dfGnRH) represents the forebrain form of GnRH that is putatively identified as the moderator of reproduction (Lovejoy et al., 1992b; Sherwood and Lovejoy, 1993). In addition to dfGnRH, cGnRH-II has been purified from the

dogfish brain. Similarly, cGnRH-II has been purified from the brains of the holocephalan ratfish, *Hydrolagus colliei*, but was the only form identified (Lovejoy et al., 1991). Another form may be isolated from ratfish brains in the future when more antisera are available. The presence of cGnRH-II in ratfish shows the continuation of this peptide in cartilaginous fishes. Dogfish GnRH has not been found in fishes outside of the sharks, although, cGnRH-II has.

In osteichthyeans, or boney fishes, cGnRH-II has been shown throughout the class from sturgeon *Acipenser spp.*, (Sherwood et al., 1991; Lepetre et al., 1993) to cichlids, *H. burtoni* (White et al., 1994) by using immunocytochemistry, HPLC elution position or identification of cDNA encoding cGnRH-II. In addition to cGnRH-II, mGnRH is often present in phylogenetically older species of boney fish. This was shown by immunocytochemistry in sturgeon *A. baeri* (Lepetre et al., 1993) and HPLC in reedfish *Calamoichthys calabaricus*, sturgeon *A. transmontanus*, alligator gar *Lepisosteus spatula* (Sherwood et al., 1991) and lungfish, *Neoceratodus forsteri*, (Joss et al., 1994).

The presence of mGnRH and cGnRH-II together in the brains of vertebrates appears to occur throughout the amphibians (Sherwood et al., 1986b; Conlon, et al., 1993), monotremes and marsupials (King and Millar, 1992) and in a primitive mammal, the musk shrew *Suncus murinus* (Dellovade et al., 1993). There are two notable exceptions to the rule of the paired presence of mGnRH and cGnRH-II. First, birds and

reptiles have cGnRH-I instead of mGnRH in their brains (Sherwood, et al., 1988; Sherwood and Whittier, 1988), but there is one amino acid difference between mGnRH and cGnRH-I. Also, the developmental pattern of cGnRH-I with an origin in the olfactory placode parallels that seen for mGnRH in frogs and mice (Akatsu et al., 1992). In birds as in amphibians, cGnRH-II first appears during development in the midbrain. The second exception to the dual presence of mGnRH and cGnRH-II is in eutherian mammals except the musk shrew. Here, the only identified form of GnRH is mGnRH. cGnRH-II has not been shown to be present. An explanation for the lack of cGnRH-II and the consequent lack of function in eutherian mammals has not been proposed.

### **Phylogeny of fishes**

The phylogeny of fishes is of great interest and is shown in Figures 1.2 and 1.3 to emphasize the classification of specific fish in this thesis. Some important points are listed below. First, the teleost fishes are monophyletic (see Nelson, 1984). This means there was one stem line for the teleosts and that subsequent duplication of specific genes during boney fish evolution can be deduced. Second, during the 400 million years of evolution in boney fishes, they have undergone two radiations that coincide with the Triassic Period (225 million years ago) when amphibian and reptilian species expanded and with the Cretaceous Period (65 million years ago) during the mammalian radiation. The

Figure 1.2. Branching diagram depicting the phylogenetic arrangement of the boney fish. All fish named are extant species. Common names are presented for fish mentioned in this thesis. Single asterisk (\*) denotes a species for which there is indirect evidence for the identification of GnRH peptides and double asterisk (\*\*) denotes a species for which there is identification of GnRH peptides by primary structure, including novel GnRH peptides. Adapted from Nelson (1984).

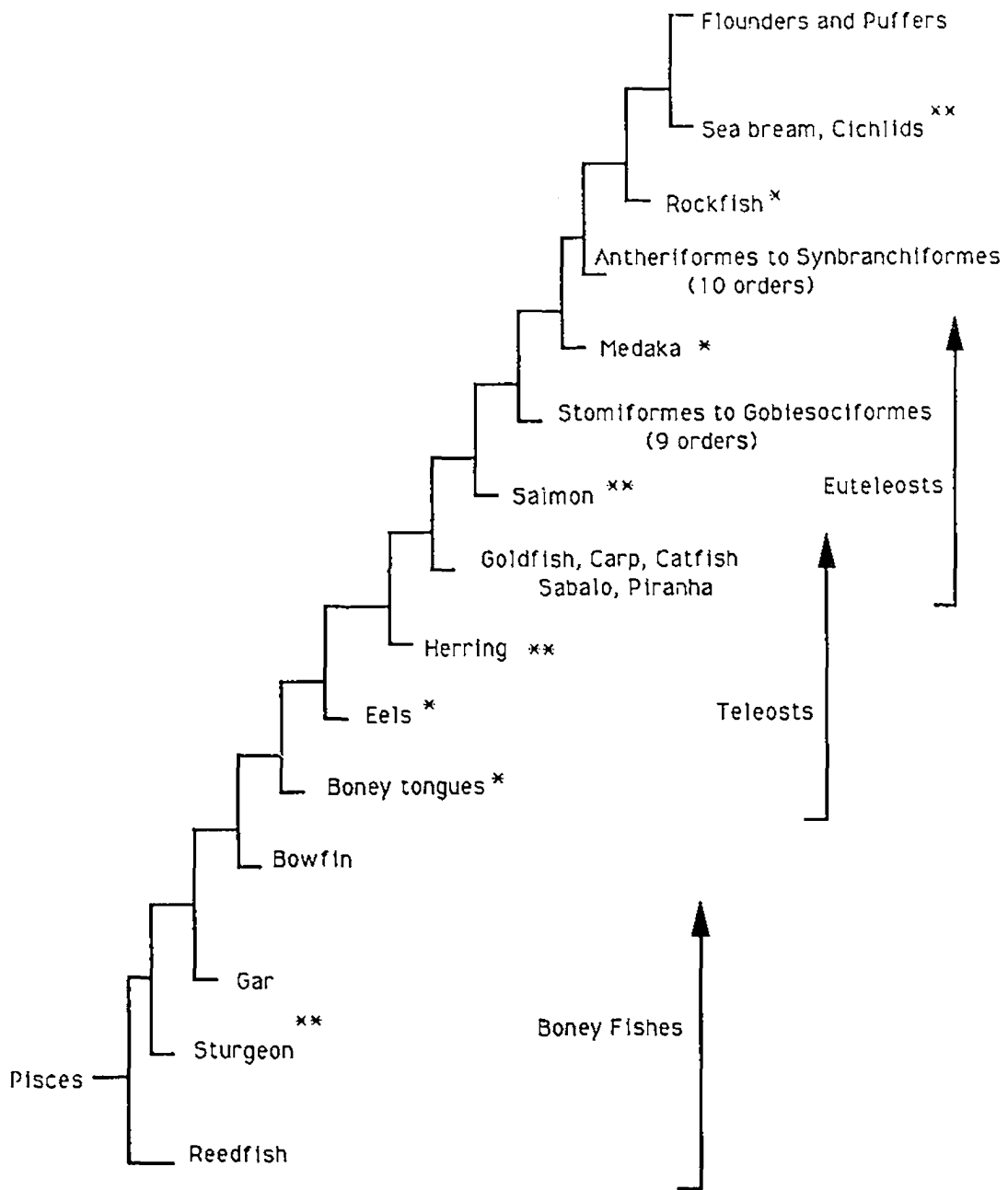
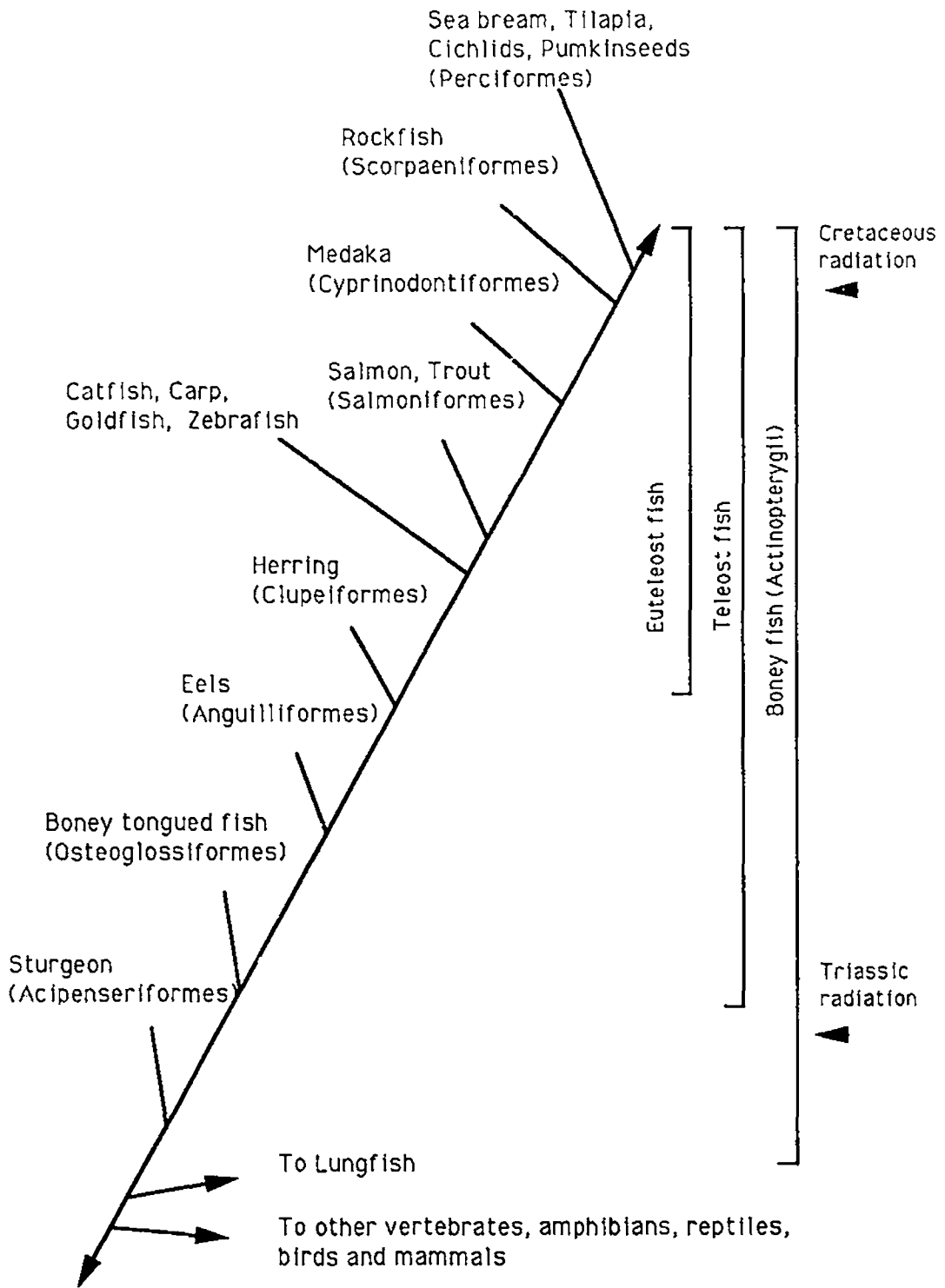


Figure 1.3. Cladistic arrangement of the groups and orders of living boney fishes studied in the research presented in this thesis. Fish are identified by their common name with the order in parenthesis. The relationship for the time of divergence from the stem line is approximate. Classification of the fishes into boney (Class), teleost (Subdivision) and euteleost (Infradivision) are indicated. Approximate time corresponding to radiations of fish species are indicated on the right. Adapted from Nelson (1984), Lauder and Liem (1983a,b) and Walker and Liem (1994).



first radiation of fishes gave rise to many species, most of which are extinct, but a few such as the reedfish and sturgeon still exist. The second radiation of fishes in the Cretaceous Period gave rise to the perciform or perch-like fishes, which today are the most abundant vertebrate species (Carroll, 1988; Walker and Liem, 1994). Third, some fishes, such as the perciforms have evolved the ability to become protogynous, or change sexes, responding to social cues or behaviour, an aspect of reproduction not seen in tetrapods.

#### **Evolution of GnRH in fishes**

In ancestral fish that gave rise to the boney fish, mGnRH must have appeared very early as it is present along with cGnRH-II in all living, but phylogenetically ancient fish. Thus, mGnRH has been indirectly identified in the phylogenetically ancient reedfish *C. calabaricus*, sturgeon *A. transmontanus*, alligator gar *L. spatula* (Sherwood et al., 1991) and bowfin, *Amia calva*, (Crim, 1983; Crim et al., 1985). These species are living representatives of early boney fishes that were closely related to both the cartilaginous fishes and the predecessors of tetrapods.

The presence of mGnRH among the teleost fishes is represented by the indirect identification of this form in the moray eel, *Gymnothorax fimbriatus*, (Shih, et al., 1988) and silver eel, *Anguilla anguilla*, (King et al., 1990). Ancestors of these fish were among the first fish to be recognised as teleosts (Nelson, 1984). To date, the forms

of GnRH present within the osteoglossomorphs, or honey tongued fish, has not been investigated. However, as the most primitive order of teleosts, they represent a key point in the evolution of fish. Therefore, it is important to identify the forms of GnRH in the brains of these early-evolving teleost fishes in order to examine the process of further GnRH evolution.

In living herring, *Clupea harengus pallasii*, mGnRH has disappeared and sGnRH has appeared (Sherwood, 1986) as a form that is predominant in the forebrain and hypothalamus of most teleost species (Sherwood et al., 1993a). The exception is the presence of cfGnRH among the genus *Clarias* that replaces sGnRH. Other than the catfish, sGnRH has been identified in all euteleosts examined (see Sherwood et al., 1993a). The location of sGnRH is predominantly in the forebrain and hypothalamus as detected by HPLC, immunocytochemistry and *in situ* hybridization (Amano et al., 1991; Davis and Fernald, 1990; White et al., 1994).

cGnRH-II has been detected or identified in all teleost fishes examined. Immunocytochemistry, HPLC and RIA analysis of discrete brain areas and *in situ* hybridization have located cGnRH-II-secreting cell bodies primarily in the mesencephalon of all fishes examined (Miller and Kreibel, 1986; Yu et al., 1988; Coe et al., 1990; Davis and Fernald, 1990; Oka, 1992; Schulz et al., 1993; Francis et al., 1994; White et al., 1994). The presence of cGnRH-II throughout the teleost lineage implies that this peptide has an

important function. Evidence to support this conjecture comes from the observations of Francis and coworkers (1994) using fish and Muske and Moore (1994) using an amphibian. They showed that the cGnRH-II neurons do not contact the pituitary and immunoreactive staining does not vary with maturational status contrary to that observed for GnRH cells of the forebrain (Davis and Fernald, 1990). However, cGnRH-II has only been identified by primary structure or cDNA in two teleost fish species (Ngamvongchon et al., 1992b; Bogerd et al., 1994; White et al., 1994). Definitive proof of the presence of cGnRH-II is essential to establishing a common thread of form and function throughout the fishes.

Three forms of GnRH in one species have emerged at least twice within the teleosts. Firstly, Sherwood and coworkers (1993b) noted that a perciform fish, the snook *Centropomis undecimalis*, had three distinct forms of GnRH as identified by HPLC and radioimmunoassay (RIA). The relative amounts of immunoreactive GnRH (irGnRH) detected in RIA varied with gender and maturational status. Secondly, Somoza and coworkers (1994) used HPLC and RIA to identify a third form of irGnRH, in addition to sGnRH and cGnRH-II, in the brains of the sabalo, *Prochilodus lineatus*. Further, this third form was present with sGnRH, but not cGnRH-II, in pituitaries. No other assemblages of fishes were reported to have three forms of GnRH within a single brain. Investigation and identification of three forms of GnRH

within a single species is of interest to both the field of molecular evolution and peptide function.

#### **Function of GnRH forms deduced from ontogenetic studies**

Developmental studies have been crucial in differentiating the function of different forms of GnRH. These studies have established that GnRH cells located in the preoptic area of the adult brain arrive at this position by migration (Schwanzel-Fukuda and Pfaff, 1989). GnRH cells originating in the olfactory placodes migrate during an early stage of development along the terminal and vomeronasal nerves (Wray et al., 1989). Some GnRH cells remain in the anterior terminal nerve region, whereas most others continue the migration to the preoptic nucleus, just anterior to the hypothalamus. Remarkably, the migrating GnRH cells express GnRH as shown by immunocytochemistry. The reason for the expression of GnRH, which can be detected as early as embryonic day 11.5 in mice is enigmatic (Wray et al., 1989). However, some evidence exists to suggest that one function of forebrain irGnRH may be to induce differentiation of the pituitary gonadotrophs (Aubert, et al., 1985). Thus, GnRH secretion may be the first step in the establishment of the hypothalamic-pituitary-gonadal axis (Schulz et al., 1994).

In fishes and amphibians, the ganglion of irGnRH cells that is associated with the terminal nerve persists throughout adulthood. This ganglion, called the terminal

ganglion, is established as noted above by the migration of irGnRH cells to this location. In contrast to the preoptic GnRH neurons, cells of the terminal ganglion neither reflect the reproductive status (Oka, 1991) nor mediate the action of pheromones via the olfactory nerves (Fujita et al., 1991). A neuromodulatory role for these cells is more likely as it has been shown that they have connections to the retina (Stell et al., 1988). Both ipsilateral and contralateral terminal ganglion cells affect rod proliferation (Owusu-Yaw et al., 1992).

Ablation studies involving the removal of one or both of the olfactory placodes in embryonic newts (Murikami et al., 1991; Muske and Moore, 1994; Northcutt and Muske, 1994) and chickens (Akatsu et al., 1992) or studies that block the migration of GnRH cells in mice (Schwanzel-Fukuda et al., 1991) prevent the establishment of preoptic-hypothalamic GnRH cells in adult animals. Moreover, Muske and Moore (1994) have unequivocally shown that the olfactory placode does not play a part in the establishment of cGnRH-II-producing cells in the newt midbrain. This corroborates the observations of Millam and coworkers (1992b) who noted differential patterns of development for both forebrain and hindbrain populations of GnRH-producing cells. Additional evidence to support an alternative role other than gonadotropin release for cGnRH-II is that eutherian mammals reproduce despite an absence of a second form in their midbrains. Clearly, cGnRH-II-producing cells are derived

from different tissues than those which regulate the control of gonadotropin release in mature animals.

The best evidence indicating a function for cGnRH-II is from the observations of Jan and coworkers (1979) who recorded late slow excitatory post-synaptic potentials from sympathetic ganglia of the bullfrog. Although the form of GnRH was not identified by Jan and coworkers (1979), later work by Hayes and coworkers (1994) identified the midbrain form of GnRH to be cGnRH-II. Further, Miller and Kreibel (1986) observed that hindbrain neurons containing GnRH, presumably cGnRH-II, had axons that terminated on the caudal neurosecretory system in mollies (*Poecilia sphenops* and *P. latipinna*). Together, these studies indicate that the role of cGnRH-II is most likely that of a neuromodulator rather than a neurotransmitter. Although cGnRH-II has a gonadotropin-releasing potency that is greater than most GnRH forms and some analogues (Ngamvongchon et al., 1992a), the developmental pattern, localization and anatomical connections of neurons with cGnRH-II suggest a different function compared to the forebrain-pituitary form of GnRH.

#### **Function of GnRH in non-neural tissues**

GnRH has been identified outside the nervous system. GnRH peptides have been detected in reproductive tissues such as the ovary, oviduct and uterus of swine (Li et al.,

1992). In the ovary, mGnRH cDNA was isolated and sequenced (Oikawa et al., 1990). As well, the cDNA encoding GnRH has been identified in the human placenta (Seeburg and Adelman, 1984), where GnRH acts in a paracrine fashion to regulate the release of human chorionic gonadotropin (see Li et al., 1992).

Other extrahypothalamic sources of GnRH are the immune system including rat spleen lymphocytes (Emanuele et al., 1990; Azad et al., 1991) and mast cells of the dove (Silverman et al., 1994). Rat splenic GnRH is bioactive in pituitary cell cultures and coelutes on HPLC with hypothalamic GnRH. The irGnRH mast cells in the dove were identified by immunocytochemistry within the brain. This irGnRH in mast cells indicates a connection between the central nervous system and the immune system. The role of GnRH in extrahypothalamic tissues is intriguing given the central role of the peptide in reproduction.

#### **Function of GnRH as deduced by location and activation of receptors**

Only one GnRH receptor has been characterized in most vertebrates (see Knox, et al. 1994). Investigations have concentrated on the mammalian pituitary cells in regard to ligand/receptor activation. However, GnRH receptors are located throughout the central nervous system and in mammalian ovary, testis and placenta (Clayton et al., 1979; Hsueh et al., 1979a,b; Mounni et al., 1994; Zhou and Selfon,

1994). These receptors are identical to those of pituitary gonadotropes and are encoded by one gene (Moumni et al., 1994).

The pituitary GnRH receptor has been most thoroughly studied. This receptor binds the hormone that has adopted a U-shaped conformation, bringing amino acids one and ten in close proximity at the molecule's lowest conformational state (Karten and Rivier, 1986). Activation of the receptor and hence, the physiological effect is determined by amino acids 1-3 (see Conn, 1986). This agrees with the conservation of the first three amino acids in the GnRH peptides, whereas variability occurs in positions 5,7 and 8. Occupancy of 20% of available receptors is sufficient to induce 80% of maximal release of LH from rat pituitary cells in culture (Naor, 1990).

The GnRH receptor was identified and cloned by Manami and coworkers (1992). This receptor belongs to a family of G-protein coupled receptors that are characterized by seven transmembrane domains. The GnRH receptor has three potential N-terminal glycosylation sites on the extracellular portion of the receptor. Two of these sites are on the N-terminal region; the other is located on the first loop between transmembrane helices two and three instead of the usual location on the C-terminus for the other seven transmembrane receptors. This may account for the observed differences in second messenger pathways.

The pathway of GnRH-induced mediation is through G-protein coupled activation of adenylate cyclase and/or diacylglycerol, which then activate protein kinases to enable a further cellular cascade (see Naor, 1990). The end result of the cellular cascade is the release of LH or FSH.  $Ca^{++}$  has also been implicated in the intracellular response to GnRH receptor activation as a second messenger (see Conn, 1986). The two pathways, protein kinase C and  $Ca^{++}$ /calmodulin, appear to be involved in the autoregulation of the gonadotrophs. The gonadotrophs down-regulate receptors in response to  $Ca^{++}$ /calmodulin mediated events, whereas protein kinase C-mediated events initiate gonadotropin biosynthesis (McArdle, et al. 1987). In fish, initial studies suggest that both forms of GnRH in goldfish (sGnRH and cGnRH-II) can bind to a single type of receptor, but activate different intracellular pathways (Chang et al., 1990; Khakoo et al., 1994). However, the critical question is whether both forms of GnRH are delivered to the pituitary gonadotroph receptors in the natural situation.

### **Research objectives**

The identification of GnRH peptides in the brain is usually accomplished using a method of indirect measurement such as HPLC elution position and/or antisera cross-reactivity. However, confusion about the forms of GnRH within a single species can arise in HPLC methodology. This is due to identification by comparison of elution position

of tissue extracts and standards that have been applied separately to the HPLC column. The many proteins in the tissue, especially the brain, can cause small shifts of one or two fractions between the tissue and standard runs. This shift could lead to misidentification. Also, polyclonal antisera may detect several forms of GnRH and may not be directed to a single epitope on the GnRH molecule. This cross-reactivity pattern is advantageous for detecting multiple forms of GnRH within a sample, but polyclonal antibodies and HPLC are most effective for screening tissue for preliminary results on GnRH peptides. The standardization of HPLC protocol and use of the same antisera can greatly strengthen the data concerning detection of GnRH forms, but the determination of primary structure is essential for final identification. Primary structure of GnRH is needed to study the evolution of GnRH in boney fishes and to begin to examine the functions of novel forms of GnRH in fishes. Therefore, the first objective of this research was to determine the primary structure of GnRH in fish that represent distinct orders and are widely separated by evolution. This work also included some collaborative studies to determine the function of GnRH. The second objective was to examine the evolution of GnRH throughout the boney fishes in finer detail by screening a number of fish species in key assemblages of boney fishes for the GnRH forms present. The third objective was to investigate the origin of vertebrate GnRH

peptides by studying an invertebrate, the tunicate *Chelyosoma productum*. This protochordate is a living representative of the group whose ancestors may have led to vertebrates.

I used the comparative approach to study the phylogenetic relationship between fish on the basis of GnRH peptide structure in which the fish are arranged (Fig. 1.2) as described by Nelson (1984) and supported by others (Lauder and Liem, 1983a,b; Walker and Liem, 1994). I have studied GnRH in fish that are living representatives of the primitive boney fishes (sturgeon) and the earliest evolved group of teleost fishes (boney tongues and eels). I then examined representative species that span the transition from teleost (herring) to euteleost (salmon). Finally, the most recently evolved assemblage of fishes, the percimorph fishes were studied. Wherever possible, primary structure was used to chart the evolution of GnRH in fishes. However, in many cases, the number of fish brains available was limited. Therefore, HPLC elution position coupled with antisera cross-reactivity using a standardized regimen was employed to determine the forms of GnRH present. This permitted a higher resolution of GnRH evolution.

The following chapters of this dissertation are organized to present the evolution of GnRH in a phylogenetic manner. The boney fishes are discussed first followed by a study on the invertebrate origin of GnRH in the tunicate, *Chelyosoma productum*.

Chapter 2 Primary structure of mammalian GnRH in the  
sturgeon Acipenser gueldenstaedti

A version of this chapter has been published and is reworked here:

Mammalian gonadotropin-releasing hormone (GnRH) identified by primary structure in the Russian sturgeon, *Acipenser gueldenstaedti*. Lescheid, D.W., Powell, J.F.F., Fischer, W.H., Park, M., Craig, A.G., Bukovskaya, O., Barannikova, I.A., and Sherwood, N.M. Regul. Pep. 55: 299-309.

#### INTRODUCTION

There were seven forms of GnRH identified by primary structure when I began my thesis research (Fig. 1.1). An additional form (lamprey GnRH-III) was determined by Sower and coworkers (1993) during my studies. One of the most interesting of these GnRH forms is the one identified in mammals, including humans: mammalian GnRH (mGnRH). mGnRH is also reported to be present in primitive boney fishes based on indirect methods (Sherwood et al., 1991). It seemed important to obtain definitive proof that mGnRH was present in the early boney fishes because the ancestors of these fish are thought to have given rise to two divergent groups of vertebrates, the teleost fishes and tetrapods. Hence, the first appearance of mGnRH in evolution is important not only because it is the sole form of GnRH in most eutherian

mammals, but is also present among boney fishes and amphibians (Sherwood et al., 1986a; 1991).

mGnRH has only been identified by primary structure in mammals and in two species of ranid frogs (Rivier et al., 1981; Conlon et al., 1993). However, other indirect evidence using immunocytochemistry and high performance liquid chromatography (HPLC) with radioimmunoassay (RIA) have shown mGnRH to be present in marsupials *Macropus eugenii*, *Isoodon macrourus* and *Dasyurus viverrinus* (King et al., 1989), amphibian newt *Taricha granulosa*, salamander *Ambystoma gracile* (Sherwood et al., 1986b), two other ranid frogs *Rana pipiens* and *R. esculenta* (Licht et al., 1994) and fishes, as discussed below.

The lungfishes (Sarcopterygii) are an important group because they have many characteristics that are associated with tetrapod evolution such as: opening of the nares to the pharynx, a developed lung and fleshy skeletonized fins (see Walker and Liem, 1994). Not surprisingly, the lungfish, *Neoceratodus forsteri*, contain mGnRH as evidenced by immunocytochemistry (Joss et al., 1994). Additionally, the lungfish bear many structural and physiological similarities to the early ray-finned fishes (Actinopterygii). As in lungfish, mGnRH has been identified by HPLC from the brains of the sturgeon *Acipenser transmontanus* (Sherwood et al., 1991) and by immunohistochemistry for *A. baeri* (Lepetre et al., 1993). Other fishes that evolved with the early ray-finned fishes

such as the reedfish, *Calamichthys calabaricus*, alligator gar, *Lepisosteus spatula* (Sherwood et al., 1991) and bowfin *Amia calva* (Crim et al., 1985) also contain mGnRH as evidenced by data from HPLC and RIA.

This chapter presents the isolation and primary structure of mGnRH from the brains of the Russian sturgeon, *Acipenser gueldenstaedti*. The identification and confirmation of mGnRH in the brains of early boney fishes is important because the ancestors of sturgeon were close to the bifurcation of boney fish and tetrapods. The primary structure of sturgeon GnRH is valuable in providing evidence about the evolution of GnRH in vertebrates. This work was done in collaboration with: David Lescheid, M. Park and Drs. W. Fischer, A.G. Craig, Drs. O. Barannikova and A. Bukovskaya.

## **MATERIALS AND METHODS**

### **Fish**

Brains were collected from post-spawned male and female Russian sturgeon, *Acipenser gueldenstaedti*. The collection of brains occurred during the period of anadromous migration in the Volga River, April, 1993. The size of the fish ranged from 110 to 130 cm in males and from 120 to 150 cm in

females. The age of the fish is unknown. Brains were frozen, shipped on dry ice to Victoria and stored at  $-90^{\circ}\text{C}$  on arrival. Pituitaries were not collected with the brains.

#### **Extraction of peptides**

Frozen brains (485g) were pulverized with a mallet and then powdered with liquid nitrogen in a Waring Blendor. The powdered material was treated as described (Sherwood *et al.* 1986b). Briefly, the material was added to 2,360ml 1N HCl/acetone (3:100 v/v), stirred for 3h and filtered through a #1 Whatman filter. The filtered matter was resuspended in 730ml 0.01N HCl/acetone (1:5 v/v) and stirred for 3min. Acetone, lipids and other hydrophobic substances were removed by five successive extractions using petroleum ether (20% v/v). The final aqueous phase (800ml) was evaporated in a vacuum centrifuge to 200ml.

#### **Preliminary investigation of irGnRH**

Sturgeon brains (11g) were treated as above for extraction of peptides. After evaporation of solvents, four aliquots of  $800\mu\text{l}$  extract were applied at two minute intervals to a  $\text{C}_{18}$  HPLC column through a 1 ml injection loop. The column was connected to a Beckman 125 HPLC and Beckman model 166 detector. Initial conditions for injections were 1 ml/min of 5% solution B (80% acetonitrile, 20% 0.1M heptafluorobutyric acid; HFBA) and 95% solution A (0.1M HFBA). After 10 min of these conditions, the gradient

was increased at 1.4%/min solution B for 50 min. Fractions of 1ml were collected for 65 min. Aliquots (100 $\mu$ l) were assayed for irGnRH (see below). Immediately after the sample was eluted from the HPLC column, the column was equilibrated at the starting conditions and seven synthetic GnRH standards were combined and applied to the column at a concentration of 200 ng/ml each. Fractions were assayed using three antisera (see below) for irGnRH to determine elution position.

#### **Sep-Pak high performance liquid chromatography (HPLC)**

The aqueous brain extract from the 485g sample of brains was pumped through 10 Sep-Pak cartridges connected in series using a peristaltic pump at a flow rate of 1.5 ml/min. The cartridge column was washed with 6 ml Milli-Q water and treated as described elsewhere (Ngamvongchon et al. 1992a). Briefly, the cartridge column was connected to the HPLC apparatus, but bypassed the detector. Initial conditions of solvent flow through the column were 95% solution A (0.05% trifluoroacetic acid in water) and 5% solution B (80% acetonitrile diluted with 20% solution A) at a flow rate of 1 ml/min. A gradient of 1% solution B per minute was applied to the cartridge column for 60 min. Fractions of 1ml were collected for 60 min and assayed for GnRH-like immunoreactivity with antisera GF-4 and Bla-4. The cross-reactivities of these antisera have been previously reported (Table 2.1; Kelsall et al. 1990; Sherwood et al., 1991).

Table 2.1. Relative percent cross-reactivity of antisera with native GnRH peptides. Mammalian GnRH was used as reference peptide and  $^{125}\text{I}$ -labeled trace in the calculation of relative activity. The final dilutions of the antisera were 1:250,000 (R-42); 1:5000 (B-6 and Bla-4); and 1:25,000 (GF-4). Adapted from Sherwood et al., 1991.

Peptide	R-42	B-6	GF-4	Bla-5
Mammalian GnRH	100.00	100.00	100.00	100.00
Chicken GnRH-I	100.00	0	44.00	39.23
Chicken GnRH-II	54.54	0	3.89	0.63
Salmon GnRH	94.79	0	68.75	87.93
Lamprey GnRH-I	1.58	0	0	318.75

### Purification of GnRH

Procedural steps for the purification of GnRH or GnRH-like peptides include three successive HPLC stages after Sep-Pak HPLC. These steps utilized a C<sub>18</sub> Supelco column with varying solvents and ion-pairing agents (Table 2.2). The last step of the purification utilized a change in column type from a C<sub>18</sub> (Supelco) to a phenyl column (Vydac) in order to further separate peptides.

Aliquots of 10 $\mu$ l were used to determine the amount of immunoreactive GnRH (irGnRH) in each fraction collected. Fractions that contained irGnRH were selected for further purification in successive steps. Only one peak from the initial Sep-Pak was detected and further purified. Aliquots (10 $\mu$ l) of fractions from the final step of the purification (phenyl column) were additionally assayed with antiserum B-6.

### Radioimmunoassay (RIA)

Aliquots of 10 $\mu$ l from fractions collected at each successive stage in the purification were assayed for irGnRH by standard RIA. Briefly, 300 $\mu$ l of 10mM phosphate buffered saline (pH 7.0) with 0.1% gelatin was added to the sample in a 5ml borosilicate test tube. A standard curve from 1ng to 250ng was made by serially diluting a stock solution of synthetic mGnRH to a final volume of 300 $\mu$ l in buffer for each point of the curve. References for zero and maximal binding were prepared in triplicate. Reference tubes

Table 2.2. Steps in the HPLC purification of sturgeon GnRH. Solvent and column types are listed for each successive step. Immunoreactive areas identified by radioimmunoassay were reduced in volume, combined and applied to the next step of purification.

HPLC Step	Solvent A	Solvent B	Column Type
1	0.05% TFA	0.1M TFA in 80% ACN	Sep-Pak
2	0.1M HFBA	0.1M HFBA in 80% ACN	C <sub>18</sub>
3	1.2mM TEAF	ACN	C <sub>18</sub>
4	1.2mM TEAP	ACN	C <sub>18</sub>
5	0.05% TFA	0.1M TFA in 80% ACN	Phenyl

**Abbreviations:**

ACN: acetonitrile

HFBA: heptafluorobutyric acid

TEAF: triethylammonium formate

TEAP: triethylammonium phosphate

TFA: trifluoroacetic acid

received 400 $\mu$ l of buffer for the zero binding reference and 300 $\mu$ l of buffer for the maximal binding reference. Tubes containing samples, standard curve and maximal binding reference received an aliquot of 100 $\mu$ l of diluted antiserum. Antiserum GF-4 (raised against salmon GnRH) was used in a dilution of 1:50,000 resulting in 22-32% binding of  $^{125}\text{I}$ -mGnRH. Antiserum Bla-4 (raised against lamprey GnRH-I) was used at a dilution of 1:10,000 resulting in 9-17% binding of  $^{125}\text{I}$ -mGnRH. Seven of the known GnRH forms are recognized by these two antisera.  $^{125}\text{I}$ -labeled mGnRH, approximately 6000 counts per minute, was added to all tubes. This included an additional three reference tubes containing 400 $\mu$ l buffer for later use to determine total radioactivity and percent maximal binding ( $B/B_0$ ).

Tubes containing antiserum and/or labeled hormone were incubated overnight at 4°C. After incubation all tubes, except the total counts per minute reference, received 1ml of 2.5% charcoal (w/v), 0.25% dextran (w/v) in 10mM phosphate buffered saline. The tubes were agitated using a vortex mixer and incubated at 4°C for 10min. After incubation the tubes were centrifuged for 15 min at 3000g and 4°C. The supernatants were then decanted into an additional set of labeled tubes and placed in a LKB Minigamma model 1275 gamma counter for determination of radioactivity. Amounts of sample irGnRH were determined by comparison of radioactivity to the standard curve.

Limits of detection for each assay ( $B/B_0=80\%$ ) averaged 10.4 pg for GF-4 and 47.6 pg for BLA-4. Although a polyclonal antiserum, B-6 is specific for mGnRH and does not cross-react with any other of the known forms of GnRH, although lamprey GnRH-III has not been tested for cross-reactivity. B-6 (1:5000 dilution) resulted in a binding of 52% of  $^{125}\text{I}$ -mGnRH and a detection limit ( $B/B_0=80\%$ ) of 9.4pg. Fractions with high immunoreactive GnRH (tracer binding,  $B/B_0$ , of 20% or less) were serially diluted 1:2 and reassayed. The value closest to 50% tracer binding is reported.

#### **Characterization of the primary structure**

The characterization of peptides in this and subsequent experiments was done by M. Park and Dr. W. Fischer at the Salk Institute. Sequence analysis was attempted on 10% of the purified sample before digestion. The lack of sequence data suggested that the peptide possessed a blocked N-terminus. Fifty percent of the sample was dried and digested with calf liver pyroglutamyl aminopeptidase (Boehringer-Mannheim Biochemicals). The details of this procedure have been reported elsewhere (Lovejoy et al., 1991). Briefly, aliquots containing approximately 250ng of sturgeon GnRH were concentrated in a Savant Speed Vac system. Reaction buffer (10 $\mu$ l) containing 100mM TES [N-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid, pH 8.0], 10mM EDTA, 5M dithiothreitol, 5% glycerol and 40  $\mu$ g

pyroglutamyl aminopeptidase lyophilizate. The solutions were incubated at 37C for 30 min. The digested GnRH was separated from the mixture using a Hewlett Packard HP 1090L HPLC fitted with a Vydac C<sub>18</sub> microbore column. The initial solvent mixture was 95% solution A (0.05% trifluoroacetic acid in water) and 5% solution B (90% acetonitrile diluted with 10% solution A) through the column to which the digested peptides were added. The rate of increase of solution B was 5% to 40% in 30 min. Fractions that coincided with peaks on the chromatograph were collected and saved for peptide sequencing. The digested peptide was sequenced using an Applied Biosystems Protein Sequencer (Model 470A) equipped with an on-line phenylthiohydantoin analyser. A sample of the purified peptide was analyzed on the mass spectrometer by Dr. A. Craig of the Salk Insitute using a JEOL JMS-HX110 double focussing mass spectrometer fitted with a Cs<sup>+</sup> gun. An accelerating voltage of 10kV and a Cs+ gun voltage of 25kV were employed. An accelerating/electric field voltage scan from m/z 1100 to m/z 1500 was used. The mass accuracy of the scan was +/- 20ppm.

## **RESULTS**

### **HPLC**

Preliminary investigation of sturgeon brain extract using the HFBA-HPLC method and RIA detected two forms of irGnRH (Fig. 2.1). This material was tentatively assigned as mGnRH

Figure 2.1. HPLC elution of sturgeon brain extract assayed for immunoreactive GnRH. Top: Assayed with antiserum GF-4. Bottom: Assayed using antiserum BLA-4. One immunoreactive area was present with both antisera. A second immunoreactive area that is not detected by BLA-4 can be deduced by noting a relative decrease in the quantity of GnRH in fraction 54 compared to that detected by GF-4. The solid lines indicate percent acetonitrile. The elution positions of seven GnRHs are shown from right to left: cI= chicken GnRH-I; cf= catfish GnRH; l= lamprey GnRH-I; m= mammalian GnRH; cII= chicken GnRH-II; s= salmon GnRH; df= dogfish GnRH.

and cGnRH-II based on elution position compared to standards and antisera cross-reactivity.

In the large-scale purification, assay of fractions from the Sep-Pak HPLC detected irGnRH in fractions 14-41 with antiserum BLA-4 and fractions 19-29 with antiserum GF-4. Fractions 20-28 (37-44% acetonitrile) were selected as having the highest amount of irGnRH (Fig. 2.2). The acetonitrile was evaporated in a vacuum centrifuge and the fractions combined before applying them to the HPLC. Elution of these fractions on a Supelco C<sub>18</sub> analytical column using the HFBA-HPLC method yielded irGnRH activity in fractions 43-62 (Fig. 2.2). One major immunoreactive area (2,488ng) was observed in fraction 50 and detected by both antisera. Fraction 50 of this HPLC was reduced in volume and injected onto the same column with triethylammonium formate, TEAF (Rivier, 1978). The material eluted as one immunoreactive area in fractions 29 and 30 (30% ACN) with an irGnRH content of 2,360ng/2ml (Fig. 2.2).

The acetonitrile in the two fractions from TEAF-HPLC was removed before they were combined and applied to the same HPLC column in the fourth step of the purification. Fractions 26 & 27 (26% ACN) from the TEAP-HPLC were found to contain 924ng of irGnRH activity by antiserum GF-4 (Fig. 2.2). The acetonitrile was removed from these fractions and they were applied to a phenyl (Vydac) column using the solvents described for Sep-Pak HPLC. Fractions 21 and 22 were found to contain a total of 1,415ng irGnRH with GF-4,

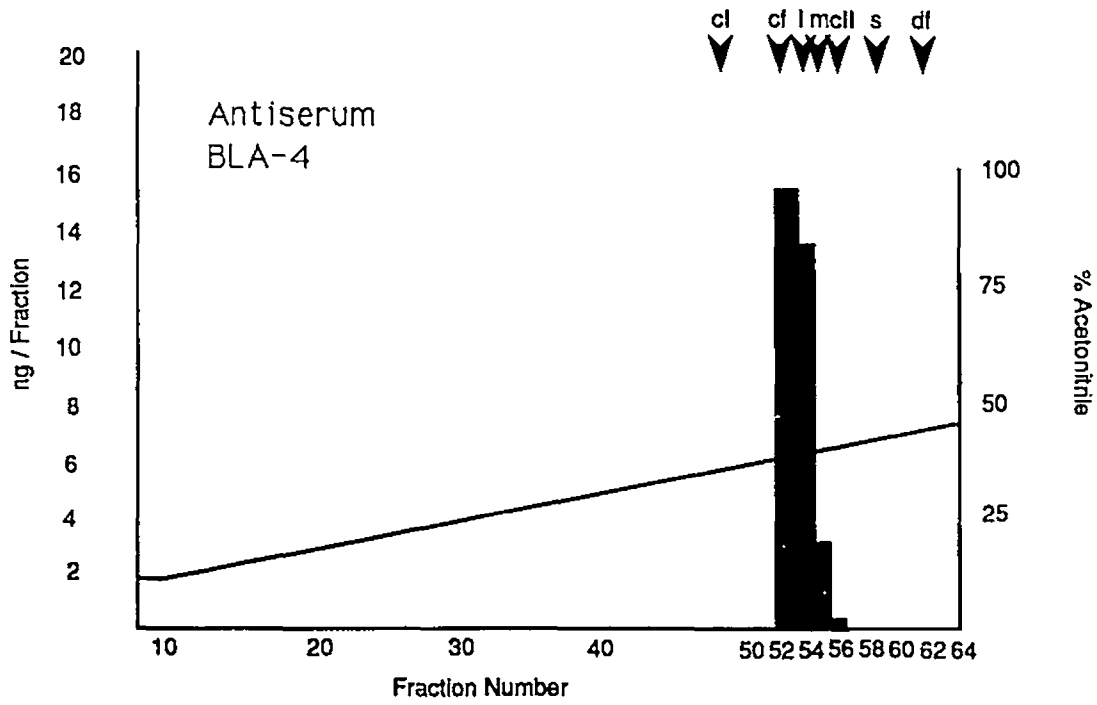
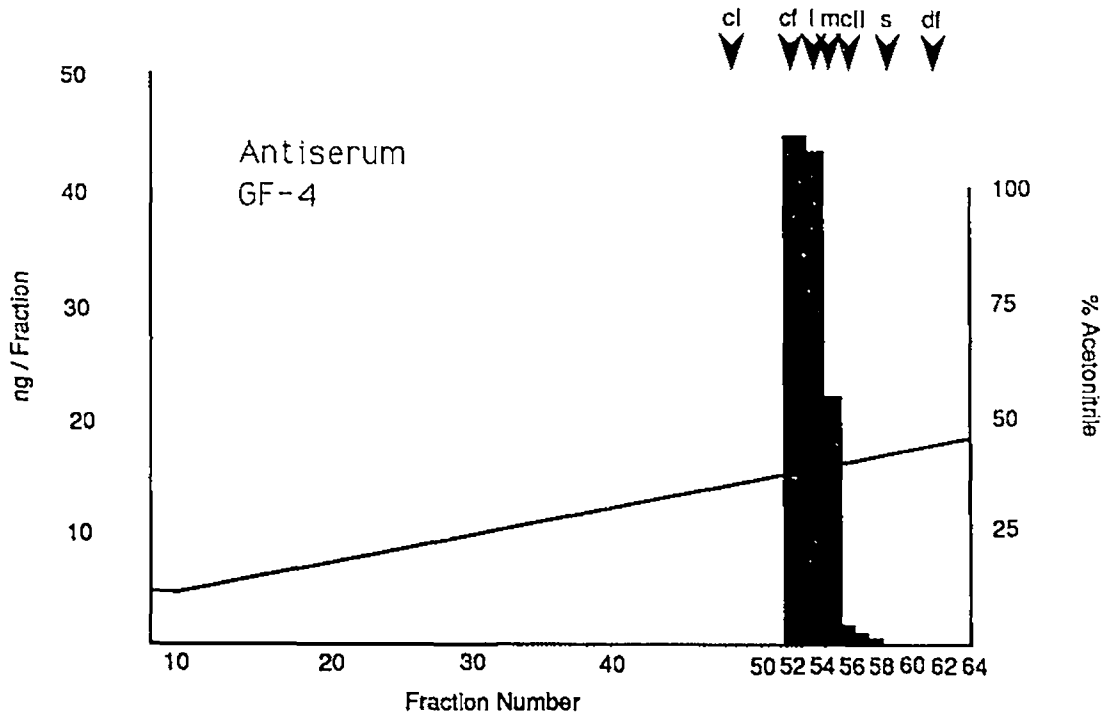
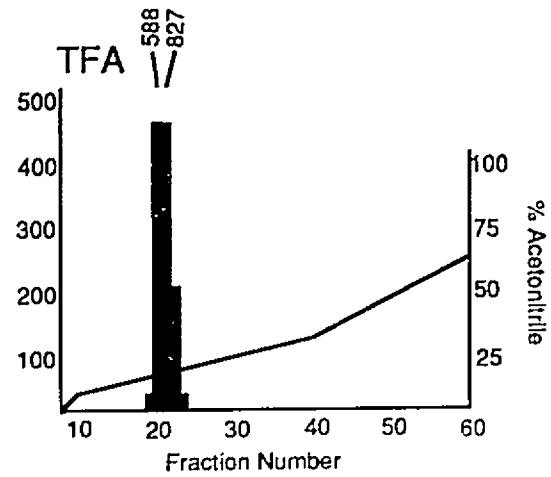
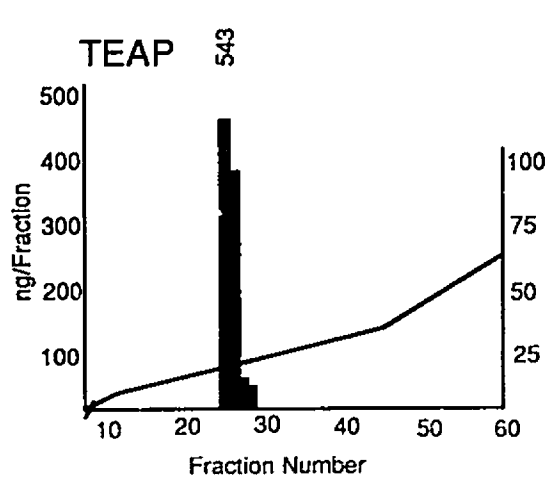
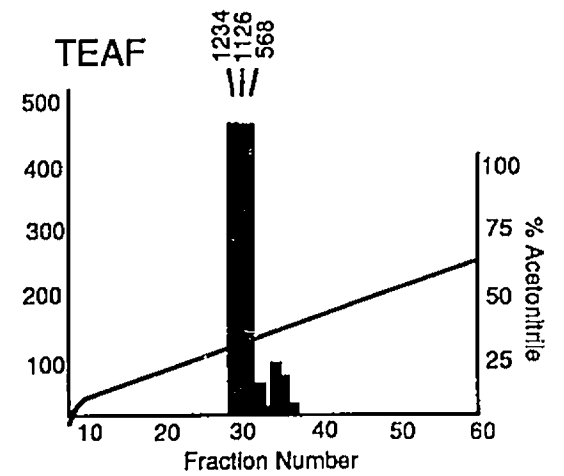
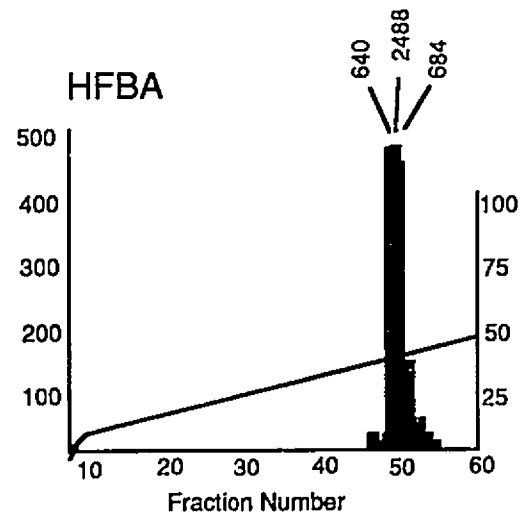
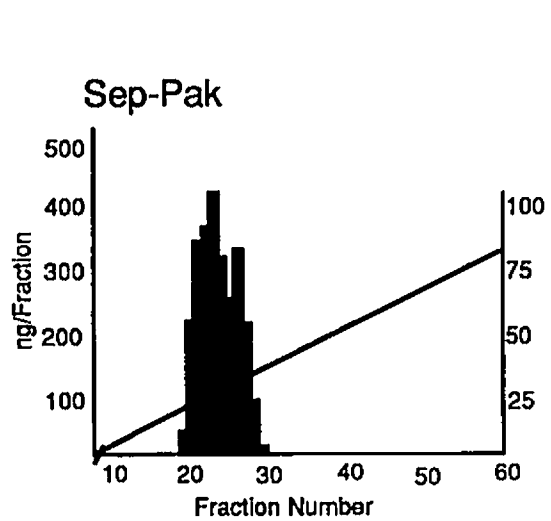


Figure 2.2. HPLC purification of sturgeon GnRH. Antiserum GF-4 was used to detect immunoreactive GnRH (irGnRH) depicted as solid bars. irGnRH detected in each step of the purification was combined and applied to the next step. Top, left to right: irGnRH detected in Sep-Pak HPLC eluates; irGnRH in the HFBA-HPLC method; irGnRH in the TEAF-HPLC method. Bottom, left to right: irGnRH in the TEAP-HPLC method; irGnRH in TFA-phenyl column HPLC. Solid lines indicate percent acetonitrile. Refer to Table 2.1 for the HPLC solvents, their abbreviations and column type.



(Fig. 2.2), 1,845ng with BLA-4 and 1,400ng with B-6. Total efficiency of the first 4 steps of the purification was 60%.

#### Sequence and mass spectral analysis

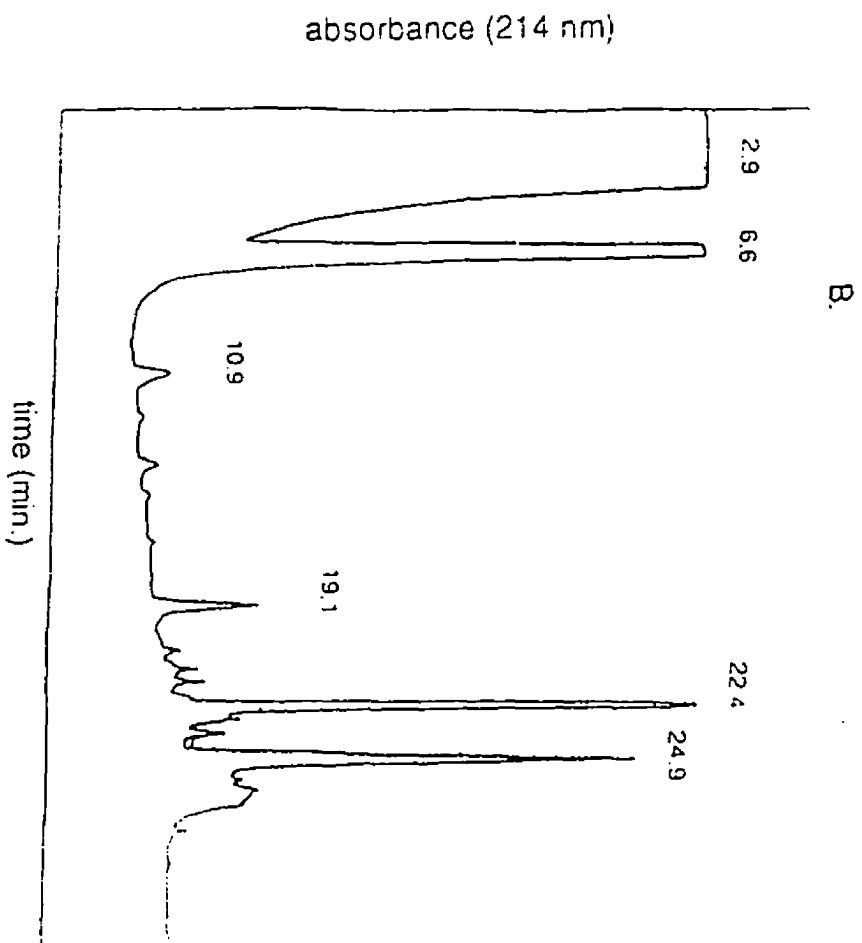
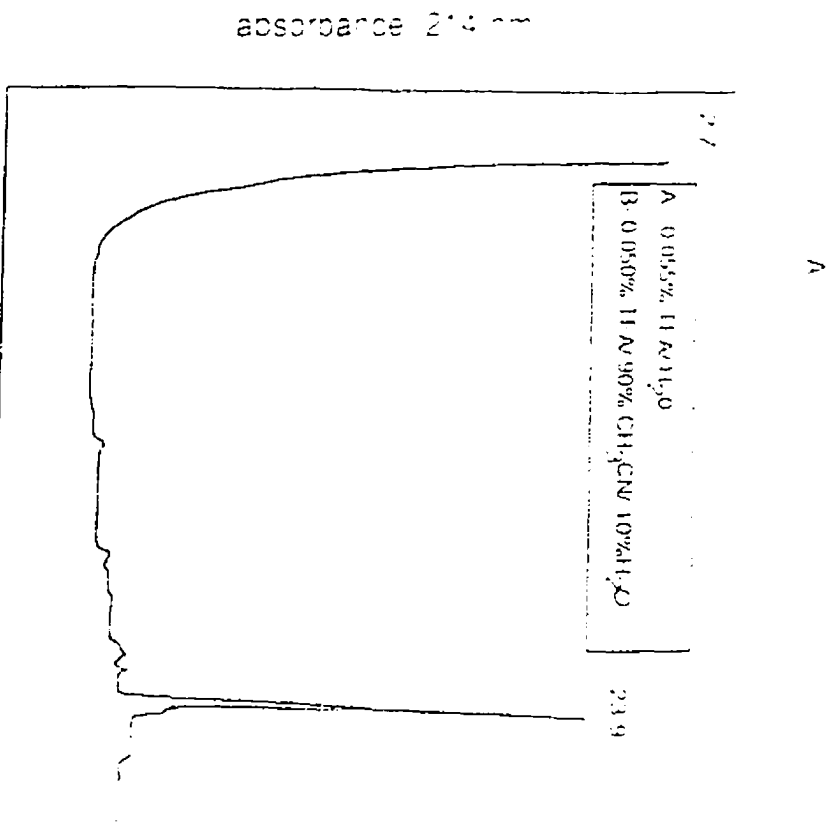
A major peak eluted at 23.9min from a narrow-bore HPLC system (Fig. 2.3) after application of a 10% aliquot of the irGnRH material from fraction 21 onto a phenyl column. Another aliquot (50%) of the immunoreactive material from the HPLC separation on the phenyl column was concentrated to dryness and subjected to pyroglutamate aminopeptidase digestion as described (Fischer and Park, 1992) and above. The digested sample eluted at 22.4min, whereas the remaining undigested sample eluted at 24.9min (Fig. 2.3). Edman degradation of the digested material that eluted at 22.4min was applied to an automated gas phase sequenator and yielded the following sequence: His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.

Another aliquot was further purified by HPLC and analysed by liquid secondary ion mass on a double focussing magnetic sector instrument (Jeol HX 110). The determined mass ( $m/z$  1182.5) is consistent with the C-terminally amidated form of mammalian GnRH ( $MH^+ = 1182.58$  Da). The complete sequence assignment is: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH<sub>2</sub>.

#### DISCUSSION

This is the first report of the primary structure for mGnRH in a fish. The confirmation of structure including

Figure 2.3. Absorbance spectra of sturgeon GnRH eluted from a narrow-bore C<sub>18</sub> HPLC column. (A) HPLC profile of undigested material. The intact sturgeon GnRH eluted at 29min. (B) HPLC profile of sturgeon GnRH after digestion with pyroglutamyl aminopeptidase. Digested peptide eluted at 22.4min, whereas the remaining undigested peptide eluted at 24.9min. The fraction containing the digested peptide was used for peptide sequencing.



the pyroglutamic modification of the N-terminus indicates that the peptide is identical to that found in tetrapods. Further, the amidation of the C-terminus provides support that this peptide is bioactive.

Identification of mGnRH in sturgeon is significant in that it suggests that ancestral fishes contributed the mGnRH form to the tetrapod line. It cannot be discounted that the mGnRH gene was modified sometime during the evolution of the present day sturgeon to give rise to identical forms of GnRH in these two divergent groups, fish and tetrapods. However, Hayes and coworkers (1994) have shown that the structure of the mGnRH gene in the frog, *Xenopus laevis*, shares a high identity with the mammalian mGnRH gene, thus suggesting that the mGnRH gene was present at the emergence of the amphibians.

The identification of mGnRH in sturgeon also supports indirect evidence that mGnRH was widely distributed among the early-evolved boney fishes and in the early teleosts such as the eels. In addition, this study indicates by HPLC elution and antisera cross-reactivity that cGnRH-II is present in the brains of sturgeon. This confirms the earlier work of Sherwood and coworkers (1991) who likewise demonstrated the presence of cGnRH-II in another species of sturgeon.

The confirmation of cGnRH-II is important for two reasons. First, this provides evidence that cGnRH-II, like mGnRH in sturgeon, was present in the brains of ancestral

species that were at the critical juncture of fish and tetrapod divergence. Second, whereas mGnRH disappears in the late-evolving fish and in reptiles and birds, cGnRH-II remains (Miyamoto et al., 1984; Sherwood and Whittier, 1988; Sherwood et al., 1988). This may also indicate that the role of cGnRH-II may not be as flexible as that of the gonadotropin-releaser form of GnRH and that the primary functions of the two forms are indeed different.

The mGnRH cells in the sturgeon are mainly located in the telencephalon, including the preoptic region, although ramifications of mGnRH-containing axons penetrate other brain areas (Lepetre et al., 1993). In a comparative sense, this is the same approximate location as mGnRH in mammals (Schwanzel-Fukuda and Pfaff, 1986). The forebrain location of mGnRH is the same location as the gonadotropin-releasing form in most vertebrates (Conlon et al., 1993; Hayes et al., 1994; Muske and Moore, 1994). Similarly, cGnRH-II cells are located in the mesencephalon of sturgeon (Lepetre et al., 1993), the teleost cichlid *Haplochromis burtoni* (White et al., 1994), amphibians (Conlon et al., 1993; Hayes et al., 1994; Muske and Moore, 1994) and in the primitive musk shrew, *Suncus murinus* (Dellovade et al., 1993). This telecephalon/mesencephalon arrangement of the two forms of GnRH present in the brains of vertebrates provides evidence linking the evolution of GnRH between groups.

In the present study, sturgeon brains were collected during the spawning migration of fish. As such, it is

reasonable to conclude that the form of GnRH present would be in relative abundance given the role of GnRH on gonadotropin release. This may, in part, explain the difference in relative abundance of mGnRH compared to cGnRH-II; the latter was difficult to detect during the purification of sturgeon GnRH.

The abundance of mGnRH detected in sturgeon brains during the spawning season implies that this form plays an important role in reproduction. Indeed, mGnRH has been shown to affect the reproductive physiology of sturgeon. Injections of synthetic mGnRH or a mGnRH analogue (D-Ala<sup>6</sup>, Pro<sup>9</sup>-NET) mGnRH into the stellate sturgeon, *A. stellatus*, and Russian sturgeon, *A. gueldenstaedti*, caused activation and exhaustion of pituitary gonadotropic cells and a corresponding rise in plasma levels of gonadotropins, spermiation and ovulation (Barannikova et al., 1982, 1989; Barannikova and Bukovskaya, 1990, 1991). Likewise, Doroshov and Lutes (1984) demonstrated that injection of an analogue of mGnRH (D-Ala<sup>6</sup>) induced ovulation in the white sturgeon, *A. transmontanus*. Together, these data indicate that the structure and probably the function of mGnRH has persisted through 200 million years of evolution.

Chapter 3 Primary structure of three forms of GnRH from the brain of the Pacific herring Clupea harengus pallasii and the appearance of salmon GnRH.

#### INTRODUCTION

In both the sturgeon *Acipenser gueldenstaedti* and in an early-evolving teleost, chum salmon *Oncorhynchus keta*, there are two forms of GnRH present, cGnRH-II and another form. The second form in sturgeon is mGnRH and in salmon is salmon GnRH (sGnRH), both of which have been identified by primary structure (Chapter 2; Sherwood, et al., 1983). cGnRH-II producing neurons in both sturgeon and salmon have been identified in the midbrain by using immunohistochemistry (Suzuki et al., 1992; Lepetre et al., 1993). Likewise, mGnRH producing cells in sturgeon, *A. baeri*, and sGnRH producing cells in masu salmon, *O. masou*, are located in the preoptic area of the forebrain in each species (Amano et al., 1992; Suzuki et al., 1992; Lepetre et al., 1993). The form of GnRH located in the preoptic area is generally accepted to be the form that releases pituitary gonadotropins despite the change in the structure of the GnRH peptide. This is supported by studies in which ovulation was induced by injection of both native peptides and analogues in salmon, *O. kisutch*, and sturgeon, *A. transmontanus* (Van der Kraak et al., 1978; Doroshov and Lutes, 1984).

Salmon and sturgeon represent two types of fish that have primitive characteristics thought to be reminiscent of ancestral species. They also represent two species on either side of the transition from early boney fish to teleosts. Therefore, an event in which mGnRH disappeared and sGnRH appeared must have occurred between the emergence of the sturgeon and the salmonids, although the function and cytoarchitectural arrangement of GnRH did not change. Further, a comparison of the mGnRH and sGnRH structures and phylogenetic distribution suggests nucleotide substitutions occurred in the mGnRH gene, resulting eventually in the salmon GnRH gene. Meanwhile, cGnRH-II remained unchanged in structure and location in both groups of fish (Amano et al., 1990; Lepetre et al., 1993).

Using the primary structures of mGnRH in sturgeon and sGnRH in salmon as evolutionary points, I investigated the GnRH forms in fish that are thought to have evolved between the two groups. Support for this approach comes from a number of observations that suggest teleosts are a monophyletic group (Nelson, 1984; Lauder and Liem, 1983a,b; Walker and Liem, 1994). Although identification of primary structures for each species is preferred, HPLC elution position and antisera cross-reactivity are important tools for this investigation.

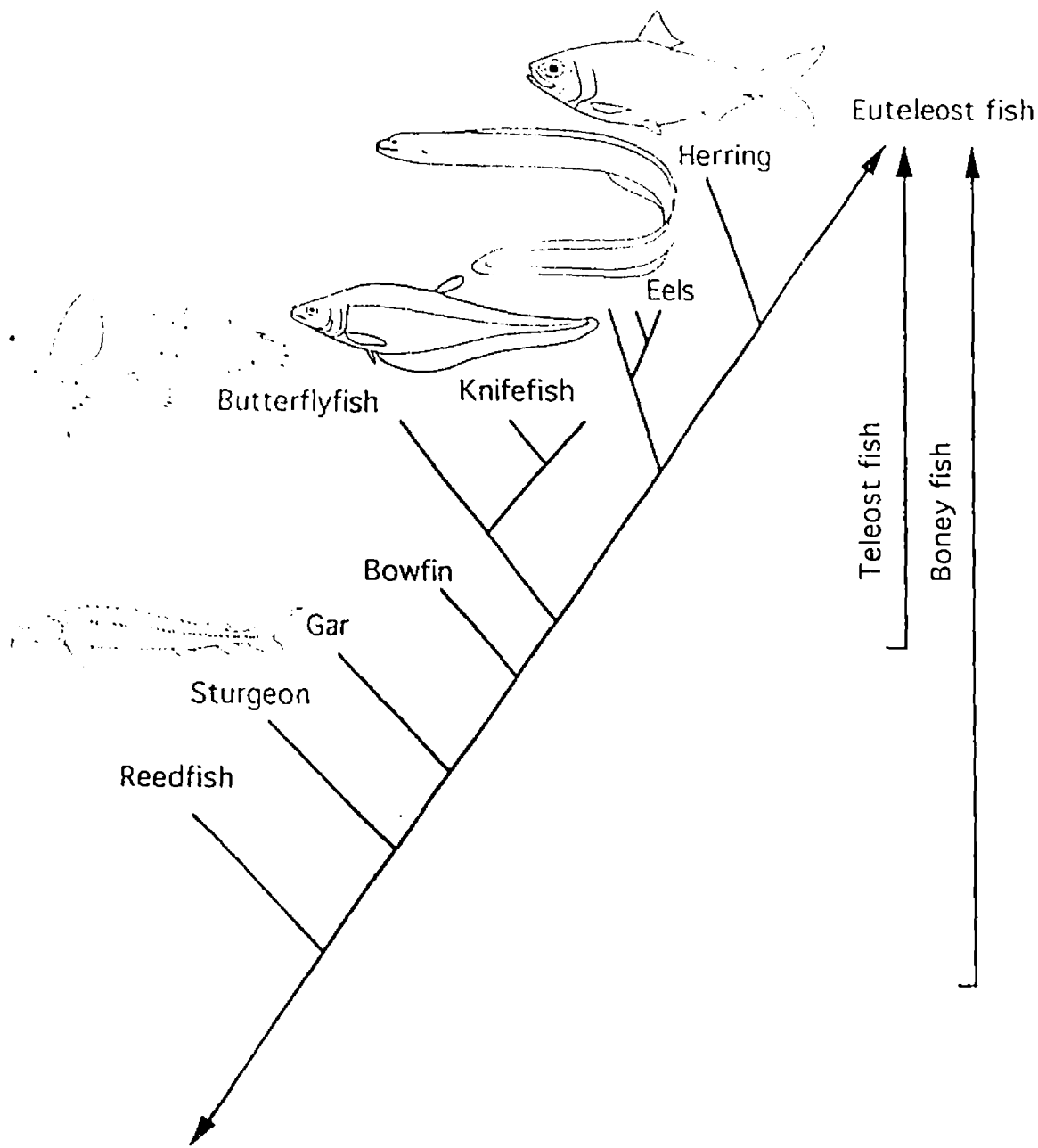
To simplify taxonomic identification of groups and species of fish, common names will be used and the classical taxonomic description of the group placed in parenthesis at

first use. The phylogenetic infradivisions or orders of fish that arose between the sturgeon and herring in order of emergence are: 1) early ray finned fish, represented by the bowfin and gar (*Neopterygii*) 2) the boney tongued fish, including knifefish and butterflyfish, (*Osteoglossiformes*), 3) the eels (*Anguilliformes*) and 4) the herring (*Clupeiformes*; Fig. 3.1).

The forms of GnRH present in the brains of a few of the early ray-finned fishes have been investigated by using HPLC, RIA and immunocytochemistry methods. mGnRH was shown indirectly to be present in the brains of reedfish, *Calamichthys calabaricus*, alligator gar, *Lepisosteus spatula* (Sherwood et al., 1991) bowfin, *Amia calva*, (Crim et al., 1985), American eel, *Anguilla rostrata*, (Grober et al., 1987), European eel, *A. anguilla*, (King et al., 1990) and moray eel, *Gymnothorax fimbriatus* (Shih et al., 1988). Sherwood (1986) detected two forms of GnRH, sGnRH and cGnRH-II in the brains of Pacific herring, *Clupea harengus pallasi*. There are no data on the forms of GnRH in the brains of boney tongued fish. These studies also established the presence of cGnRH-II in the reedfish, sturgeon, gar, eel, herring and salmon despite the change in the forebrain-associated GnRH peptide.

The purpose of the investigation of this chapter is to determine in more detail where the sGnRH peptide emerged in evolution. If changes in the mGnRH gene gave rise to the

Figure 3.1. A cladistic scheme for the phylogenetically ancient boney fishes. Investigated in the present study are the sturgeon, butterflyfish, knifefish, eel and herring. Adapted from Nelson (1984) and Walker and Liem (1994).



sGnRH form, then it is unlikely that sGnRH and mGnRH will ever appear in one species. If sGnRH arose from changes in a duplicated gene that is either the mGnRH or cGnRH-II gene, then both mGnRH and sGnRH may be expressed in a single species. Boney tongued fish are represented by the butterflyfish, *Pantodon buchholzi*, and by the knifefish, *Xenomystus nigri*. Only small numbers of the boney tongued fishes were available and hence, were investigated by HPLC and antisera cross-reactivity to determine the forms of GnRH present in their brains. As well, the brains of gold-spotted moray eels, *Muraena miliaris*, were used in a HPLC study to compare the forms of GnRH reported in another species of moray eel (*Gymnothorax fibriatus*) in abstract form only (Shih et al., 1988). Pacific herring, *Clupea harengus pallasii*, are of great interest as their spawning pattern is an example of a primitive mating behaviour in which schooling rather than pairing occurs (Carolsfeld et al., 1992). In collaboration with J. Carolsfeld, we collected brains of Pacific herring during the spawning season and identified the GnRH peptides in brain tissue by amino acid sequencing. My hypothesis was that sGnRH emerged in the teleost brain in the ancestors of one of these three groups: the boney tongued fish, eels or herring. The study of boney tongued fish and eels was done in collaboration with Emily Standen.

## MATERIALS AND METHODS

### Extraction of peptides from tissues

Brains with and without pituitaries (515g) were removed from 1200 post-spawning male and female Pacific herring, *Clupea harengus pallasii* and immediately frozen on dry ice. Likewise, 11 brains (0.4g) were removed from butterflyfish, *Pantodon buchholzi*, and 11 brains (0.4g) from gold-spotted moray eels, *Muraena miliaris*. Brains and pituitaries were removed from 11 knifefish *Xenomystus nigri*; the brains weighed 1.2g, the pituitaries weighed 10mg. Tissues were treated for the extraction of peptides as detailed in Chapter 2. The last three species of fish were obtained from a local pet store in Victoria. The classification of the fish was confirmed at the University of Victoria using physical characteristics against a key.

### Purification of GnRH peptides from herring brains

Methods for purification of peptides were as described in Chapter 2 with the following changes. Only one Sep-Pak column (10 individual Sep-Paks) was used for all the herring brain extract. After Sep-Pak HPLC, the heptafluorobutyric acid HPLC step was not used. Aliquots of 10 $\mu$ l from the Sep-Pak HPLC fractions were assayed for irGnRH and the fractions with irGnRH were combined. This pool of fractions was reduced in volume using a vacuum centrifuge and applied to a Supelco C<sub>18</sub> analytical HPLC column in 6 aliquots of 800 $\mu$ l at 2min intervals. Synthetic GnRH standards had not been

previously applied to this column. Initial elution conditions, maintained for 10 min after the start of the sample application, consisted of 17% solution B (acetonitrile) and 87% solution A (0.25M triethylammonium formate, TEAF, pH 6.5) pumped at a flow rate of 1ml/min. Subsequently, a gradient increase of 1%/min solution B was applied for 7min followed by a 42min isocratic elution with 24% solution B. Fractions of 1ml were collected for 60min and an aliquot of 10 $\mu$ l of each fraction assayed for irGnRH.

Three groups of fractions with irGnRH from the herring (H) extract were designated H-I, H-II and H-III according to elution time. Each group was pooled separately, reduced in volume using a vacuum centrifuge, and further purified.

Instead of HPLC with a TEAF gradient used in sturgeon GnRH purification, a TEAP step was used for the individual pools of irGnRH-containing fractions. Fractions with irGnRH were combined, reduced in volume, and individually reapplied again to the same column. The elution program for this step was equivalent to that of the last step, except that solution A consisted of 0.05% TFA in water and solution B consisted of 80% acetonitrile and 0.5% TFA in water. Initial conditions were a flow rate of 1 ml/min 5% solution B and 95% solution A. After 5min, a gradient of 1.5% per min solution B was applied for 10 min followed by a gradient of 1% per min solution B by for an additional 30 min. Fractions of 1 ml were collected for 50min and assayed for irGnRH.

Fractions containing irGnRH were combined for each HPLC run, reduced in volume and applied to a phenyl (Vydac) analytical column and eluted with the same program and solvents as in the previous step. Fractions of 1 ml were collected and 10 $\mu$ l aliquots were assayed for GnRH.

Both the C<sub>18</sub> and phenyl columns were tested for residual contamination before use in the purification procedure in which 800 $\mu$ l solution A was injected. The fractions were dried and assayed for irGnRH. All HPLC purification steps were carried out on a Beckman model 125 apparatus.

Fractions from the phenyl column that contained HPLC-purified GnRH were reduced in volume using a vacuum centrifuge, frozen on dry ice and shipped to the Salk Institute, La Jolla, California. Fractions were further purified and sequenced as described in Chapter 2. The intact molecular mass of the purified material was also determined with matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). In this method, the ionized mass of the sample is determined by the time of flight between dipoles of the spectrophotometer as measured by a laser. The measured time of flight is compared to standard samples and the mass determined. Accuracy of this method is +/- 0.1Da.

#### **Identification of GnRH peptides by HPLC-RIA only**

After extraction of peptides from tissues of butterflyfish, knifefish and moray eel, the extracts were

reduced in volume using a vacuum centrifuge. Material was applied to a C<sub>18</sub> HPLC column and the TEAF isocratic method was employed to separate GnRH peptides. Aliquots (10-100 $\mu$ l) were removed from each fraction for the detection of irGnRH by RIA. Blank HPLC runs were done between samples to ensure residual contamination was not on the column. After the last extract was applied to the column, the column was equilibrated to the starting condition. Synthetic standards (200ng each) of mGnRH, cGnRH-II and sGnRH were combined and applied to the column; the HPLC method was repeated. Fractions of 1 ml were collected and an aliquot removed for confirmation of elution position by RIA.

#### **Radioimmunoassay (RIA)**

During the purification of GnRH from herring extracts, 10 $\mu$ l aliquots of each 1ml fraction were assayed for irGnRH with RIA. Methods used for detection of irGnRH in fractions were as described previously in Chapter 2. The limit of detection ( $B/B_0=80\%$ ) for the assay was 10pg using GF-4. Antiserum R-42 was used in a dilution of 1:100,000 to determine if purified GnRH peptides were complete, intact molecules by a topographically assembled epitope (Copeland et al., 1978). Additionally, antiserum B-6 was used in a final dilution of 1:5000 to determine whether mGnRH was present in HPLC samples of butterflyfish, knifefish and moray eel. Antiserum BLA-5 was used in a final dilution of 1:5000 in a RIA for samples other than herring. Where

tracer binding exceeded detection limits ( $B/B_0=20\%$ ),  $10\mu\text{l}$  aliquots were diluted serially and the value closest to  $B/B_0=50\%$  is reported.

#### **Characterization of GnRH in the herring pituitary**

Herring pituitaries ( $n=100$ ) were powdered and subjected to extraction of peptides as described (Chapter 2). The extract was reduced in volume and purified by procedures described above except that Sep-Pak HPLC and TFA-HPLC using the  $C_{18}$  column were omitted. Semi-purified extract was sent to the Salk Institute, further purified using narrow-bore HPLC and sequenced. The mass of the purified peptide was determined.

### **RESULTS**

#### **Purification of GnRH from herring brain extract**

Sep-Pak purification of the extract resulted in a total of  $10.9\mu\text{g}$  irGnRH (Fig. 3.2). HPLC purification of this material with TEAF resulted in three areas of irGnRH designated H-I, H-II and H-III (Fig. 3.2). Each area was individually purified. Amount of irGnRH detected and pooled for further steps in the purification of GnRH peptides is shown in Table 3.1.

Figure 3.2. Immunoreactive GnRH from herring brain as detected by antiserum GF-4 in eluates of: (A) Sep-Pak HPLC and (B) isocratic TEAF-HPLC. Vertical bars indicate immunoreactive GnRH. In TEAF fractions, stippled bars indicated fractions combined and designated herring GnRH-I (H-I); solid bars indicate fractions combined and designated herring GnRH-II (H-II); and hatched bars indicate fractions combined and designated herring GnRH-III (H-III). Numbers appearing above a vertical bar indicate the ng of immunoreactive GnRH present. Designated areas were individually purified in subsequent steps. Solid lines indicate percent acetonitrile.

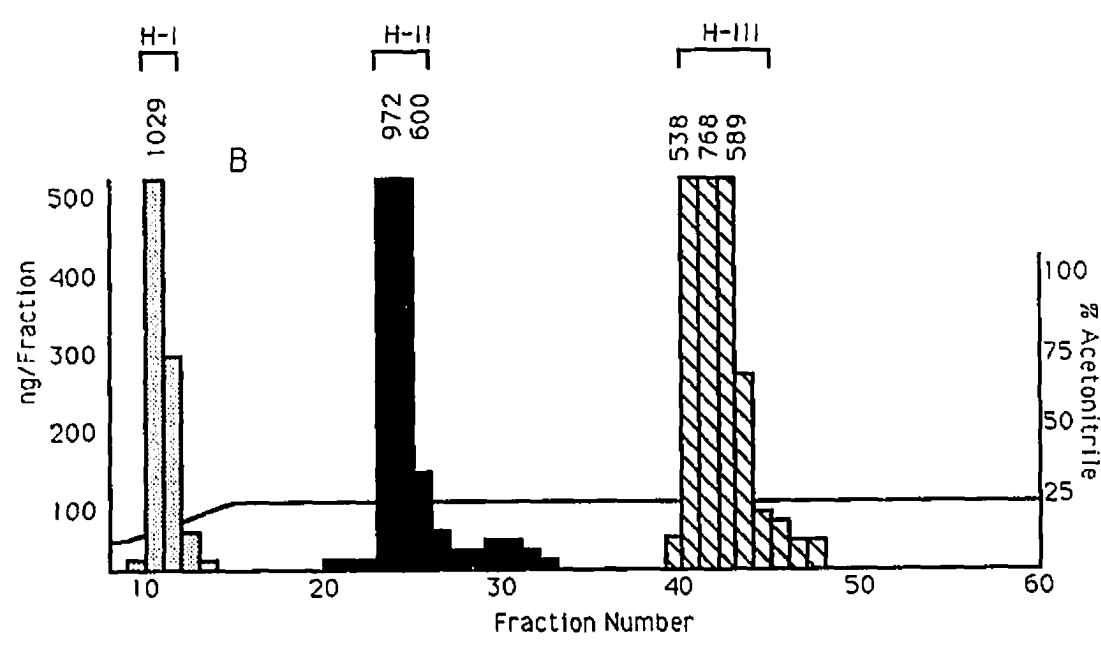
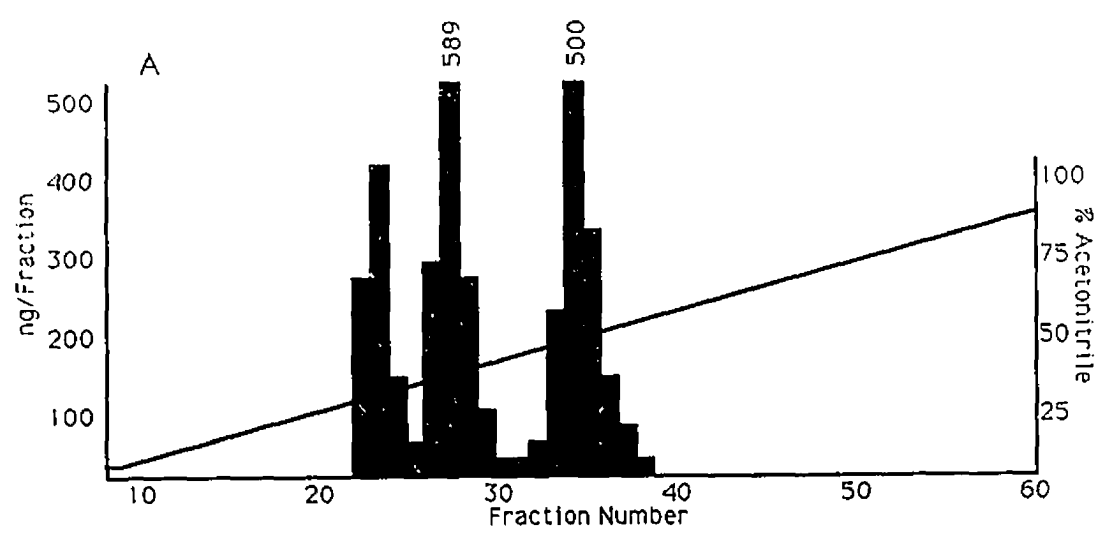


Table 3.1. Amount of irGnRH detected in HPLC fractions during the purification of GnRH peptides from herring brains. Areas of distinct irGnRH from the TEAF step of HPLC were designated H-I, H-II and H-III according to elution time and were purified separately in subsequent steps. TEAF: triethylammonium formate, TEAP: triethylammonium phosphate, TFA: trifluoroacetic acid. See text for HPLC conditions.

TEAF		TEAP		TFA		TFA	
Column C <sub>18</sub>		C <sub>18</sub>		C <sub>18</sub>		phenyl	
Frac.	Amt.	Frac.	Amt.	Frac.	Amt.	Frac.	Amt.
	$\mu\text{g}$		$\mu\text{g}$		$\mu\text{g}$		$\mu\text{g}$
H-I	11-13 1.3	30-32	1.1	50-53	0.9	22	0.5
H-II	24-26 1.7	23-24	0.8	35-39	0.4	14	0.1
H-III	41-44 2.2	38-40	1.9	39-42	1.4	27	0.9

### Characterization of primary structures from brain

Edman degradation sequencing of GnRH that was digested with pyroglutamate amino peptidase indicated that the structures of the three GnRH molecules are:

H-I pGlu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly,

H-II pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly,

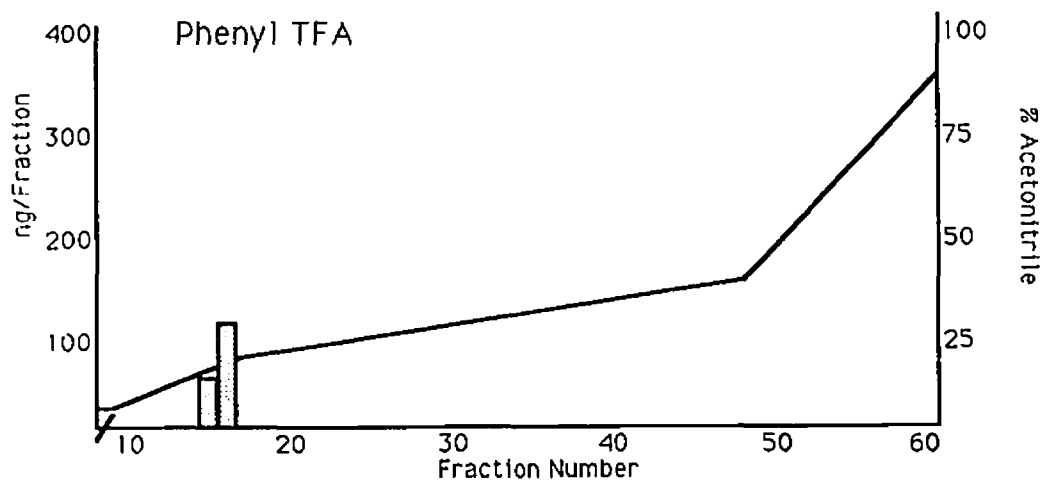
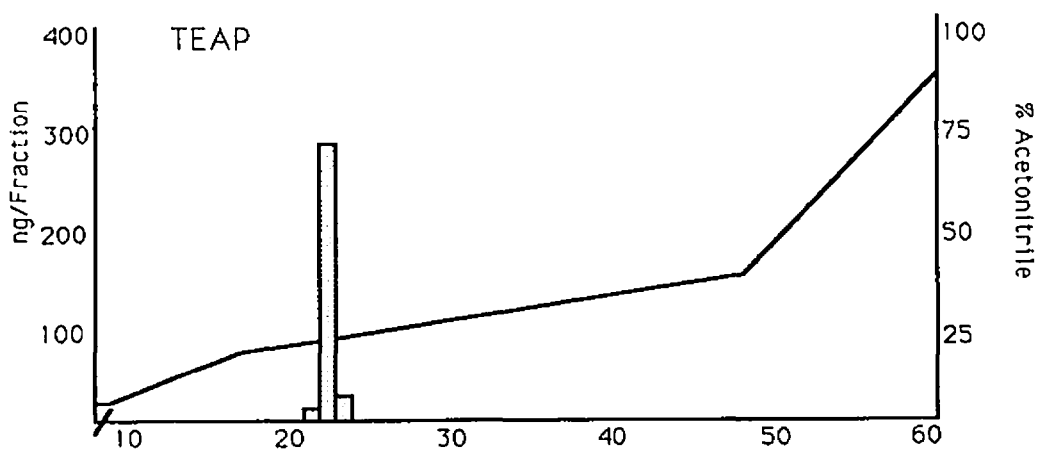
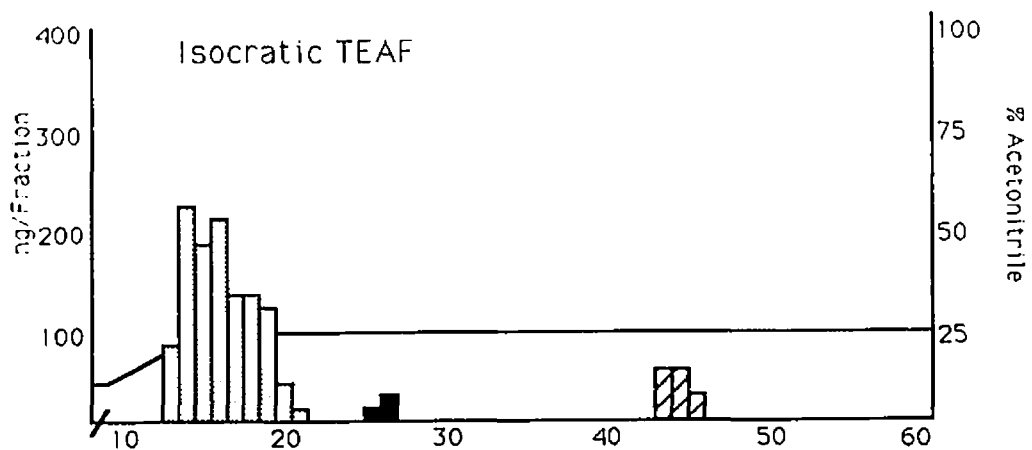
H-III pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly.

H-I GnRH is a novel form of GnRH that we named herring GnRH (hGnRH). The mass of intact hGnRH was determined to be 1088Da. The structures of H-II and H-III correspond to known GnRH peptides: cGnRH-II (Miyamoto et al., 1984) and sGnRH (Sherwood et al., 1983).

### Characterization of GnRH structure from herring pituitary

Three forms of irGnRH were detected in the fractions of the isocratic TEAF-HPLC method (Fig. 3.3). The early-eluting fractions containing irGnRH (12-18) corresponded to those in which hGnRH was purified from herring brain extract. Likewise, two other immunoreactive areas were detected that corresponded to cGnRH-II and sGnRH from herring brain. The first-eluting irGnRH area was the most abundant in the pituitary as detected by antiserum GF-4. This area was further purified, sequenced and identified as the amidated form of hGnRH with a mass of 1088.55Da.

Figure 3.3. Purification steps of herring pituitary GnRH. Top: initial separation of immunoreactive GnRH forms using the isocratic TEAF method. Stippled bars indicate fractions with immunoreactive GnRH that were combined for further purification. Middle: Purification of herring pituitary GnRH using the TEAP method. Stippled bars indicate fractions with immunoreactive GnRH that were combined for the next step of purification. Bottom: Further purification of herring pituitary GnRH using the TFA phenyl column method. Stippled bars indicate fractions with immunoreactive GnRH that were combined for further purification by narrow-bore HPLC prior to sequencing.



#### HPLC of boney tongued fish and eel brains

In the butterflyfish brains, two forms of GnRH were identified by HPLC elution position and antisera cross-reactivity (Fig. 3.4). The forms were mGnRH and cGnRH-II. Antiserum B-6, which is specific for mGnRH detected the first peak, but not cGnRH-II (Fig. 3.4). HPLC eluates of knifefish brain extract had two forms of GnRH identified by elution position with synthetic cGnRH-II and sGnRH standards (Fig. 3.5). In contrast, the knifefish pituitary extract had only one form of GnRH present that eluted in a similar position to the sGnRH standard (Fig. 3.5). Moray eel brain extract contained two immunoreactive areas that eluted in the same positions as synthetic mGnRH and cGnRH-II (Fig. 3.6). There was not sufficient material to check cross-reactivity with antiserum B-6.

#### DISCUSSION

This is the first report of a novel GnRH form, herring GnRH (hGnRH). As well, this is one of the first reports of three forms of GnRH identified by primary structure from the brains of a single species (the other is presented in Chapter 5). This investigation also demonstrates that sGnRH emerged before hGnRH; sGnRH is first detected in the boney tongued knifefish, whereas hGnRH is first detected in the herring, known to evolve later than knifefish (see Lauder and Liem 1983b). Therefore, not only did mGnRH disappear, but two new GnRHs appeared, sGnRH and hGnRH.

Figure 3.4. HPLC and RIA analysis of GnRH from butterflyfish brain extract using the isocratic TEAF method. Top: Immunoreactive GnRH as detected by antiserum GF-4. Bottom: Immunoreactive GnRH as detected by antiserum B-6, an antiserum specific for mGnRH. Elution positions of synthetic standards are indicated by arrowheads: M= mammalian GnRH; CII= chicken GnRH-II; S= salmon GnRH. Numbers appearing adjacent to vertical bars indicate amount of GnRH detected. Solid lines indicate percent acetonitrile.

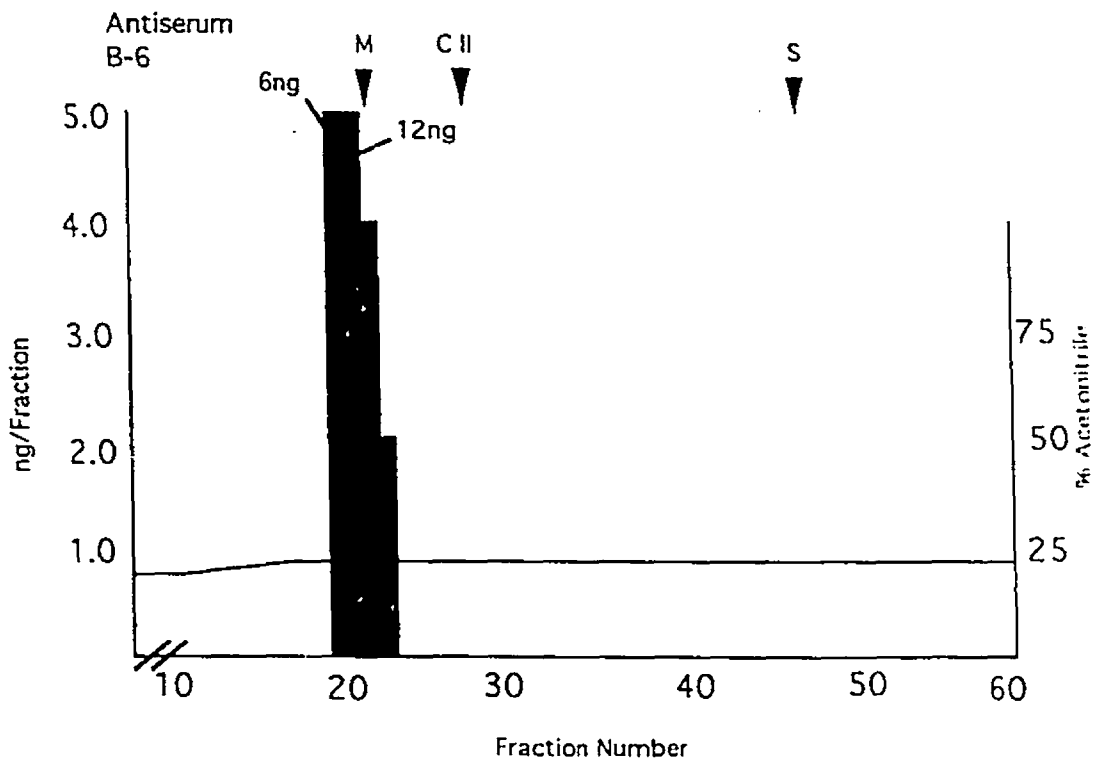
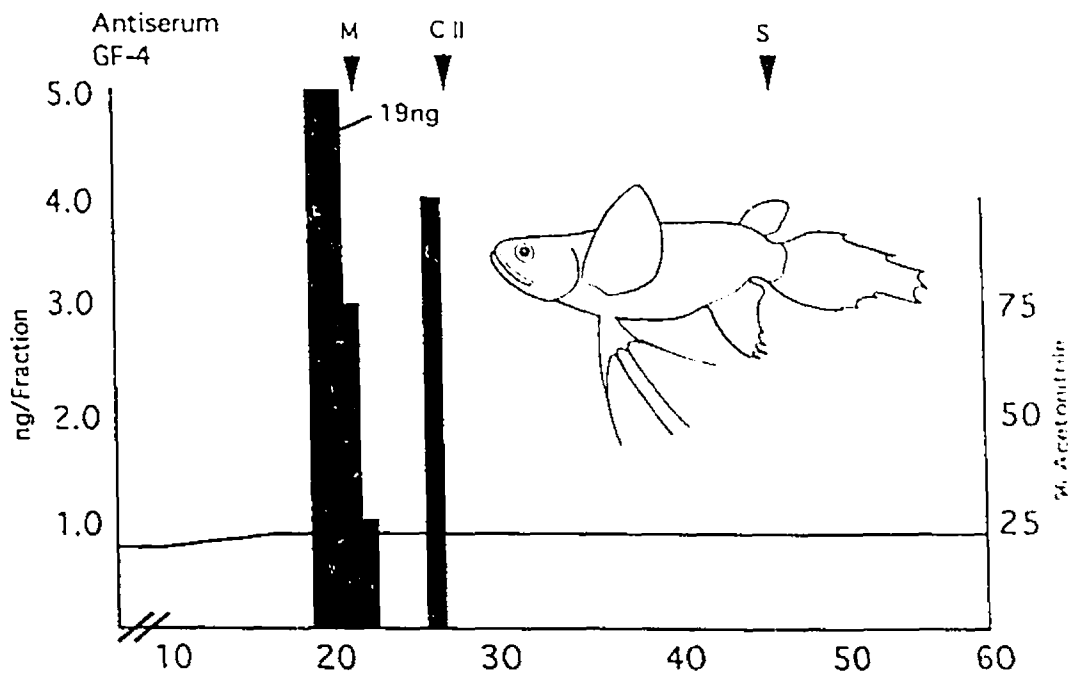


Figure 3.5. HPLC and RIA analysis of GnRH from knifefish brain and pituitary extracts using the isocratic TEAF method as detected by antiserum GF-4. Top: Immunoreactive GnRH detected in brain extract. Bottom: Immunoreactive GnRH detected in pituitary extract. Elution positions of synthetic standards are indicated by arrowheads: M= mammalian GnRH; CII= chicken GnRH-II; S= salmon GnRH. Numbers appearing adjacent to vertical bars indicate amount of GnRH detected. Solid lines indicate percent acetonitrile.

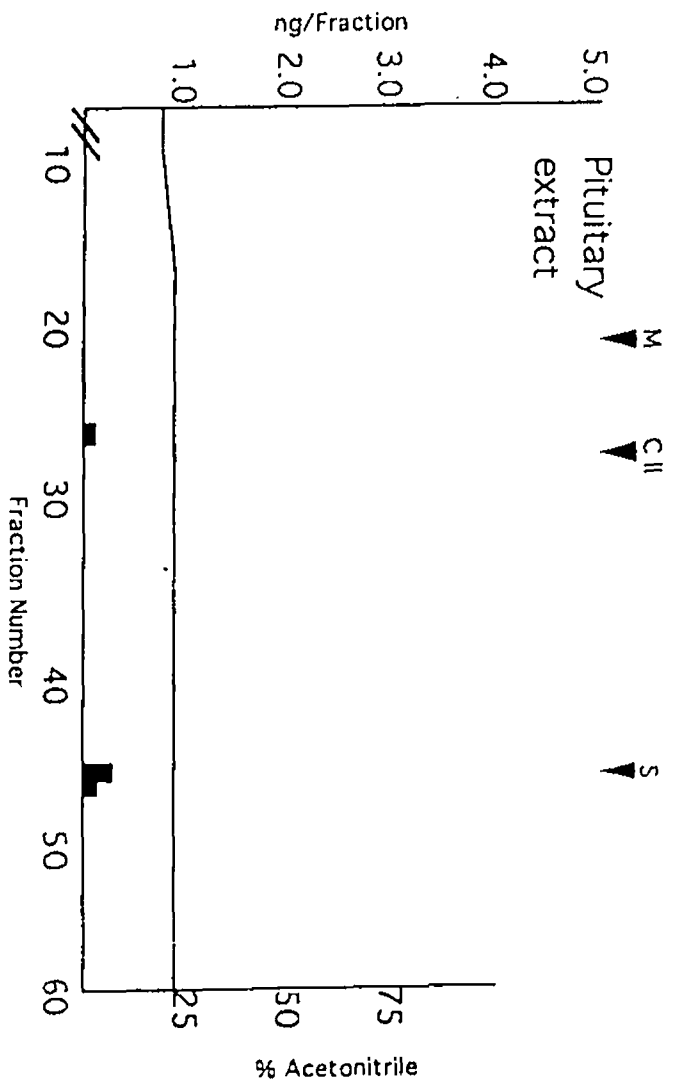
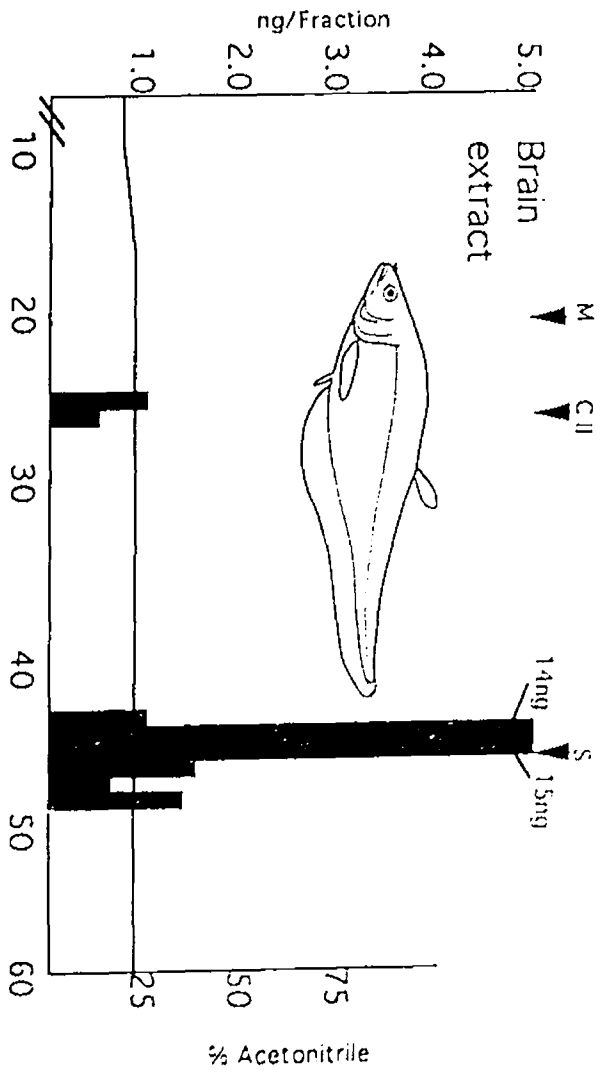
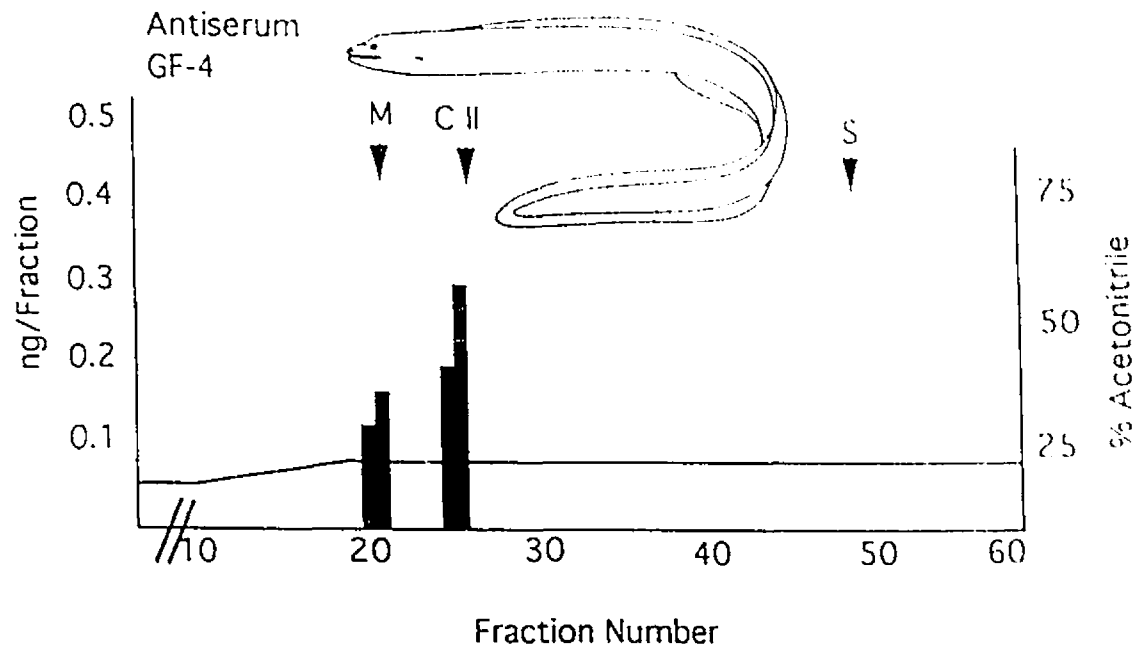


Figure 3.6. HPLC and RIA analysis of GnRH from moray eel brain extract using the isocratic TEAF method. Immunoreactive GnRH was detected by antiserum GF-4. Elution positions of synthetic standards are indicated by arrowheads: M= mammalian GnRH; CII= chicken GnRH-II; S= salmon GnRH. Numbers appearing adjacent to vertical bars indicate amount of GnRH detected. Solid line indicates percent acetonitrile.



Three forms of GnRH have been observed in two other teleost species that evolved after herring, the sabalo *Prochilodus lineatus* (Somoza et al., 1994) and snook *Centropomus undecimalis* (Sherwood et al., 1993b), which are in different orders. Other studies have indicated that multiple forms of GnRH may be present in the brains of other fish species. However, many of these studies have not combined HPLC elution position with characterized antisera. This may lead to the misidentification of the number and types of GnRH forms present. The identification of hGnRH, cGnRH-II and sGnRH by primary structure represents definitive proof that three forms of GnRH are present within the herring brain.

In the pituitary of herring, three forms of irGnRH were also detected. The most abundant form detected was purified, sequenced and identified as hGnRH. The confirmation of C-terminal amidation of pituitary hGnRH by MALDI-MS shows that this form is present as a bioactive form. The other forms of GnRH detected in pituitary extract coincide with the elution positions of cGnRH-II and sGnRH that were purified from herring brain by the same procedure. hGnRH may be the gonadotropin releasing form because it is present in abundance, that is, hGnRH is approximately 40 fold greater than cGnRH-II and 10 fold greater than sGnRH (as detected by antiserum GF-4). Alternatively, the form of GnRH that releases the gonadotropins in the herring pituitary may have been released or degraded before

collection, resulting in the erroneous assignment of hGnRH to this role. Location of the hGnRH producing cell bodies by *in situ* hybridization and location of their axons containing hGnRH by immunohistochemistry will help delineate the role of hGnRH in the pituitary.

As three forms of GnRH are present in herring brains, but not in boney fish that evolved before herring, a gene duplication event may have occurred for one of the two existing GnRH genes. It is possible that a duplication of the mGnRH gene was followed by nucleotide substitutions in both genes resulting in hGnRH and sGnRH. Alternatively, the mGnRH gene may have been changed to sGnRH due to nucleotide substitutions and later it was duplicated and one of the genes was altered to code for hGnRH, while the other remained as sGnRH.

The phylogenetic distribution of sGnRH shows it is present in both the pituitary and brain extracts of the osteoglossomorph knifefish (Fig. 3.5). This latter evidence strongly indicates that the transformation of mGnRH to sGnRH occurred earlier than the herring. This does not preclude that knifefish are incorrectly classified and actually evolved after herring or that the form detected in knifefish brains and pituitary has been misidentified. Analysis of the cDNA encoding hGnRH, sGnRH and mGnRH in fish will provide more definitive proof of the relationship of these three forms because the cDNA encodes the longer GnRH precursor form for each peptide.

The present study gives the first evidence of mGnRH and sGnRH in the boney tongued fishes and confirms the presence of mGnRH in the eels (Fig. 3.6). Further, the identification of sGnRH in the knifefish pituitary indicates that sGnRH, and not cGnRH-II, controls the release of the gonadotropins. The appearance of sGnRH in the boney tongued fishes, but retention of mGnRH in the later-evolving eels is curious. The analysis of GnRH peptides in a sister group to the knifefish, the mooneyes, will aid in the elucidation of sGnRH origin. Again, comparison of the cDNA for GnRH among species will refine the pathway of evolution for sGnRH.

Chapter 4 Primary structure of sGnRH and cGnRH-II in chum salmon Oncorhynchus keta brain; distribution of both GnRH peptides in eight species of salmonids and the function of a sGnRH analogue in chinook salmon O. tshawytscha.

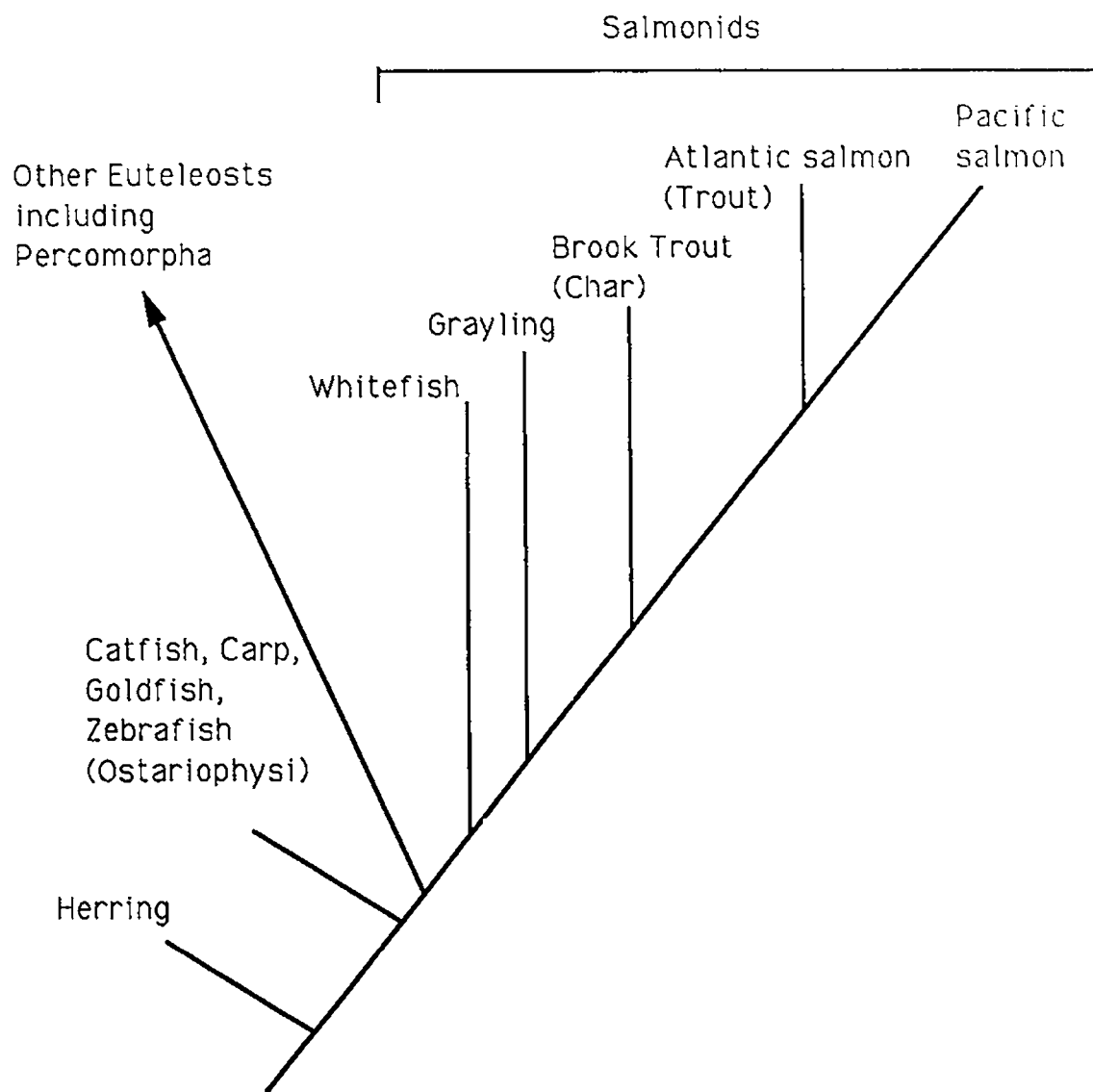
#### INTRODUCTION

For salmon, two forms of GnRH are reported to be present, but only sGnRH has been identified by primary structure (Sherwood et al., 1983). sGnRH was found to be the only form present in the pituitary (Amano et al., 1992, Suzuki et al., 1992) and to release one form of the gonadotropins, gonadotropin-II (GTH-II), although GTH-I was not measured. The second form of GnRH present in the brains of salmon has been hypothesized to be cGnRH-II as shown by the indirect evidence of HPLC elution position and cross-reactivity with antisera (Sherwood, 1986; Okuzawa et al., 1990; Suzuki et al., 1992). sGnRH, cGnRH-II and their analogues stimulate the release of GTH-II from goldfish pituitaries (Peter et al., 1987). As well, exogenous application of GnRH peptides *in vivo* has been used to increase GTH-II levels in various fish (Van der Kraak et al., 1987). However, these studies have not measured the effect of GnRH on the levels of GTH-I. Development of a suitable RIA for chum salmon (Oncorhynchus keta) GTH-I and GTH-II by others is a necessary first step in investigating the physiological effect of GnRH in salmon. Here, the role of sGnRH in releasing GTH-I and GTH-II in

mature fish is investigated. Of the various boney fish, I selected salmon to do a more detailed study of the forms of GnRH in the brains of eight species of salmonids and the physiological effects of GnRH on plasma GTH levels in one species. Salmon are of particular interest to me because I have been involved in their culture for a number of years. Also, salmon GnRH (sGnRH) was the first GnRH to be identified in a teleost fish (Sherwood et al., 1983) and the reproduction of salmonids has been the focus of much study, particularly after the development of a RIA to measure plasma GTH-I and GTH-II in coho salmon *Oncorhynchus kisutch* (Swanson et al., 1989; 1991). However, a number of questions remain such as: 1) What is the primary structure of the second form of GnRH in the brains of salmon? 2) Do all salmon have these same two forms? 3) What is the physiological effect of GnRH on GTH-I?

Our recent identification of hGnRH in herring opens the question of whether this peptide is present in salmonids and other fish, but was not detected in earlier studies that reported only two forms for salmonids, sGnRH and cGnRH-II (Sherwood et al., 1983, 1984; Okuzawa et al., 1990; Amano et al., 1992; Suzuki et al., 1993). It is also possible that early-evolving, but not later-evolving salmonids have herring GnRH. The herring and salmonids are considered to be related in that an ancestral fish line may have given rise to herring-like fish and later to a salmonid ancestor (Fig. 4.1) such as the whitefish (*Coregonus spp.*) and

Figure 4.1. Phylogenetic representation of the extant groups of salmonids. Adapted from Nelson (1984) and Stearly and Smith (1993).



grayling (*Thymalus spp.*; Stearly and Smith 1993). The later-evolving salmonids such as chars (*Salvelinus spp.*), trout (*Salmo spp.*) and Pacific salmon (*Oncorhynchus spp.*) came from the same stem line ancestors as the whitefish and grayling (Stearly and Smith, 1993).

The most recently-evolved salmonids are the Pacific salmon of the genus *Oncorhynchus*, which has eight species (Stearly and Smith, 1993). In salmonids, sGnRH has been identified by primary structure in chum salmon, *O. keta*, (Sherwood et al., 1984) and by the gene sequence in sockeye, *O. nerka*, (Coe et al., 1995) and Atlantic salmon, *Salmo salar*, (Klungland et al., 1992) and by cDNA sequence in masu salmon, *O. masou* (Suzuki et al., 1993). In each of these species, hGnRH was seen neither in the HPLC-RIA nor in the cDNA. Therefore, hGnRH, if present in salmonids, would be located in the genus *Salvelinus* that evolved before *Salmo* or *Oncorhynchus*.

This chapter details studies to determine: 1) the primary structure of the second form of GnRH in the brains of chum salmon, 2) the distribution of the forms of GnRH in the brains of closely related char, trout and salmon and 3) the effect of a sGnRH analogue on plasma GTH-I and GTH-II levels.

## **MATERIALS AND METHODS**

### **Fish used for purification of GnRH**

Approximately 3,000 (3Kg) chum salmon brains were collected from mature salmon that had returned to freshwater to spawn. Fish from the Big Qualicum River hatchery were anesthetized, the gametes and brains removed and the brains frozen and stored at  $-80^{\circ}\text{C}$ . Pituitaries were not included in the samples.

### **Fish used for detection of GnRH forms**

Seven species of salmonids in addition to chum salmon were obtained from commercial hatcheries or aquatic facilities at the University of Victoria (Table 4.1). In each case, brains were removed as described, or for immature fish, the fish was decapitated and the brain removed. Brains were immediately placed on dry ice or in liquid nitrogen. In contrast to the chum salmon samples, pituitaries were removed with the brains for the seven species described here.

### **Extraction of peptides from tissues**

Tissues were treated for the extraction of peptides as detailed in Chapter 2.

### **Purification of GnRH peptides from chum salmon**

Purification steps for identification of salmon GnRH peptides were as detailed in Chapter 2 with the following

Table 4.1. Species of salmonids used in determining the number and elution position of GnRH forms from brain tissue. Number of brains and weight of brains is indicated in addition to the gender of the fish and source of the tissues. SW = fish in saltwater, FW = fish in freshwater. Fish were sampled after gametes were taken for rearing in commercial hatcheries. Immature fish refers to fish that are not reproductively mature.

Name	Weight	Number	Gender	Source
Sockeye <i>Oncorhynchus nerka</i>	5g	25	Immature	FW
Chinook <i>O. tshawytscha</i>	7	11	Immature	FW
Coho <i>O. kisutch</i>	8	10	Mixed	SW
Pink <i>O. gorbuscha</i>	5	6	Mixed	SW
Masu <i>O. masou</i>	3	6	Immature	FW
Atlantic <i>Salmo salar</i>	4	15	Immature	FW
Brook trout <i>Salvelinus fontinalis</i>	3 2	10 10	Male Immature	FW FW

exceptions. After evaporation of the solvents used in the extraction of peptides, the extract was divided into nine equal portions. Each of these portions was applied to a Sep-Pak cartridge column as described earlier. Material was eluted from the nine Sep-Pak cartridge columns as described using the TFA/acetonitrile method. irGnRH fractions from each Sep-Pak HPLC were combined within a given set of eluted fractions. Eight of these pooled eluates were individually applied to a C<sub>18</sub> column and treated as described for sturgeon GnRH purification using the HFBA/acetonitrile method. The pooled eluates from the ninth Sep-Pak HPLC were applied to the C<sub>18</sub> column and the isocratic TEAF method of HPLC was used to elute the applied extract. Therefore, of eluates from the nine Sep-Pak cartridge columns to be eluted, eight were further purified using eight individual HFBA-HPLC runs, and one was further purified using the isocratic TEAF-HPLC method. The latter method was used to separate the early-eluting cGnRH-II-like irGnRH from sGnRH (Sherwood and Lovejoy, 1989).

Three similar regions of irGnRH activity were identified for all eight HFBA steps. Fractions from these regions, called S-I, S-II and S-III, were individually pooled from the eight HFBA eluates. Regions S-I, S-II and S-III were treated separately for further purification. Aliquots (800 $\mu$ l) of S-I, S-II and S-III were applied to the HPLC column in 5 repeated injections using a flow rate of 1 ml/min of 5% solution B (acetonitrile) and 95% solution A

(0.25M triethylammonium formate, TEAF). The gradient TEAF method detailed in Chapter 2 was then followed. After purification of chum salmon GnRH, samples were treated as described (Chapter 2) for sequencing of the peptide and determination of mass.

Fractions with irGnRH from the ninth Sep-Pak HPLC were combined, reduced in volume and applied to the C<sub>18</sub> HPLC column using the isocratic TEAF method described in Chapter 3. Two distinct regions of irGnRH were identified, one early eluting, the other, late eluting. Fractions with irGnRH in the early-eluting fractions were combined, reduced in volume and applied to the last two steps of purification as described in Chapter 3, sequenced and the mass determined.

#### **HPLC for indirect identification of GnRH in salmonids**

Determination of GnRH forms in the brain was done using the HFBA-HPLC method for chinook salmon, masu salmon and brook trout. The isocratic TEAF method of HPLC was used for coho, pink, sockeye and Atlantic salmon. A C<sub>18</sub> column similar to the one used for purification was used for HPLC. In each method, HPLC fractions of 1 ml were collected and an aliquot assayed for irGnRH. To compare the elution positions of the native peptides to synthetic GnRH peptides, seven standards (sGnRH, cGnRH-II, lamprey GnRH, chicken GnRH-I, mammalian GnRH, dogfish GnRH and catfish GnRH) were

combined and applied (200ng each) to the column for each of the methods used above.

#### **Radioimmunoassay (RIA) for GnRH**

For the purification of GnRH in chum salmon, 10 $\mu$ l aliquots were used in RIA, whereas for other species, HPLC fractions of 100 $\mu$ l were dried before determination of irGnRH. The method for detection of irGnRH in HPLC fractions was as described (Sherwood, et al. 1984, 1986a). Aliquots of plasma (100 $\mu$ l) were assayed in triplicate. Antisera GF-4 (1:25,000 final dilution) and BLA-4 or BLA-5 (1:5,000) were used to detect irGnRH with iodinated mGnRH used as tracer and mGnRH used as standards. The cross-reactivity of the two antisera for several known forms of GnRH has been reported (Kelsall et al., 1990; Sherwood et al., 1991). Limits of detection ( $B/B_0=80\%$ ) for the assay were approximately 10pg for GF-4 and 50pg for BLA-4 and BLA-5. All HPLC fractions were assayed with both antisera because GF-4 detects cGnRH-II and BLA-4 does not. In fractions with tracer binding that exceeded detection limits ( $B/B_0=20\%$ ), 10 $\mu$ l aliquots were diluted serially and the value closest to  $B/B_0=50\%$  was recorded.

#### **RIA for Gonadotropin (GTH)**

Plasma GTH-I and GTH-II samples were analyzed as previously described (Swanson et al., 1989, 1991). A heterologous assay was used with purified coho salmon GTH-I

and GTH-II as the labeled hormone and standards. This assay is appropriate for chum salmon GTH because parallel lines were generated in a dose-response curve for coho and chum GTH (Swanson, unpublished data). Percent binding ranged from 29-38% for GTH-I and 26-34% for GTH-II. The sensitivity was 0.32 and 0.07ng/ml for GTH-I and GTH-II, respectively. Interassay differences for pooled coho plasma were 7.4 and 7.7% for GTH-I and GTH-II assays, respectively.

#### **Fish used for in vivo effects of a sGnRH analogue**

Forty mature, domesticated female chinook salmon (*Oncorhynchus tshawytscha*) with an average weight 5.6kg were sedated in 0.5 ppm metomidate (Syndel Laboratories, Vancouver) and sampled for blood (see below) one week prior to transfer to freshwater. Immature (silver) siblings of mature fish (n=20) were also sedated and a blood sample taken. For transfer to freshwater, fish were sedated and placed in a 1 cubic meter bucket of oxygenated dilute seawater (1 part seawater, 2 parts freshwater). The bucket and fish were transported by helicopter to a freshwater hatchery (Sea Spring Salmon Farms, Chemainus, B.C.) in 20 minutes. Upon arrival, 20 females were selected at random and sampled for blood before transfer to freshwater (10°C). The fish were allowed to acclimate for 3 days in the freshwater.

After acclimation (Day 0), the fish were sedated (0.5 ppm, metomidate), sampled for blood and tagged in the

operculum with tags of different color and numbers to identify fish. The fish were checked for ovulated eggs and injected intraperitoneally with 0.1ml/kg body weight saline (0.6%) or Ovaprim (sGnRH analogue; sGnRH<sub>a</sub> D-Arg<sup>6</sup>-Pro<sup>9</sup>-NET; 10µg/ml and domperidone; 20µg/ml, Syndel Laboratories, Vancouver, B.C.). The fish were returned to the communal tank to recover. On Day 3, a blood sample was taken and a second injection of saline or analogue (0.4 ml/kg) was given. Subsequently, fish that had ovulated were examined for eggs and sampled for blood on days 14, 17, 20, 24, 31 and 38. Three control fish ovulated on day 50, but blood samples were not collected.

#### **Blood samples**

A heparinized syringe with a 22 gauge needle was used to withdraw blood from caudal vessels or, in some ovulated fish, from the heart. The syringe was immediately placed on crushed ice until the samples were transferred to 1ml Eppendorf vials and centrifuged for 4 min. The plasma was placed in a 0.5ml Eppendorf vial, frozen on dry ice, and stored at -80°C.

#### **Ovulation and eggs**

Complete ovulation was defined as a steady stream of eggs expressed from the urogenital papillae in response to slight pressure on the anterior abdominal cavity. Diameters of eggs from ovulated fish were compared between treatments.

The average diameter was determined from a random sample of 300 eggs.

### **Statistical analysis**

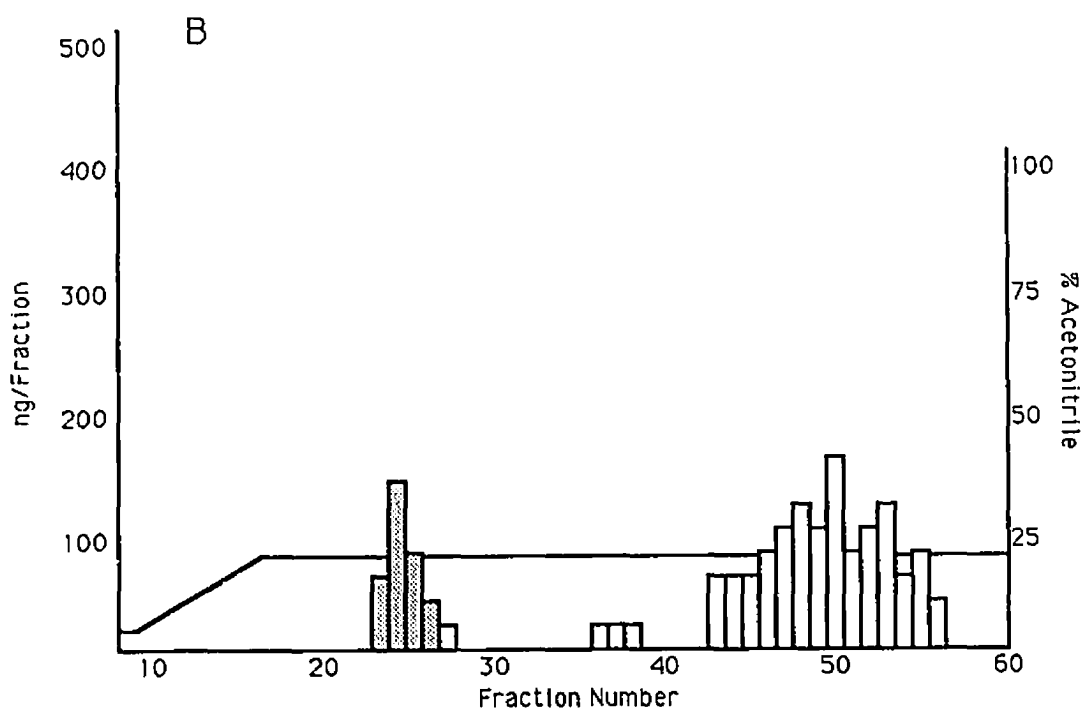
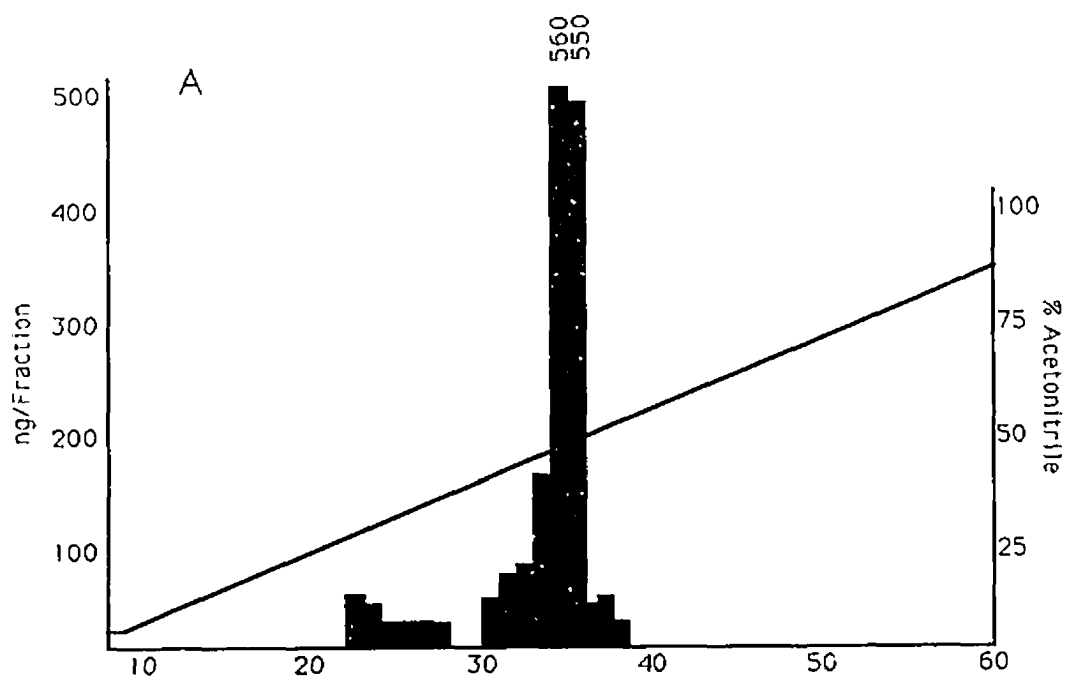
Student's t-test was used to determine differences at a given time between treatments. A paired two-tailed t-test was used to determine difference within treatments. A ranked Mann-Whitney test was used to determine the difference in spawning times between treatments. Significance was defined at the 95% interval.

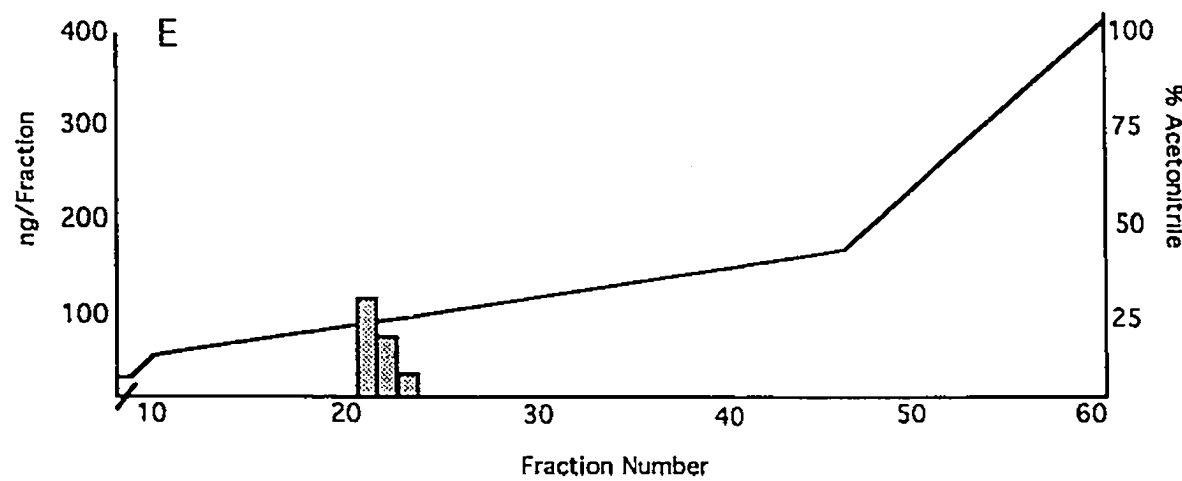
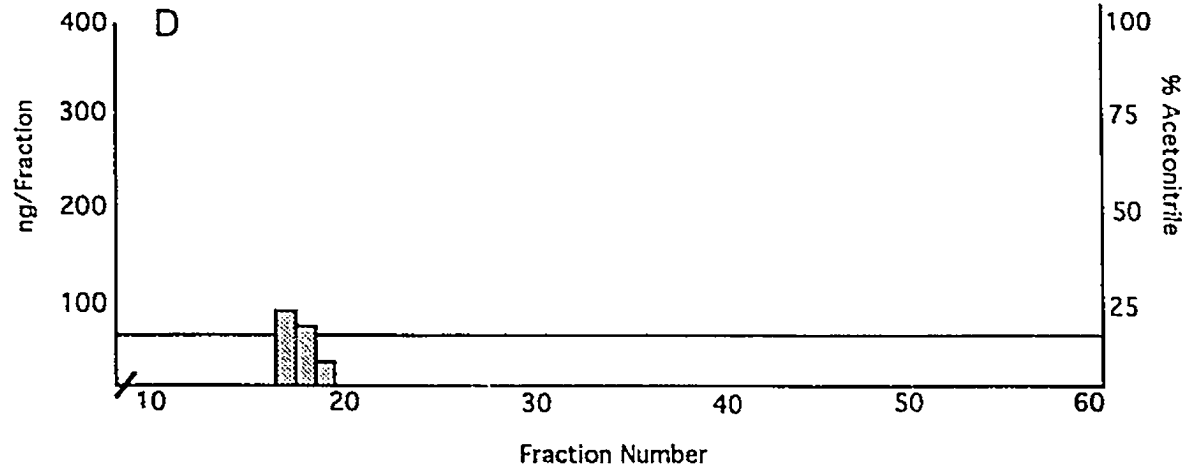
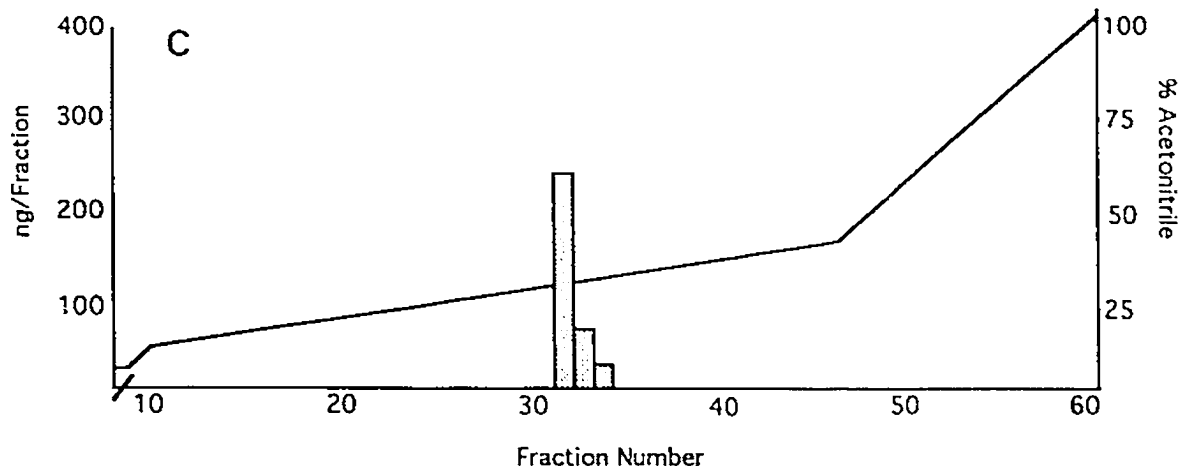
## **RESULTS**

### **Primary structure of cGnRH-II**

Approximately 1,750ng of irGnRH was detected by antiserum GF-4 in the eluate from the ninth Sep-Pak HPLC column. The irGnRH eluted between 20 and 40 min after initiation of the HPLC program (Fig 4.2A). After application of these fractions to the C<sub>18</sub> column using the isocratic TEAF method, two areas of irGnRH were identified by antiserum GF-4 (Fig 4.2B). The first of these two areas contained 352ng of irGnRH in fractions 24-27. Other irGnRH fractions that eluted later in the method were discarded as they eluted in a similar position to sGnRH (see below). A TEAP-gradient HPLC method resulted in 308ng irGnRH in fractions 32-34 (Fig. 4.2C) as detected by antiserum GF-4. After application of these fractions to the C<sub>18</sub> column and elution with the TFA method, a single area of irGnRH was detected in

Figure 4.2. Immunoreactive GnRH (irGnRH) in eluates from chum salmon brain HPLC detected by antiserum GF-4. (A) irGnRH from Sep-Pak HPLC. Numbers above fractions indicate the amount of irGnRH detected. (B) irGnRH from isocratic TEAF HPLC (C) irGnRH from TEAP HPLC. (D) irGnRH from isocratic C<sub>18</sub> TFA HPLC. (E) irGnRH from the phenyl TFA HPLC step. irGnRH from (E) was further purified by narrow-bore HPLC, digested with pyroglutamyl aminopeptidase and sequenced. Stippled bars indicate the fractions combined and further purified. Solid lines indicate percent acetonitrile.





fractions 17-20 that contained 227ng irGnRH as detected by antiserum GF-4 (Fig. 4.2D). After application of the irGnRH fractions to the phenyl column, a single area of irGnRH was detected in fractions 21-24 that contained 149ng irGnRH as detected by antiserum GF-4 (Fig. 4.2E). Fractions 22 and 23 were combined and used in the determination of sequence and mass. The peptide isolated and identified from chum salmon brains was amidated cGnRH-II which has the sequence pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH<sub>2</sub> with a mass of 1236.5Da.

#### **Primary structure of sGnRH**

Whereas the isocratic TEAF method for HPLC of chum salmon brain extracts resulted in two regions of irGnRH, the HFBA method resulted in three regions of irGnRH. The three regions of irGnRH (S-I, S-II and S-III) in the HFBA method were detected using antiserum BLA-5. These regions are not in the area associated with the elution position of synthetic sGnRH (see Fig. 3.4), but instead they either do not correspond to a standard (S-I, fraction 48) or match the elution positions of synthetic catfish GnRH (S-II, fraction 50) or mammalian GnRH (S-III, fraction 52). Fractions 48, 50 and 52 were pooled with identical fractions from all eight of the HFBA methods to yield three 8ml aliquots. Sequence identification by Edman degradation after purification yielded the sGnRH amino acid sequence for all three regions.

#### **Indirect identification of GnRH by HPLC and RIA**

In all species of salmonids examined, only two forms of GnRH were identified and characterised by HPLC elution position and antisera cross-reactivity (Figs 4.3, 4.4, 4.5 and 4.6). These forms were cGnRH-II and sGnRH in both the HFBA and the TEAF methods. Synthetic standards of sGnRH and cGnRH-II eluted in similar positions to the two forms detected for both HPLC methods. Unlike the purification of sGnRH from chum salmon brains, short exposure to HFBA in the mobile phase did not produce irGnRH in fractions that differed from the sGnRH standard elution position.

#### **Survival of injected fish**

Thirty-five out of 40 fish survived to ovulate in the saline-injected group between Day 0 and the end of the experiment. Likewise, 35 out of 40 fish in the sGnRH<sub>a</sub>-injected group survived in freshwater.

#### **Timing of Ovulation**

Salmon GnRH<sub>a</sub> decreased the time to ovulation compared with saline-injected controls ( $P < 0.05$ ). For fish treated with sGnRH<sub>a</sub>, 71.4% (25/35) ovulated in contrast to 5.7% (2/35) of the saline-injected controls by day 13 after the initial injections (Fig. 4.7). All of the fish that received sGnRH<sub>a</sub> had ovulated by day 24 after the first injection, whereas the control fish receiving saline did not complete ovulation until day 50.

Figure 4.3. RIA analysis of HFBA-HPLC eluates from brain extracts of mature (top) and juvenile (bottom) brook trout using antisera GF-4 (left) and BLA-4 (right). Solid lines indicate percent acetonitrile. Arrowheads indicate the elution position of standards: c-II= chicken GnRH-II; s= salmon GnRH.

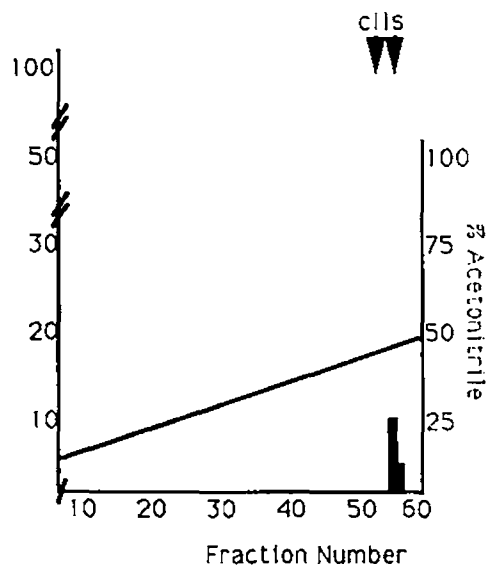
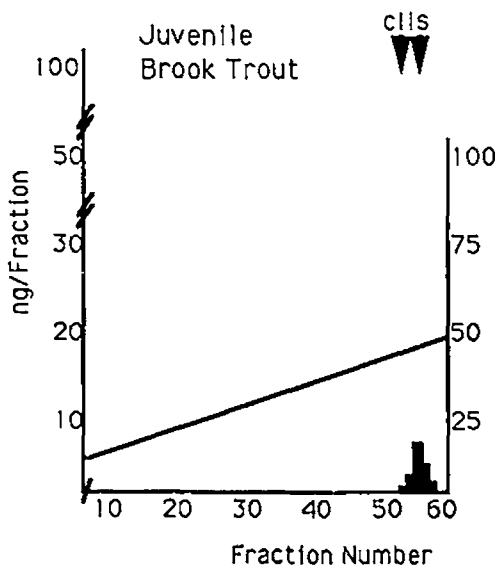
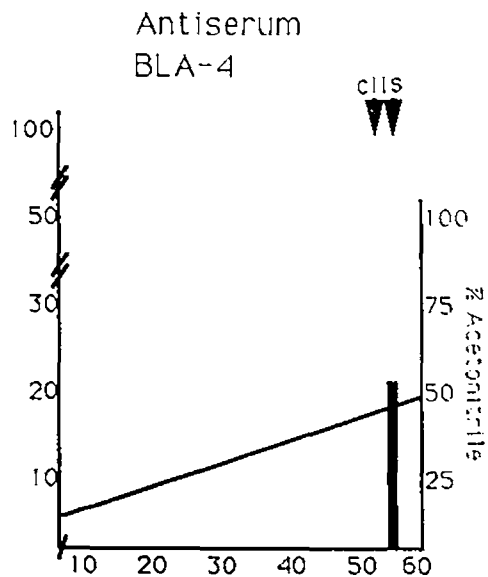
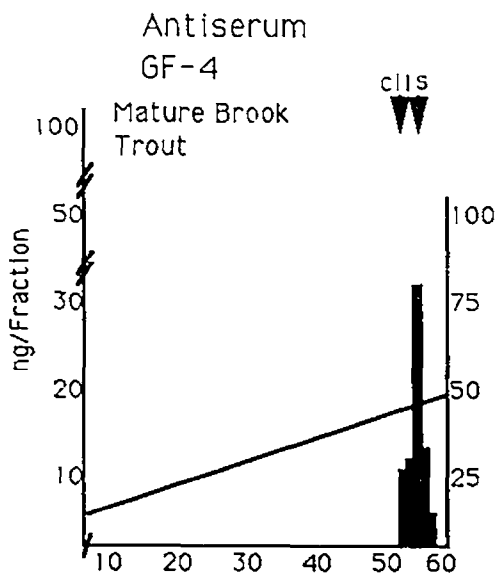
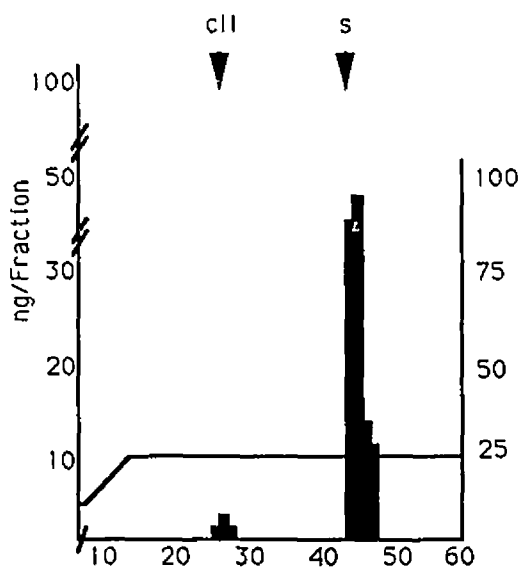


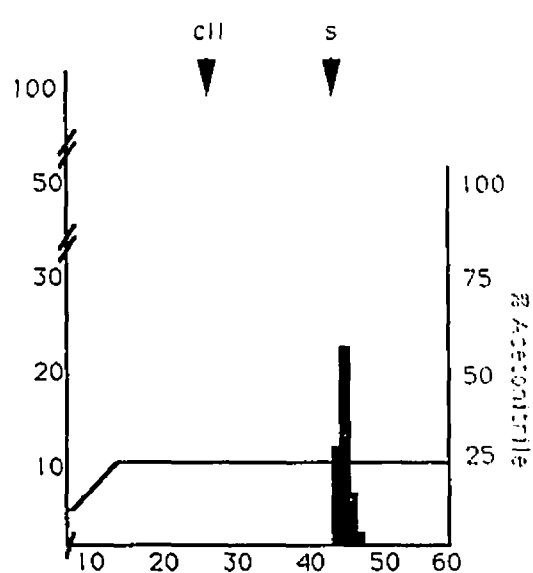
Figure 4.4. RIA analysis of isocratic TEAF-HPLC eluates from brain extracts of Atlantic salmon (top) and coho salmon (bottom) using antisera GF-4 (left) and BLA-5 (right). Solid lines indicate percent acetonitrile. Arrowheads indicate the elution position of standards: c-II= chicken GnRH-II; s= salmon GnRH.

Antiserum  
GF-4

Atlantic Salmon



Antiserum  
BLA-5



Coho Salmon

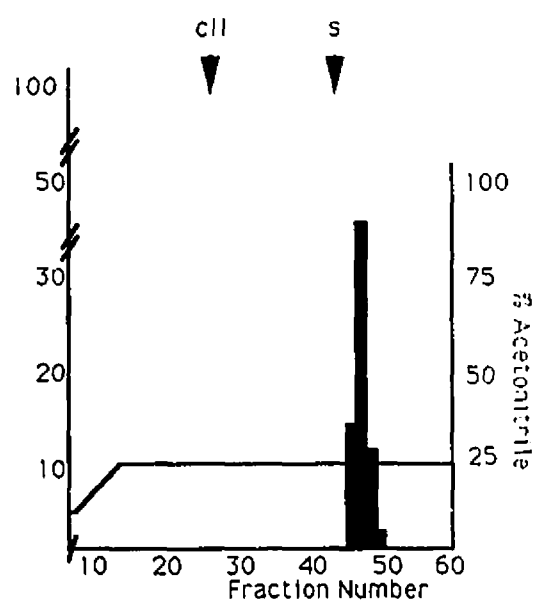
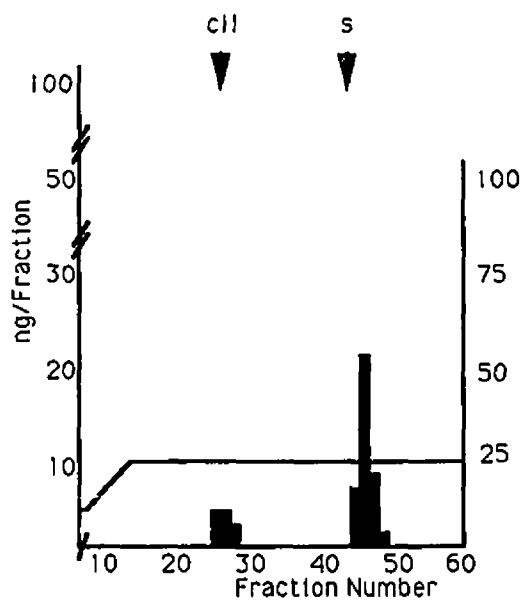
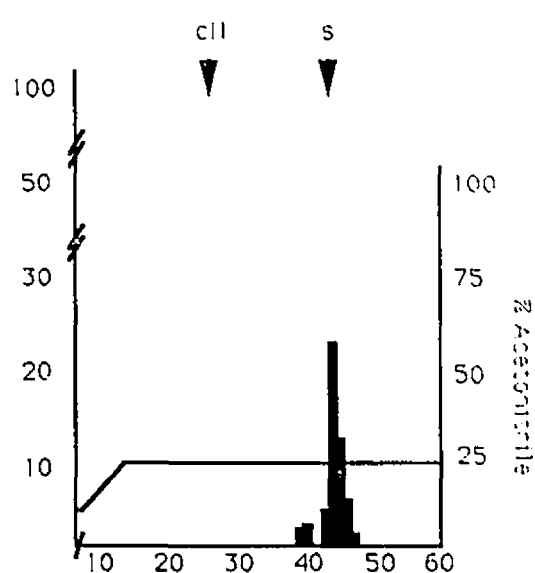
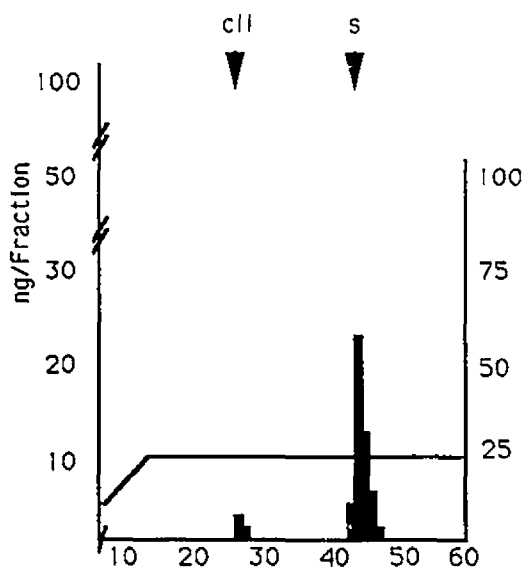


Figure 4.5. RIA analysis of isocratic TEAF-HPLC eluates from brain extracts of sockeye salmon (top) and pink salmon (bottom) using antisera GF-4 (left) and BLA-4 (right). Solid lines indicate percent acetonitrile. Arrowheads indicate the elution position of standards: c-II= chicken GnRH-II; s= salmon GnRH.

Antiserum  
GF-4

Antiserum  
BLA-5

Sockeye Salmon



Pink Salmon

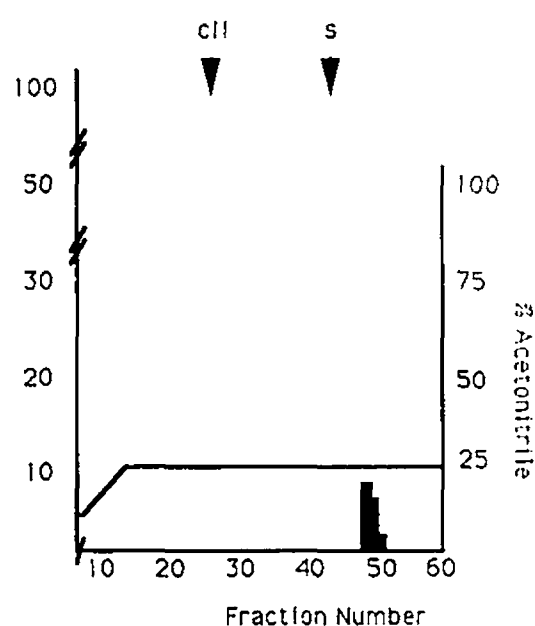
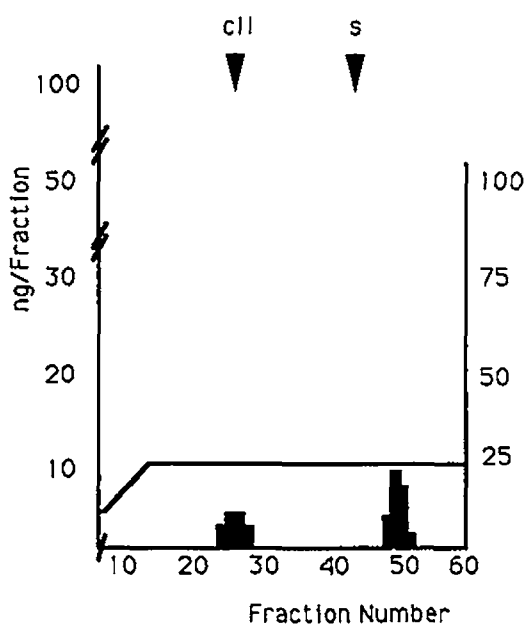


Figure 4.6. RIA analysis of isocratic TEAF-HPLC eluates from brain extracts of masu salmon (top) and chinook salmon (bottom) using antisera GF-4 (left) and BLA-4 (right). Solid lines indicate percent acetonitrile. Arrowheads indicate the elution position of standards: c-II= chicken GnRH-II; s= salmon GnRH.

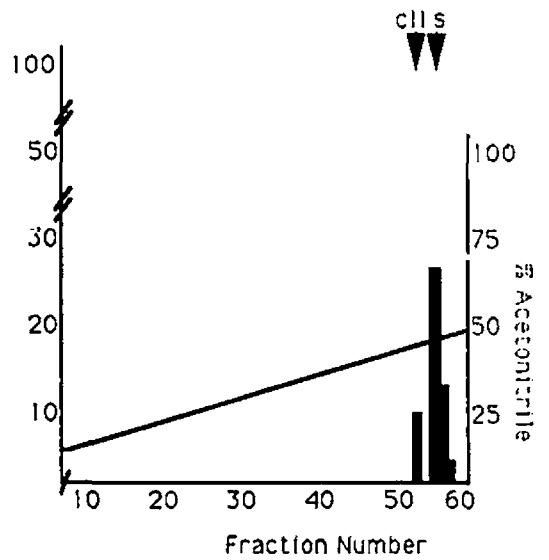
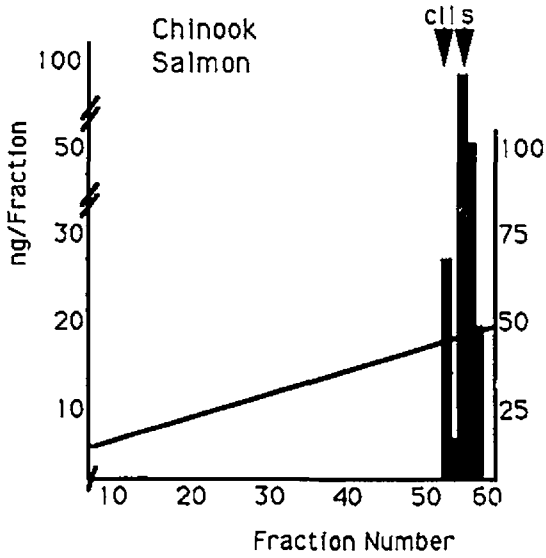
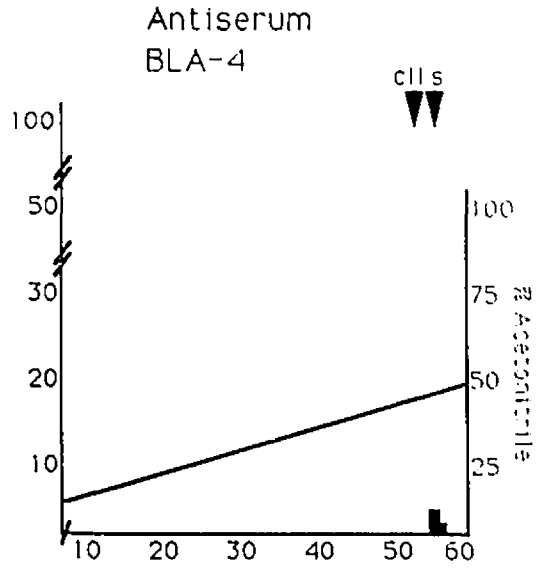
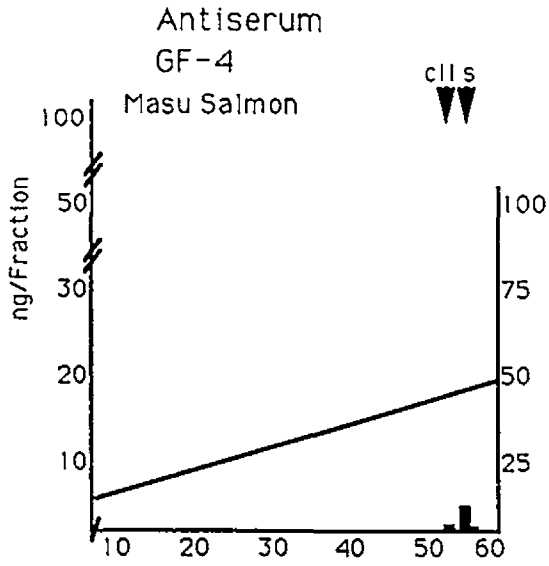
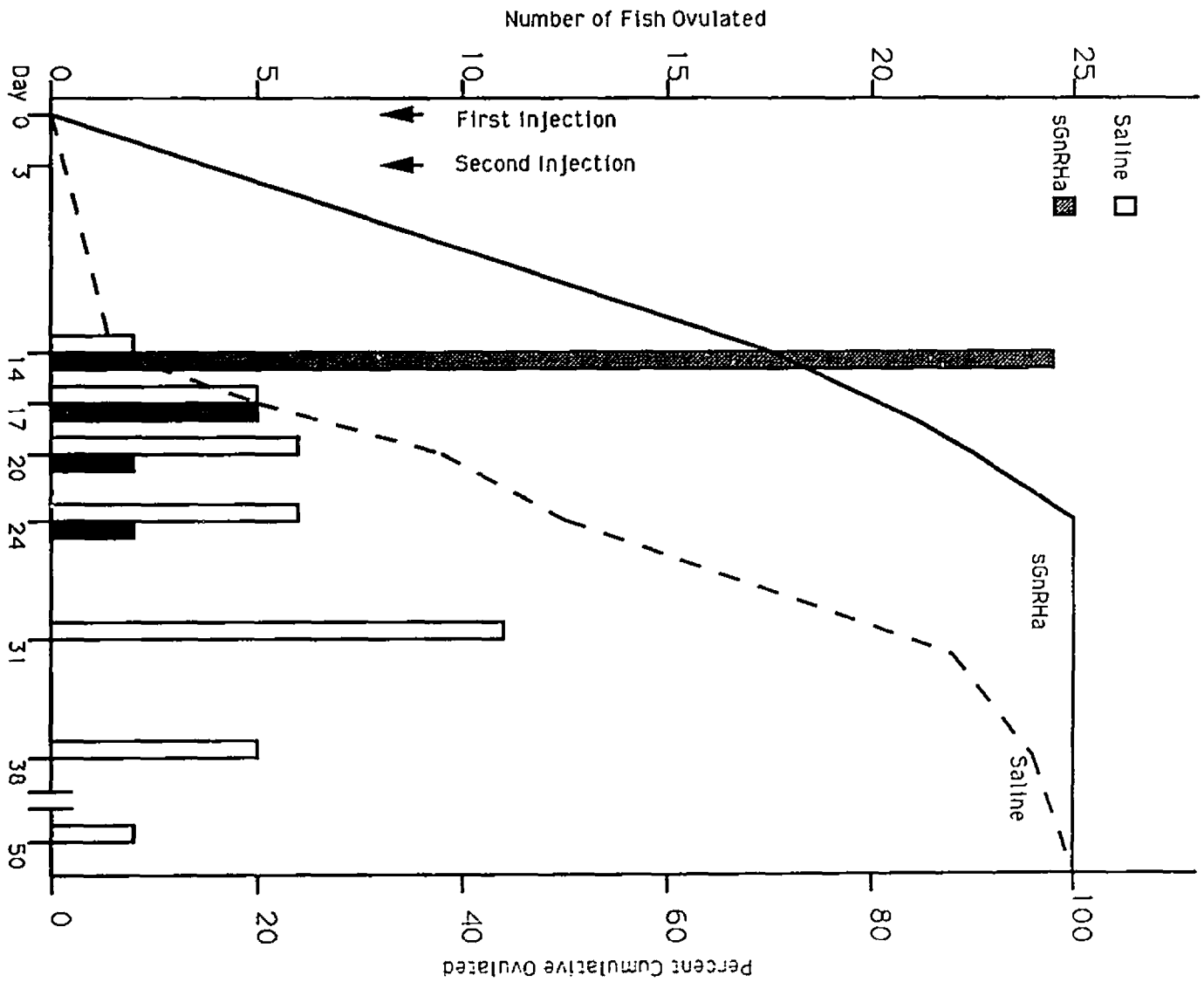


Figure 4.7. Number of fish ovulated and cumulative percentage of ovulation post-injection in female chinook salmon. Saline-injected (controls): open bars and dashed line; sGnRHa-injected: stippled bars and solid line.



### Offspring

Diameters of 300 randomly selected eggs taken after ovulation were measured to determine if vitellogenesis and egg maturation differed between treatments. No differences ( $P < 0.01$ ) in egg diameters were found between treatments. The progeny of the sGnRHa-treated fish were 16% larger (0.85 vs. 0.71g) than saline-treated fish four months after fertilization. However, the eggs of the treated group may have been fertilized in advance of the control group leading to advanced development.

### Plasma GnRH

Throughout the spawning season prior to ovulation, plasma GnRH was detected at various times in 5-42% of the mature and silver fish. Conversely, plasma GnRH was detected at the time of ovulation only in fish that had been injected with sGnRHa (Table 4.2). However, antiserum GF-4 does not detect salmon analogue (personal observation). Values were near the minimum detectable level of the assay (3pg/100 $\mu$ l). Therefore, plasma GnRH does not appear to be a reliable measure of the maturational state.

### Plasma GTH-I and GTH-II

Values for GTH-I at the time of the initial sorting of fish (7 days prior to transfer) differed for fish placed in groups for later saline injection (11.8ng) or sGnRHa injection (9.5ng). This may be due to the protocol in which

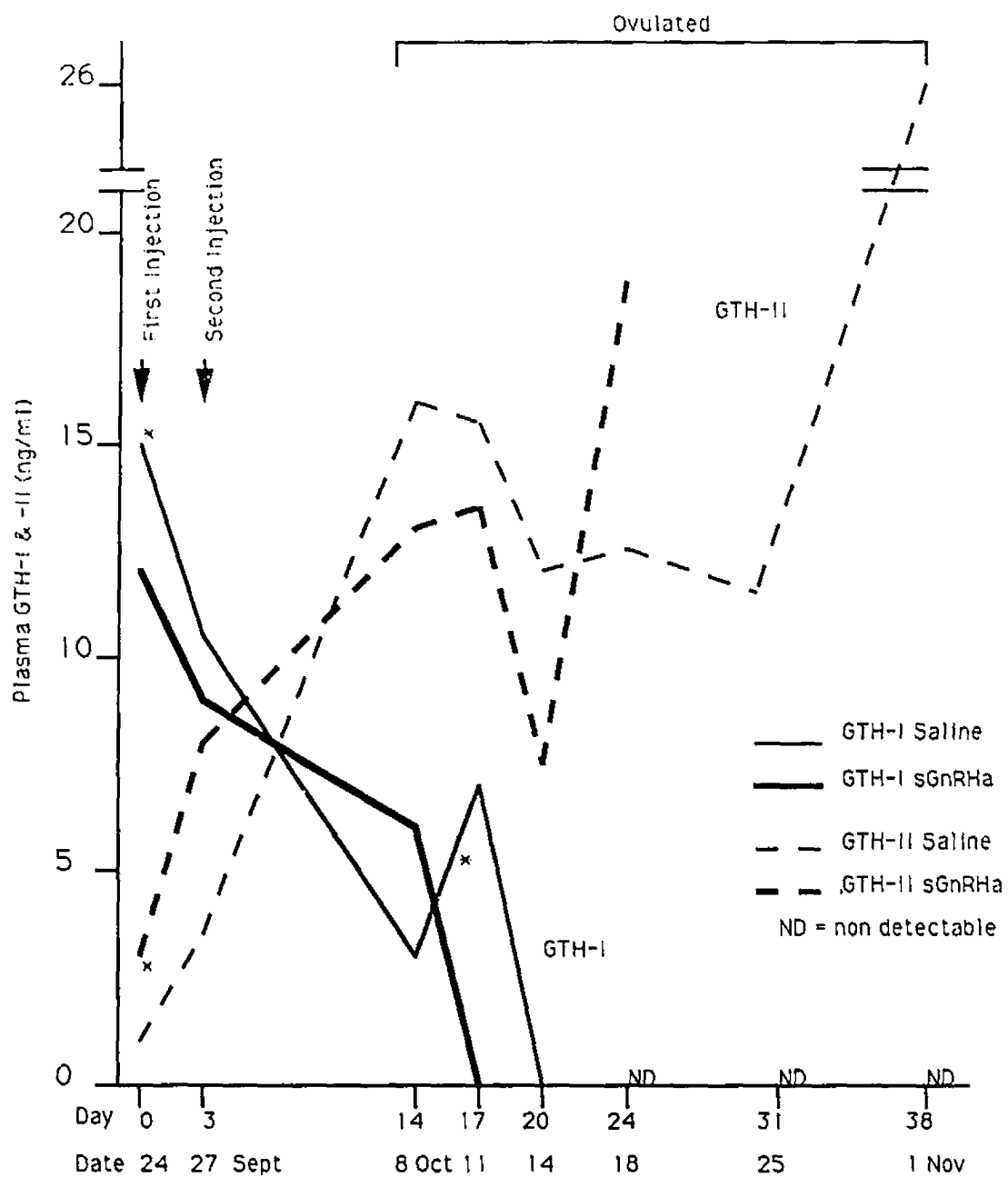
Table 4.2 Detectable levels of plasma GnRH in female chinook salmon using antiserum GF-4.

Treatment	Sample date	No. Fish	No. with detectable GnRH	Ave. GnRH detected pg/0.1ml
Pre-saline	24 Sept	40	16	7.0
Pre-sGnRHa	24 Sept	40	16	7.4
Control	27	40	2	3.1
sGnRHa	27	38	4	9.4
Control	8 Oct	25	5	5.4

sGnRHa-injected designates were tagged and sampled in the morning, whereas saline-injected designates were treated likewise in the afternoon. After transfer to freshwater, saline-injected fish were sampled for blood in the morning, whereas sGnRHa-injected fish were sampled in the afternoon. In this case, levels of plasma GTH-I and GTH-II differed between treatments at the time of the initial injections ( $P < 0.05$ , Fig 4.8). Thereafter, all sampling of blood was done in the morning. The same general pattern of change for GTH-I and GTH-II was seen in both control and experimental groups in that GTH-I decreases simultaneously as GTH-II increases. Only the timing differs between the groups. Plasma GTH-I levels in the saline-injected fish decreased from  $15 \pm 7.1$  to  $10.6 \pm 5.3$  ng/ml at 3 days after the initial injections. In the same period of time, plasma GTH-I decreased in the sGnRHa-injected fish from  $11.9 \pm 4.5$  to  $8.8 \pm 5.5$  ng/ml. By day 17, GTH-I levels decreased to nondetectable in sGnRHa-injected fish and by day 20 in saline-injected fish. Levels of GTH-I in sGnRHa-injected fish were lower (nondetectable) than control levels ( $P < 0.05$ ) on day 17 after the initial injection. GTH-I remained nondetectable in the plasma of ovulating fish thereafter.

Plasma GTH-II levels were different between the two treatments at the time of the first injections ( $P < 0.05$ , Fig. 4.8). However, 3 days later, plasma GTH-II levels could not be distinguished on the basis of treatment. Once fish had

Figure 4.8. Plasma GTH-I and GTH-II levels after the first and second injections of saline (control) or analogue (sGnRHa) in chinook salmon. Plasma GTH concentrations are shown for saline-injected fish as a narrow solid line (GTH-I) or a narrow dashed line (GTH-II). For sGnRHa-injected fish, a bold solid line (GTH-I) or bold dashed line (GTH-II) is used. Values reported from beyond day 3 are from ovulated salmon only. Significance ( $P > 0.05$ ) is denoted as an asterisk (\*).



ovulated on days 14 to 38, GTH-II values increased to an average of  $13.5 \pm 9.9$ ng/ml.

#### Observations in control fish

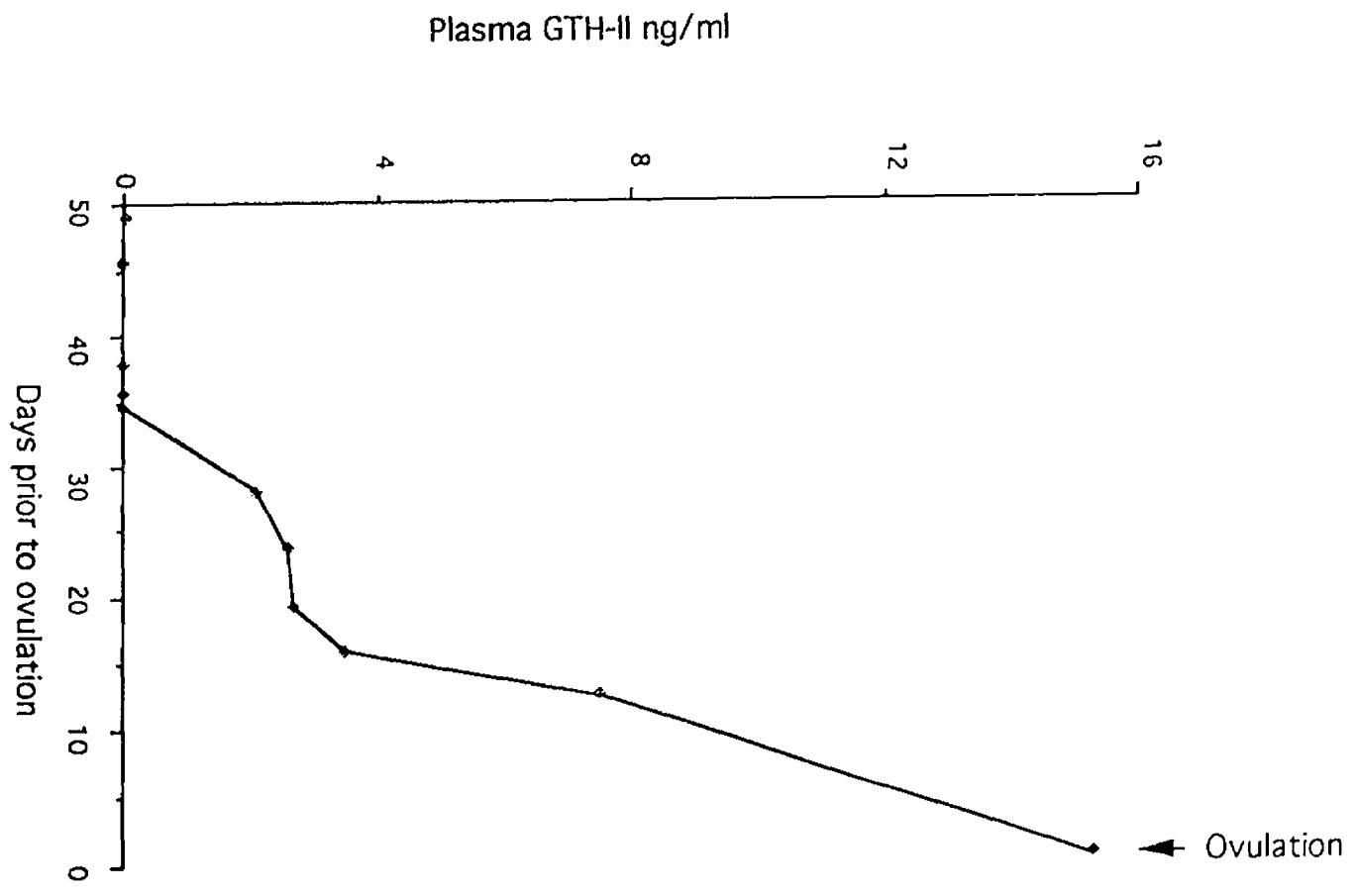
Saline-injected fish ovulated naturally without exogenous hormones. By using the date of ovulation as a reference point, the GTH-II values for fish showed a progressive increase over time, which culminates near ovulation (Fig. 4.9). Forty days before ovulation, plasma levels of GTH-II were nondetectable. By 10 days before ovulation, GTH-II levels were similar to those seen at the time of ovulation.

Non-maturing silver fish sampled at the initial sampling date (n=20) had GTH-I levels of  $2.4 \pm 0.03$ ng/ml and GTH-II levels of  $0.02 \pm 0.02$ ng/ml. GTH-I and GTH-II levels in silver fish were lower than those of maturing fish ( $P < 0.05$ ).

#### DISCUSSION

The present study identifies cGnRH-II by primary structure as the second form of GnRH in the salmonid brain. Prior to the identification by primary structure, the presence of cGnRH-II in salmonids had been indirectly demonstrated by immunohistochemistry, HPLC elution position and antisera cross-reactivity (Sherwood et al., 1984, Amano et al., 1990). The presence of cGnRH-II in the salmonids establishes a phylogenetic link with chondrichthyan fishes, the spiny dogfish and ratfish (Lovejoy et al., 1991, 1992a). As well, cGnRH-II has been shown to be present in other

Figure 4.9. Plasma GTH-II levels of saline-injected female chinook salmon prior to ovulation. The graph shows an average plasma GTH-II level for all females sampled at ovulation.



classes of vertebrates including amphibians, reptiles, birds and some mammals (see Sherwood et al., 1993b). Within the phylogeny of fishes, salmon are one of the most ancient teleosts to have cGnRH-II in their brains, a characteristic shared by more recently evolved fish such as those in the order Perciformes (Powell et al., 1994; White, et al., 1994).

The presence of sGnRH in the brains of chum salmon is reconfirmed by four purification schemes followed by amino acid sequencing. It appears that HFBA altered sGnRH so that it eluted into three different positions from the HPLC column. Each set of fractions, however, was shown to have an identical primary structure. The explanation is not clear, but HFBA should be avoided in HPLC studies of GnRH. The traditional TEAF method of HPLC resulted in the elution of only one peak of sGnRH that was confirmed by primary analysis (Sherwood et al., 1984). These studies strongly indicate that sGnRH and cGnRH-II are the only forms present in the brains of chum salmon and that HPLC elution position alone is a reliable criterion for the identification of unknown irGnRH forms only if the characteristics of the mobile phase are known.

In the three genera and eight species of salmonids examined for the forms of GnRH in their brains, only two forms, sGnRH and cGnRH-II were identified. Comparison of elution position for synthetic cGnRH-II and sGnRH with native GnRH from HPLC of brain extracts clearly indicated

the presence of these two forms. As well, antiserum GF-4 is more effective in detecting cGnRH-II than antiserum BLA-5, which supports the evidence that the first region of irGnRH is cGnRH-II. Antisera GF-4 and BLA-5 detect similar amounts of irGnRH in the later-eluting area of irGnRH, indicating that this form is sGnRH. These levels of detection for the two forms agree with data regarding the cross-reactivity of the antisera for synthetic cGnRH-II and sGnRH (Kelsall et al., 1990; Sherwood et al., 1991). Irrespective of reproductive status, mature and juvenile fish had two forms of GnRH present in their brains although the relative amounts of these two forms may vary.

The detection of only sGnRH and cGnRH-II precludes the presence of hGnRH in the salmon studied here. Among the later-evolving salmonids, (*Salvelinus*, *Salmo* and *Oncorhynchus*) hGnRH is not present in their brains. The extensive purification of irGnRH peaks from chum salmon brains likewise indicates that only two forms of GnRH are present in the salmonid brain. If hGnRH is present in salmonids, it may be present in species that are located closer to the euteleost stem line such as whitefish or grayling.

The measurement of GnRH in the blood of salmonids is not a reliable indication of maturational status. The levels of plasma GnRH are not detectable in the majority of fish. Rather, sGnRH acts on the pituitary to release gonadotropins, which can be reliably measured and

distinguished by using RIA (Swanson et al., 1991). As sGnRH is the sole form of GnRH in the pituitary of salmonids (Amano et al., 1992) and analogues are known to be more potent than native forms (Habibi et al., 1992), plasma GTH-I and II were measured in salmon induced to ovulate by using sGnRH $\alpha$ . The results indicate that GnRH acts upon the pituitary to elicit the release of GTH-II and induce ovulation. Further, in sGnRH $\alpha$ -injected fish, GTH-I levels declined and GTH-II levels increased in advance of control fish. The data clearly show that the levels of GTH-II associated with induced ovulation in chinook salmon are not unlike those seen in control fish.

In addition to increasing plasma GTH-II levels in sGnRH $\alpha$ -injected fish, GTH-I levels decreased prior to ovulation (Fig. 4.3). This is the first identification of decreasing GTH-I levels induced by exogenous GnRH application. The role of GTH-I in vitellogenesis is analogous to the role of mammalian FSH in that these hormones act on ova to prepare them for ovulation and subsequent fertilization. This period occurs before GTH-II or LH levels increase. Application of exogenous sGnRH analogue advanced the timing of a GTH-I decrease and GTH-II increase in concentration. Interestingly, Van Der Kraak and coworkers (1987) noted that exogenous application of GnRH peptides was ineffective six weeks prior to the anticipated ovulation date, but not four weeks prior.

In summary, this study shows that the brains of at least eight species of salmonids have two forms of GnRH: sGnRH and cGnRH-II. Both forms have been identified by primary structure or HPLC elution position and antisera cross-reactivity in eight species of salmonids. hGnRH appears to be unique to herring, but this cannot be confirmed until the forms of GnRH in more phylogenetically ancient salmonids are investigated. GnRH cannot be measured accurately in plasma, but clearly acts upon the pituitary to alter the ratio of GTH-I to GTH-II, which, in turn, appears to initiate ovulation as evidenced by the events following exogenous application of sGnRH<sub>a</sub>.

Chapter 5 Identification of three forms of GnRH, including the primary structure of a novel form, from the brains of sea bream Sparus aurata and tilapia Oreochromis niloticus.

A version of this chapter has been published and is reworked here:

Powell, J.F.F., Zohar, Y., Elizur, E., Park, M., Fischer, W., Rivier, J.E., Craig, A.G., Lovejoy, D.A. and Sherwood, N.M. 1994. Three forms of gonadotropin-releasing hormone characterized from the brains of one species. Proc. Natl. Acad. Sci. USA. 91: 12081-12085.

#### INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is both a releaser of gonadotropins and a neuromodulator (Jan et al., 1979; Millan et al., 1986), but the relationship between structure and function remains unclear. Most vertebrate species have more than one form of GnRH in the brain (see Sherwood et al., 1993a). The placental mammals appear to have a single form of GnRH, but even within this group there is an exception, the primitive musk shrew, *Suncus murinus* (Dellovade et al., 1993). At least two forms of GnRH within a species are characteristic of nonplacental mammals, birds, reptiles, amphibians and fish (see Sherwood et al., 1993a).

Distinct locations of the two forms of GnRH within the brain of a given species suggest that each form has a different function. In African catfish, *Clarias gariepinus*,

evidence from *in situ* hybridization shows that the cell bodies containing one form of GnRH (chicken GnRH-II, cGnRH-II) are in the midbrain, whereas cell bodies with the second type of GnRH (catfish GnRH, cfGnRH) are in the forebrain and infundibular stalk (Bogerd et al., 1994). Further, immunohistochemical or chromatographical studies support the conclusion that there is a discrete localization of cell bodies with different GnRH forms in several fish species (Yu et al., 1988, Okuzawa et al., 1990; Amano et al., 1992; Lepetre et al., 1993; Bogerd et al., 1994).

Teleost fish make a good model for determining if one or more of the GnRH peptides is the gonadotropin releaser. Known to lack a hypothalamo-pituitary portal system, teleosts have GnRH axons that grow into the pituitary and release GnRH near the gonadotrophs. Hence, the presence of a specific form of GnRH in the pituitary is a good indication of releaser function. In contrast, relative potency of the GnRH forms does not identify the gonadotropin releaser because all endogenous forms of GnRH release gonadotropin if tested in the species of origin (see Sharp et al., 1990; Habibi et al., 1992; Ngamvongchon et al., 1992b).

Localization studies to date show that the number of GnRH forms in the pituitary varies with the species. The single form of sGnRH in the salmon pituitary (Okuzawa et al., 1990) and both sGnRH and cGnRH-II in the goldfish pituitary (Yu et al., 1988) are potent releasers of pituitary gonadotropins

either *in vivo* or *in vitro* (Van der Kraak et al., 1987; Habibi et al., 1992). When two forms of GnRH are detected in the pituitary, it is not clear if the forms have different functions such as separate release of the FSH-like (GTH-I) or LH-like (GTH-II) hormones. An additional question is whether GnRH peptides in axons that terminate outside of the pituitary (Kah et al., 1986; Millan et al., 1986) have more than one neuromodulatory role.

A complication to the above story is that three forms of GnRH are present in a number of teleosts based on primary structure (herring GnRH; Chapter 3), HPLC and immunological studies. Examples of fish for which indirect evidence shows three GnRH peptides in the brain are the snook *Centropomus undecimalis* (Sherwood et al., 1993b) and sabalo *Prochilodus lineatus* (Somoza et al., 1994).

The hypothesis that the three forms of GnRH in one species have discrete locations and functions is testable, but only if the primary structure of each of the three forms is identified and synthesized. This chapter provides the first evidence that three distinct forms of GnRH are in each of the sea bream, *Sparus aurata*, and tilapia, *Oreochromis niloticus*, two species in the order Perciformes. The primary structure of one of these forms has not been previously described for any species. In addition, I examined whether only one form of GnRH is likely to be the gonadotropin releaser. In collaboration with Yoni Zohar, University of Maryland in Baltimore, the three native GnRH

peptides were tested for their effect on the release of gonadotropin in sexually mature sea bream.

In addition to releasing gonadotropins, GnRH has been shown to elicit the release of growth hormone from pituitary somatotrophs (Marchant et al., 1989). Originally, it was hypothesized that the GnRH-associated peptide (GAP) was an inhibitor of prolactin (PRL) release from mammalian pituitary lactotrophs (Seeburg et al., 1987). However, it was later observed that GnRH could also elicit PRL release (G. Weber, University of Hawaii, personal communication). With the identification of a short and long form of PRL, PRL<sub>177</sub> and PRL<sub>188</sub>, secreted from the pituitary of tilapia, *O. mossambicus*, it is important to determine whether one form is preferentially released by a specific GnRH peptide. A method to distinguish the two forms of PRL by electrophoresis and densitometry is used in this investigation. In collaboration with G. Weber and Dr. Gordon Grau at the University of Hawaii, the three GnRH peptides in the brains of tilapia are tested for their effect on the release of prolactins.

## **MATERIALS AND METHODS**

### **Collection of Brains**

Intact brains (10,000) and dissected hypothalami (2,500) weighing 1.8kg were collected from mature male and female gilthead sea bream, *Sparus aurata*, during the spawning season (January) at the Israel National Center for

Mariculture. The tissues were immediately frozen in liquid nitrogen, shipped on dry ice to the University of Victoria and stored at  $-80^{\circ}\text{C}$ . Brains were divided into four groups; lots A, B, and C were predominantly brains and some hypothalami, whereas lot D was predominantly hypothalami.

A sample of 100 brains (14g) from mature male and female tilapia, *Oreochromis mossambicus* was collected at the University of Hawaii, frozen in liquid nitrogen, shipped to the University of Victoria and stored at  $-80^{\circ}\text{C}$ . These brains were used to determine the number of forms present in tilapia and their HPLC elution positions. For the purification of GnRH from tilapia, the brains and pituitaries of 7,000 (1.25Kg) tilapia *O. niloticus*, of mixed sex were removed and frozen on dry ice. Tissues were shipped on dry ice from the National Aquaculture Genetics Research Insititute, Bangkok, Thailand to the University of Victoria and stored at  $-80^{\circ}\text{C}$  until extracted for peptides.

#### **Extraction of Peptides**

Extraction of peptides from all tissues and separation of the irGnRH peptides on HPLC using Sep-Pak columns are described in Chapter 2 with a few differences. Sea bream brains were treated individually for the four lots of brains. Brains of *O. niloticus*, used for purification of tilapia GnRH, were loaded onto two Sep-Pak HPLC columns with approximately 600g of brains per column. Sep-Pak HPLC was not done for *O. mossambicus*.

### Purification of GnRH peptides by HPLC from sea bream

Further HPLC steps for purification of sea bream irGnRH are detailed in Chapter 2 (Table 2.1). Early-eluting irGnRH fractions from all lots were pooled from HFBA-HPLC and designated sea bream GnRH-I (sbGnRH-I). Late-eluting irGnRH fractions for all lots from HFBA-HPLC runs were combined, reduced in volume and applied to isocratic TEAF-HPLC as detailed in Chapter 3. The early- and late-eluting irGnRH fractions from this step were designated sbGnRH-II and sbGnRH-III, respectively. Purification of sbGnRH-II & -III followed the methods described.

### Initial HPLC study of GnRH forms in tilapia, *O. mossambicus*

Aliquots of *O. mossambicus* brain extract (800 $\mu$ l) were applied to the C<sub>18</sub> column in 2 repeated injections and the HFBA method of HPLC was then followed as detailed in Chapter 2. Fractions of 1ml were collected and an aliquot of 100 $\mu$ l assayed for irGnRH. After the column was equilibrated again to 5% solution B, synthetic standards were applied to the column using the same method as for brain extract. A mixture of seven known synthetic GnRH peptides (catfish, dogfish, lamprey, chicken-I, chicken-II, salmon and mammalian) was injected at a concentration of 200ng each. Synthetic sea bream GnRH was not available to apply to the column. Fractions (1ml) were collected for 60 min and the elution positions of the standards were determined by assay of 10 $\mu$ l aliquots for irGnRH.

#### HPLC Purification of GnRH forms from tilapia, O.niloticus

Purification of tilapia (T) GnRH followed the procedures detailed in Chapter 3 for purification of herring GnRH with one exception. Four areas of irGnRH called T-I, T-II, T-III and T-IV were identified in distinct areas of the isocratic TEAF method and were treated separately thereafter for purification of irGnRH peptides.

After the TEAP step of tilapia GnRH purification, all four areas of irGnRH identified (T-I, T-II, T-III and T-IV) were applied to the phenyl column and eluted using TFA in the mobile phase. The first two areas of irGnRH identified in this step (T-I and T-II) were individually further purified. Fractions containing irGnRH were reduced in volume and applied to the phenyl column with a mixture of 10% solution B (80% acetonitrile and 0.5% TFA in water) and 90% solution A (0.05% TFA in water) at a flow rate of 1ml/min. After 2min, a gradient of 1%/min solution B was applied for 7min and remained isocratic for an additional 51min. Fractions (1ml) were collected for 60 min and assayed for irGnRH.

#### Radioimmunoassay (RIA)

For the purification of GnRH in sea bream and tilapia *O. niloticus* extracts, 10 $\mu$ l aliquots were used in the RIA, whereas 100 $\mu$ l aliquots were dried before determination of irGnRH for tilapia *O. mossambicus* samples. The method for detection of irGnRH in fractions was as described in Chapter

2. The limit of detection ( $B/B_0=80\%$ ) for the assay was 10pg for GF-4. Where tracer binding exceeded the detection limit ( $B/B_0=20\%$ ), 10 $\mu$ l aliquots were diluted serially and the value closest to  $B/B_0=50\%$  reported.

Antiserum GF-4 was used in a final dilution of 1:25,000 resulting in 26-37% binding of  $^{125}\text{I}$ -mGnRH. The limit of detection ( $B/B_0=80\%$ ) averaged 7.2pg. Antiserum BLA-5 was used in a final dilution of 1:10,000 resulting in 9-17% binding of  $^{125}\text{I}$ -mGnRH. The limit of detection for BLA-5 was 33pg. Antiserum R-42, which does not recognize forms of GnRH that are altered at the N- and C-termini (Copeland et al., 1987) was used after the last step in the purification of both sea bream and tilapia to verify that the peptide was intact. A dilution of 1:100,000 of R-42 had a binding of 31% and a detection limit of 2.8pg. Serial dilutions were done if fractions of 10 $\mu$ l had values of irGnRH that exceeded  $B/B_0=20\%$ .

#### **Primary structure determination**

An aliquot (10% by volume) of the sea bream and tilapia GnRH peptides purified by HPLC on a phenyl column was further purified and subjected to amino acid sequencing and mass determination as described in Chapter 2.

#### **Peptide Synthesis**

Solid phase synthesis of sbGnRH was carried out by Dr. J.E. Rivier on a methylbenzhydrylamine resin (Boc strategy)

using previously established methods (Rivier et al., 1992) and the following protecting groups: pryo-Glu(carbobenzoxy), Boc-His(tosyl), Boc-Ser(Benzyl) and Boc-Tyr(2-bromo-carbobenzoxy). sbGnRH was deprotected and cleaved from the solid support with hydrofluoric acid. After purification with reverse phase HPLC (>97% pure) in two solvent systems (Rivier et al., 1984), the structure was confirmed by mass spectral and amino acid composition analyses.

#### HPLC analysis of pituitary extracts

Sea bream pituitaries (n=100, 0.75g total) were collected during the spawning season from two-year-old females with preovulatory oocytes (500-600 $\mu$ m o.d.) by Dr. Y. Zohar and colleagues. The frozen tissue was shipped to the University of Victoria. The pituitaries were extracted for peptides as described above. The extract was applied to a separate C<sub>18</sub> HPLC column and eluted using the isocratic TEAF-HPLC method. Reference peptides of sbGnRH, cGnRH-II and sGnRH (200ng each) were applied to the HPLC column and eluted with the same method used for the pituitary extract. Fractions (1ml) were collected separately for each HPLC application and assayed for irGnRH. The blank fractions (500 $\mu$ l) did not contain detectable irGnRH.

Tilapia pituitaries (n=100) were collected from mature *O. mossambicus* by G. Weber and colleagues in Hawaii, frozen in liquid nitrogen and shipped to the University of Victoria.

Tissues were treated for the extraction of peptides as described and reduced in volume. The extract was applied to a C<sub>18</sub> column and the isocratic TEAF method employed. Fractions (1ml) were collected and assayed for irGnRH using antiserum GF-4. After completion of the HPLC method, the column was equilibrated at the starting conditions and reference peptides of sbGnRH, cGnRH-II and sGnRH (200ng each) were applied to the HPLC column and eluted with the same method used for the pituitary extract.

**Physiological studies: Release of gonadotropin from sea bream**

The *in vivo* study of GTH-II release by native GnRH peptides was done by A. Elizur, National Center for Mariculture, Eilat, Israel. Four groups of eight female sea bream were injected with 5µg/kg or 25µg/kg body weight of sbGnRH, cGnRH-II, sGnRH or saline. Solutions were injected into the dorsal musculature. Blood samples were taken at 1.5, 5 and 8h post injection. RIA for sea bream GTH-II was done by the method described by Zohar and coworkers (1990b).

**Physiological studies: Release of PRL from tilapia**

The investigation of PRL release *in vitro* and *in vivo* from pituitaries and fish was done by G. Weber, University of Hawaii. Briefly, rostral pars distalis were removed from the pituitaries of tilapia (*O. mossambicus*) and placed in 96 well culture plates. Cells were incubated for 24h with

factorial dilutions of 1000 to 0.01nM of each native GnRH peptide and a control. PRL<sub>177</sub> and PRL<sub>188</sub> concentrations of the medium were quantified using electrophoresis and densitometry. For *in vivo* investigations, 0.01µg/g body weight of D-Ala<sup>6</sup>-mGnRH analogue was injected into 12 mature tilapia and saline-injected controls. After 11h, blood samples were removed and the serum assayed for PRL<sub>177</sub> and PRL<sub>188</sub> by the above mentioned assay.

## RESULTS

### Initial HPLC analysis of sea bream brains

Assay of HPLC fractions from the 4 lots of sea bream brain extract that eluted from the Sep-Pak columns showed irGnRH activity in fractions 21-41 (Fig. 5.1A). Fractions that contained less than 15ng/fraction of irGnRH activity were not used in subsequent steps.

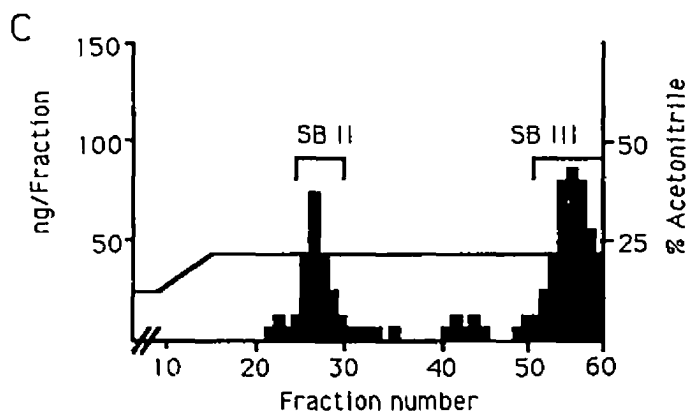
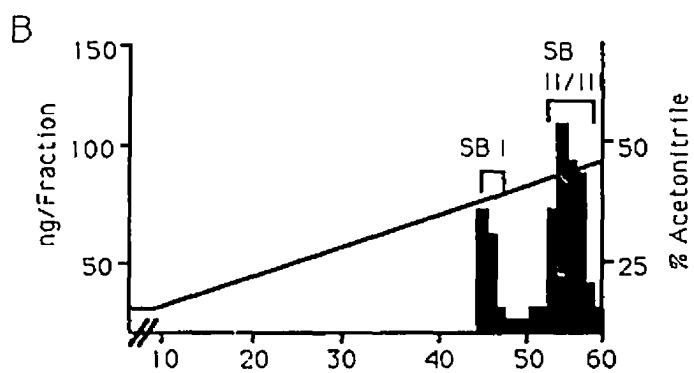
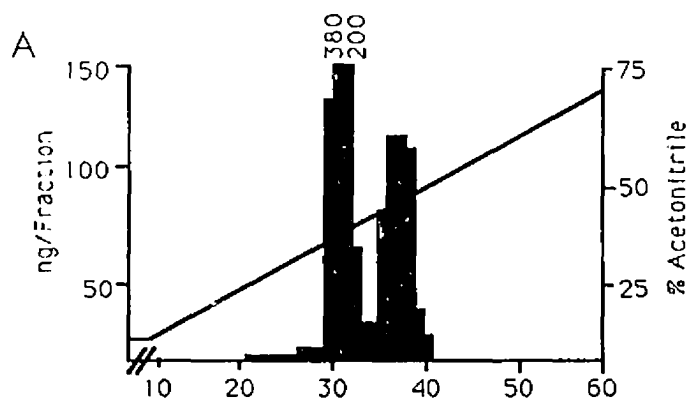
### Purification of sbGnRH-I

Each HPLC run in the HFBA method of HPLC showed an early-eluting peak at 46min (Fig. 5.1B). These irGnRH fractions (called sbGnRH-I) from the 4 lots were pooled and shown to contain a total of 153ng of irGnRH. A large proportion (110ng) of the combined irGnRH was derived from lot D containing predominantly hypothalamic tissue.

In the next step of the purification, 297ng of irGnRH was detected in fractions 33 and 34 with less than 10ng of activity in the next 4 fractions. Fractions 33 and 34 were

Figure 5.1. Purification of GnRH from sea bream brains.

A. irGnRH was eluted from Sep-Pak HPLC using TFA in the mobile phase from lot B brains. B. irGnRH from lot B brains was eluted from a C<sub>18</sub> column using HFBA in the mobile phase. C. Purification of SB-II and SB-III using a C<sub>18</sub> column with the isocratic TEAF-HPLC method. Percent of acetonitrile is shown by a solid line.



combined for TEAP-HPLC. Three fractions (28-30) were found to have a total of 220ng of irGnRH and were further purified. At the last step of the purification (TFA, phenyl column), fractions 23 and 24 contained 232ng of irGnRH (data not shown). Fraction 23 (179ng irGnRH) was selected for digestion with pyroglutamyl aminopeptidase and protein sequencing. RIA using antiserum R-42 confirmed the presence of intact irGnRH in these fractions, but detected only 82ng in fraction 23 and 120ng in combined fractions 23 and 24.

Edman degradation of the pyroglutamate aminopeptidase-treated fraction of sbGnRH-I yielded the sequence:

His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly.

Antisera GF-4 and BLA-5 were found to cross-react with synthetic sbGnRH-I 120% and 110% as compared to mGnRH in RIA. The molecular ion mass of the non-treated sbGnRH-I fraction was  $m/z$  1113.6 as determined by liquid secondary ionization. The calculated monoisotopic mass ( $MH^+$ ) for pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH<sub>2</sub> (elemental composition C<sub>52</sub>H<sub>69</sub>N<sub>14</sub>O<sub>14</sub>) is 1113.5 Da.

#### **Purification of sbGnRH-II and -III**

Late-eluting fractions (>50 min) from the HFBA-HPLC (Fig. 5.1B) appeared to contain two forms of irGnRH as indicated by our preliminary studies in which extracts were compared to synthetic GnRH standards. Likewise, in the present study, irGnRH eluted as two areas from isocratic TEAF-HPLC (Fig. 5.1C). The earlier-eluting peak was designated

sbGnRH-II and was applied to the remaining steps of purification. The later-eluting peak was designated sbGnRH-III and was separately applied to the remaining steps of purification.

#### **Identification of sbGnRH-II**

Protein sequencing and mass spectrometry showed that sbGnRH-II is identical in sequence and mass with cGnRH-II. The amino acid sequence of the pyroglutamate amino peptidase treated sbGnRH-II was determined to be:

His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly.

The molecule ion mass of this peptide was  $m/z$  1236.6 (MALDI). The calculated monoisotopic mass ( $MH^+$ ) for pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH<sub>2</sub> (elemental composition C<sub>60</sub>H<sub>70</sub>N<sub>17</sub>O<sub>13</sub>) is 1236.53 Da. These data confirm that sbGnRH-II is identical in structure to the previously described cGnRH-II (Miyamoto et al., 1984).

#### **Identification of sbGnRH-III**

sbGnRH-III shared an elution position with sGnRH identified for other perciform fish (Sherwood et al., 1993b). The late elution position of sbGnRH-III (Fig. 5.1C) is consistent with sGnRH in other species (Sherwood et al., 1984; Sherwood, 1986; Sherwood et al., 1993b). Likewise, sbGnRH-III maintained a late elution position throughout purification, but was not present in sufficient quantity to sequence. During the identification of GnRH forms in the

pituitary, one form of irGnRH eluted in the same position with the sGnRH standard (see below). In addition, the cDNA for sGnRH from sea bream has been described (Dr. Y. Zohar, personal communication).

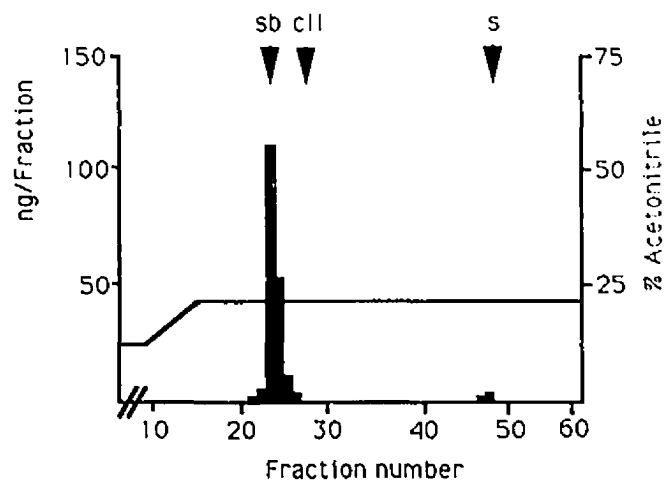
#### **Identification of pituitary GnRH from sea bream**

The most abundant form of GnRH in the sea bream pituitary eluted at the same time as the sbGnRH standard (Fig. 5.2). A total of 166ng irGnRH (antiserum GF-4) was detected in fractions 23-24. Only 0.33ng irGnRH was detected in fractions 46-47 corresponding to the elution position of sGnRH. irGnRH eluting with the cGnRH-II standard was not detected, although, antiserum cross-reactivity of GF-4 with cGnRH-II is only 4%.

#### **Initial HPLC analysis of brains from tilapia, *O. mossambicus***

Three areas of irGnRH were identified in the initial HPLC investigation of forms of irGnRH present in the brains of tilapia *O. mossambicus* (Fig. 5.3). These three areas coincided with the elution position of the synthetic standards chicken GnRH-I (21.6ng), chicken GnRH-II (3.2ng) and salmon GnRH (4.8ng). However, we demonstrated later that chicken GnRH-I and sea bream GnRH have coincident elution using this HPLC method.

Figure 5.2. HPLC analysis of GnRH from sea bream pituitaries. irGnRH was eluted from a C<sub>18</sub> column using an isocratic method with TEAF and acetonitrile as mobile phase. The elution positions of synthetic standards are indicated by arrows (sb=sbGnRH, cII=cGnRH-II, s=sGnRH). Percent acetonitrile is shown by a solid line.



### Purification of GnRH from tilapia, *O. niloticus*

RIA after Sep-Pak HPLC detected a total of 8.9 $\mu$ g irGnRH (Fig. 5.4). The isocratic TEAF-HPLC resulted in four areas of irGnRH from the tilapia (T) that were called T-I, T-II, T-III and T-IV corresponding to their elution time (Fig. 5.5). T-I had 3.5 $\mu$ g of irGnRH detected in fractions 22 and 23. T-II had 1.8 $\mu$ g irGnRH detected in fractions 25-27. Likewise, T-III had 0.35 $\mu$ g in fractions 38-40 and T-IV had 2.1 $\mu$ g irGnRH detected in fractions 46-49. Application of the individual areas to TEAP-HPLC (Fig. 5.6) resulted in irGnRH as follows: T-I 3.6 $\mu$ g (fractions 28-30), T-II 1.9 $\mu$ g (fractions 33 and 34), T-III 0.24 $\mu$ g (fractions 38-40) and T-IV 1.3 $\mu$ g (fractions 42-44). Application of the areas to the phenyl column (Fig. 5.7) resulted in the detection of irGnRH for T-I 3.4 $\mu$ g (fractions 22-24), T-II 0.72 $\mu$ g (fractions 22-24), T-III 0.1 $\mu$ g (fractions 29-34) and T-IV 0.95 $\mu$ g (fractions 30-33). Isocratic HPLC using the phenyl column for further purification resulted in irGnRH in fractions 17-21 for both T-I (1.5 $\mu$ g) and T-II (1.1 $\mu$ g; Fig. 5.8). T-I, T-II, T-III and T-IV fractions were treated as four samples for further application to the narrow bore HPLC, digestion with pyroglutamyl aminopeptidase and amino acid sequencing.

### Identification of pituitary GnRH from tilapia, *O.*

#### *mossambicus*

Three forms of irGnRH were detected in HPLC eluates of brain extracts from tilapia *O. mossambicus*. The three areas

Figure 5.3. HPLC analysis of GnRH from tilapia, *O. mossambicus* brains. irGnRH was eluted from a C<sub>18</sub> column using an isocratic method with TEAF and acetonitrile as mobile phase. Antiserum GF-4 was used to detect GnRH peptides. The elution positions of synthetic standards are indicated by arrows (sb=sbGnRH, cII=cGnRH-II, s=sGnRH). Percent acetonitrile is shown by a solid line.

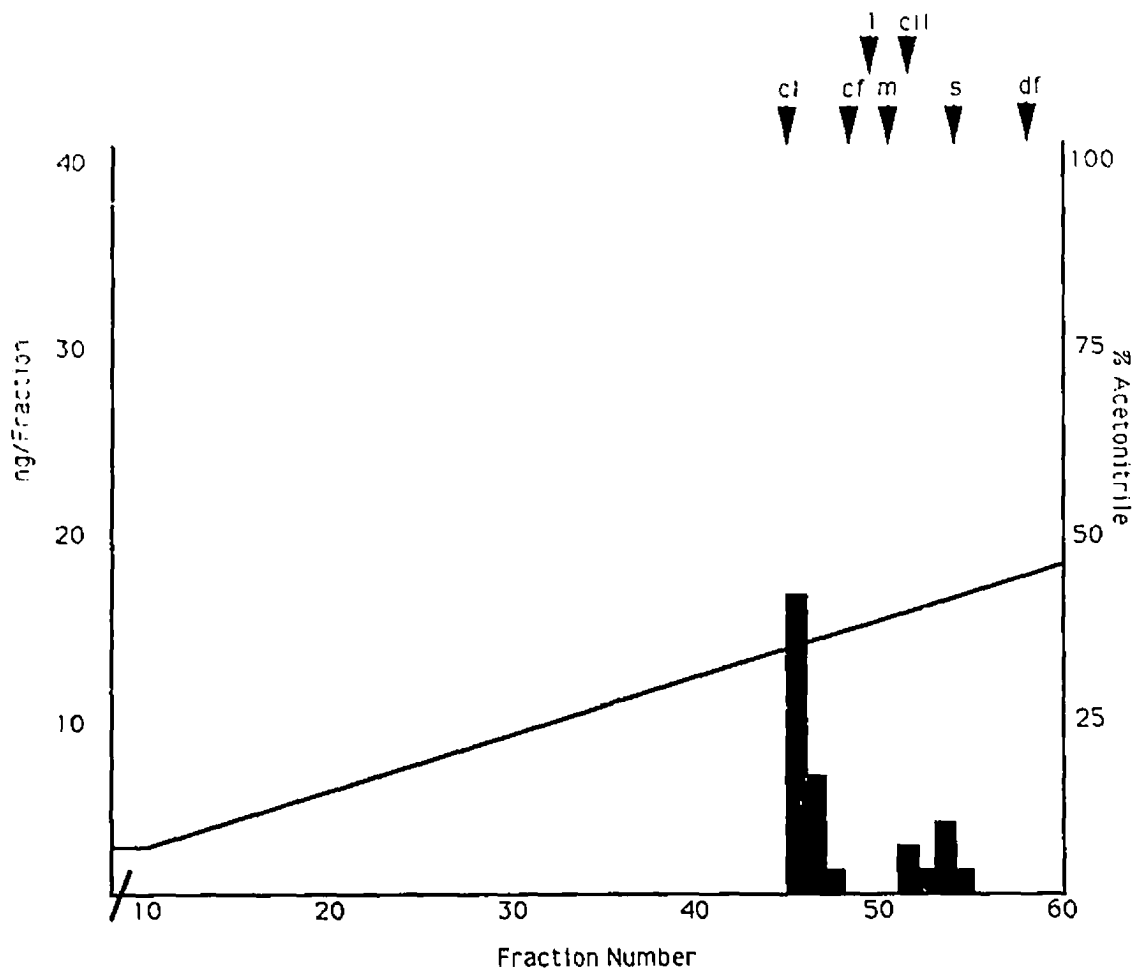


Figure 5.4. HPLC analysis of GnRH from tilapia, *O. niloticus* brains using Sep-Pak columns. irGnRH was eluted from two (A and B) Sep-Pak columns using 0.05% TFA in the solvents. irGnRH in fractions from both Sep-Pak HPLC runs was combined and applied to the next step of purification. Numbers above fractions indicate the amount of irGnRH detected by antiserum GF-4. Solid lines indicate percent acetonitrile.

Sep-Pak  
HPLC

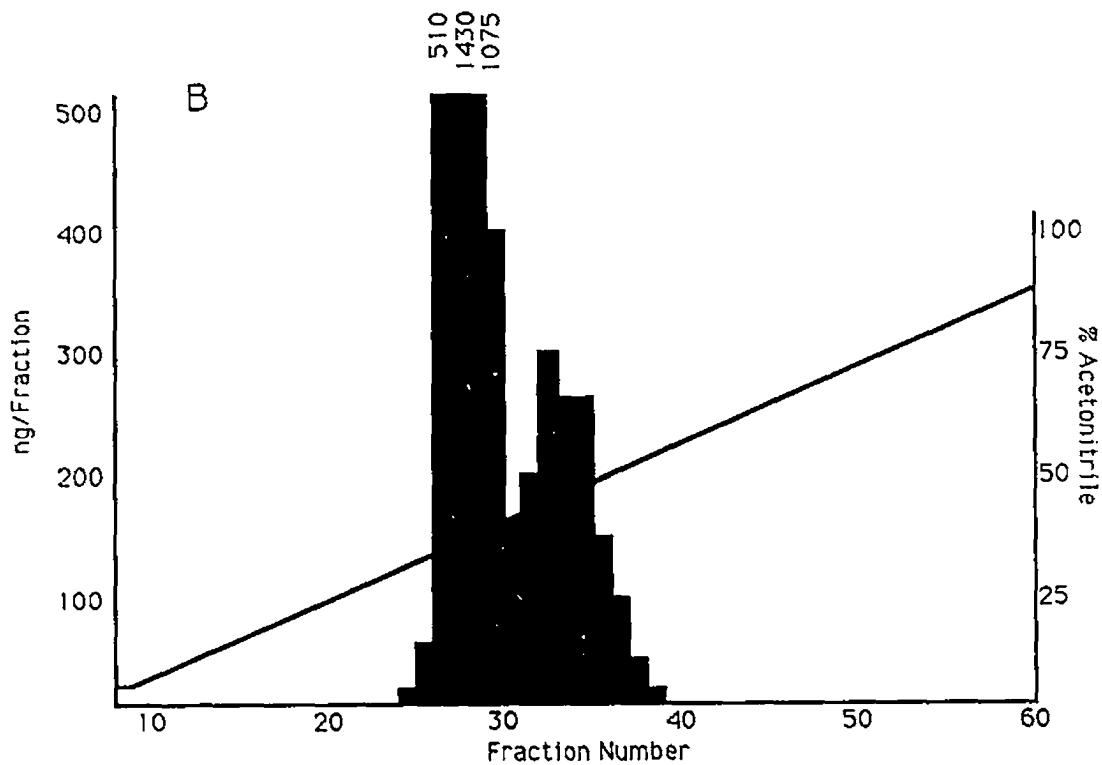
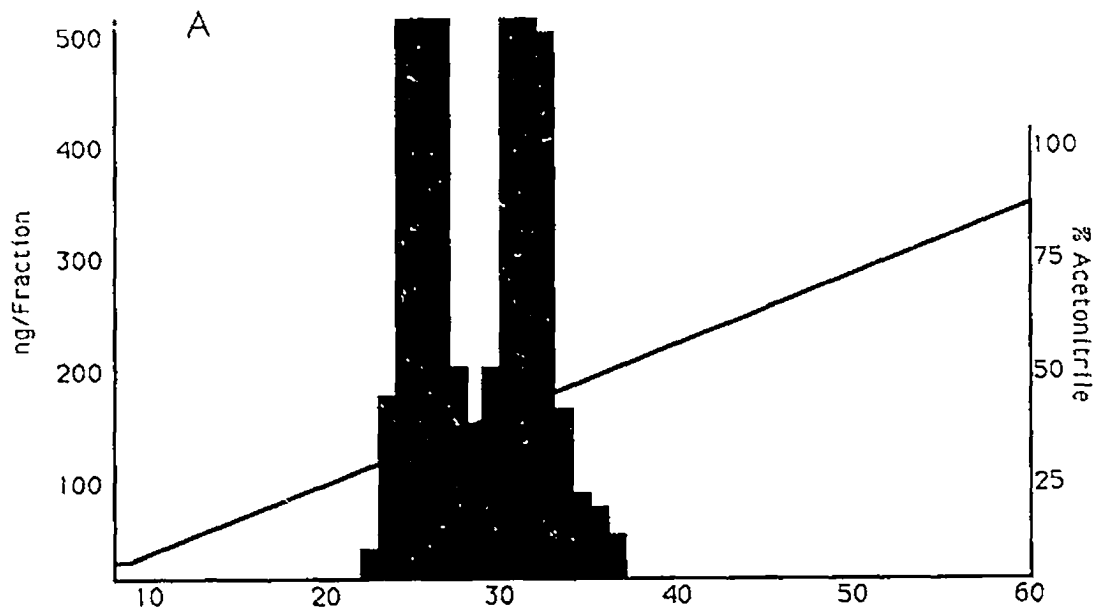


Figure 5.5. Purification of GnRH from tilapia *O. niloticus* brains using the isocratic TEAF method. irGnRH detected by antiserum GF-4 was given the designation T-I, T-II, T-III and T-IV based on elution time and peak areas of irGnRH. Individual areas of irGnRH in fractions from this step were combined and applied to the next step of purification. Numbers above fractions indicate the amount of irGnRH detected by antiserum GF-4. Solid line indicates percent acetonitrile.

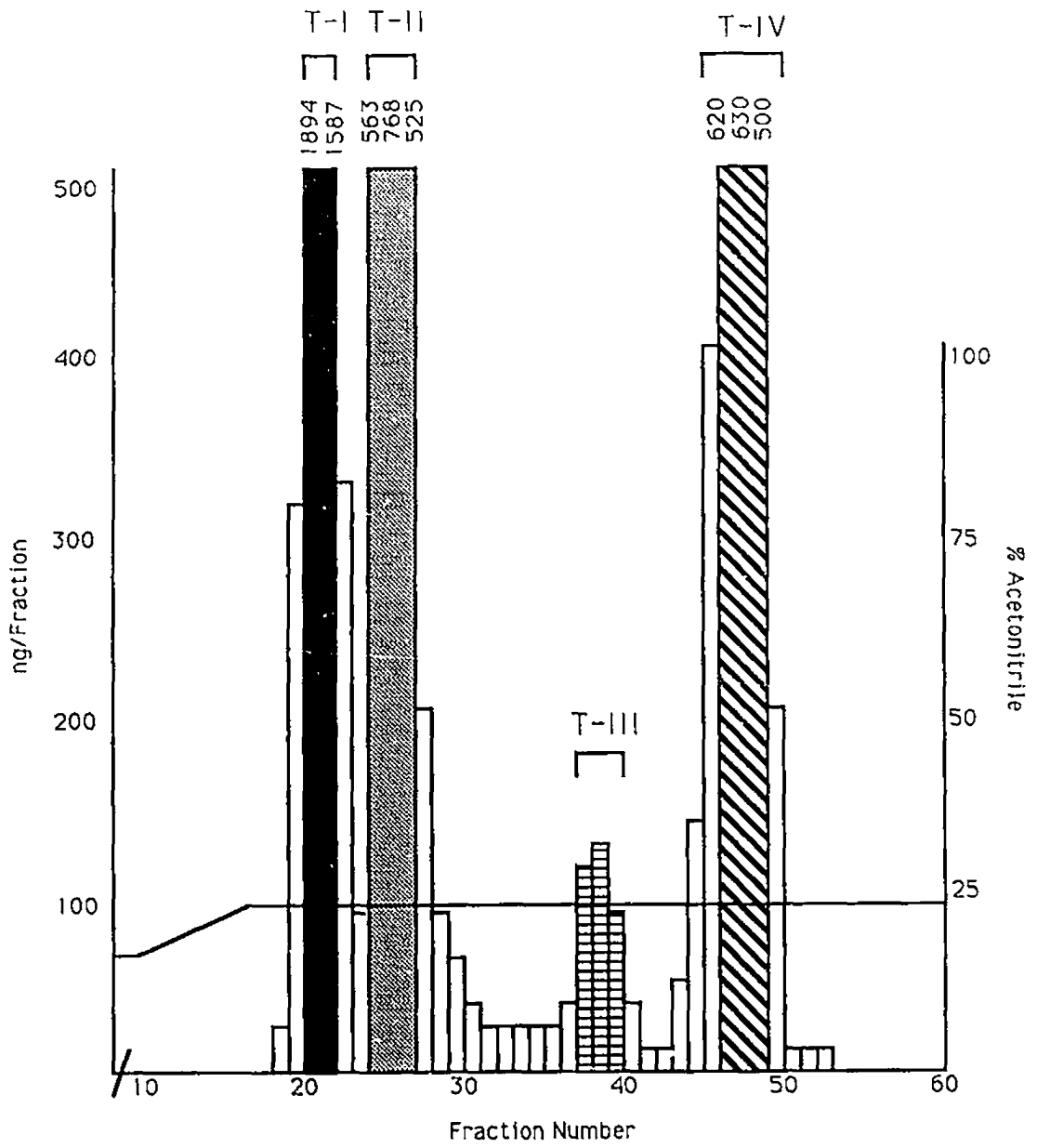


Figure 5.6. Purification of GnRH from tilapia *O. niloticus* brains using the TEAP method. Data of irGnRH detected by antiserum GF-4 in four successive TEAP-HPLC runs was used to form a composite figure. The designations T-I, T-II, T-III and T-IV based on elution time and peak areas of irGnRH in the isocratic TEAF-HPLC remain in the same order for the gradient TEAP method. Individual areas of irGnRH in fractions from this step were combined and applied to the next step of purification. Numbers above fractions indicate the amount of irGnRH detected by antiserum GF-4. A solid line indicates percent acetonitrile.

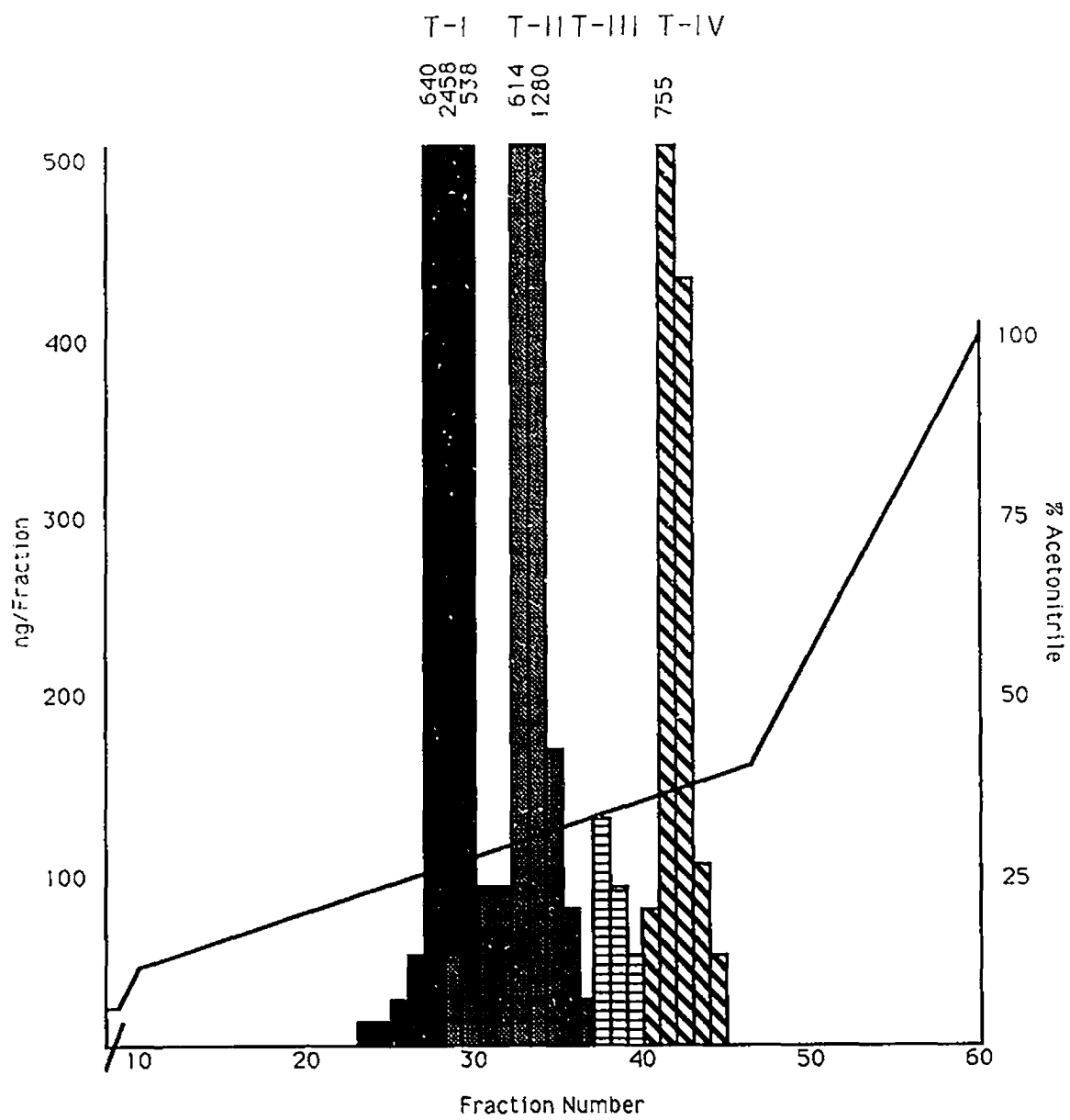


Figure 5.7. Purification of GnRH from tilapia *O. niloticus* brains using the phenyl column TFA method. Data of irGnRH detected by antiserum GF-4 in four successive phenyl column TFA HPLC runs. Combined T-I or T-II fractions from this step were separately applied to the next step of purification. Combined T-III or T-IV fractions were separately applied to narrow-bore HPLC, digested with pyroglutamyl aminopeptidase and sequenced for amino acids. Numbers above fractions indicate the amount of irGnRH detected by antiserum GF-4. Solid lines indicate percent acetonitrile.

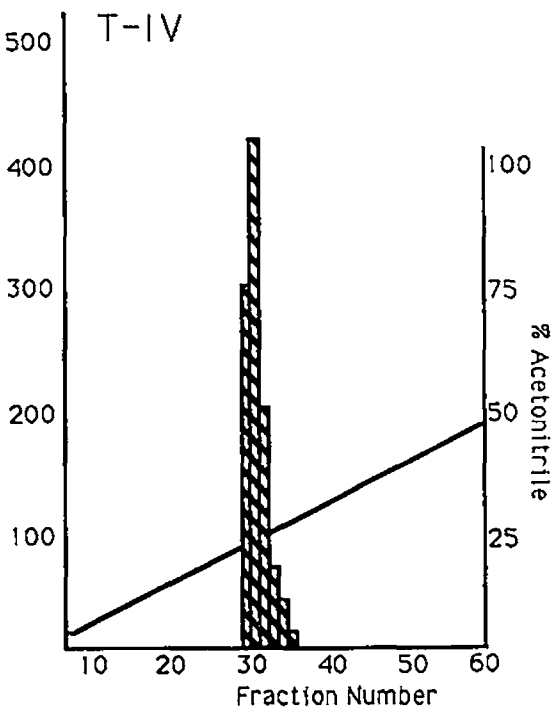
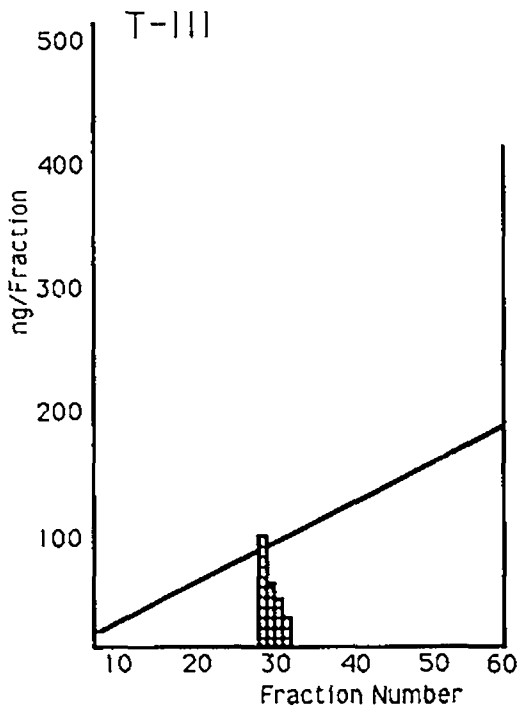
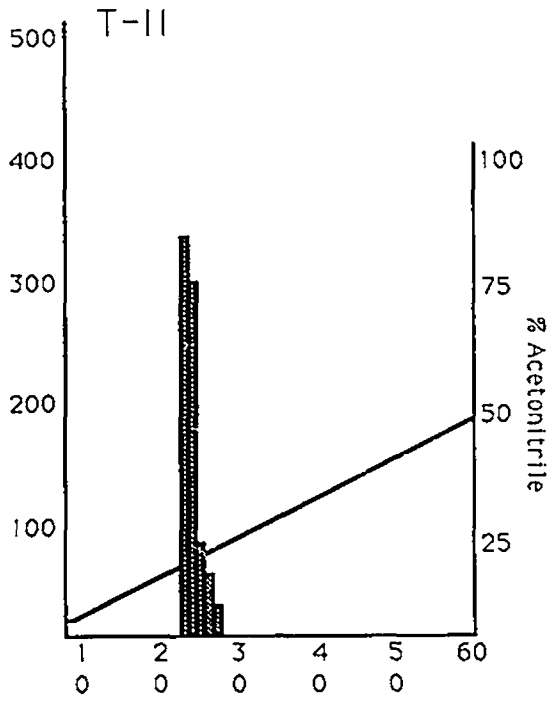
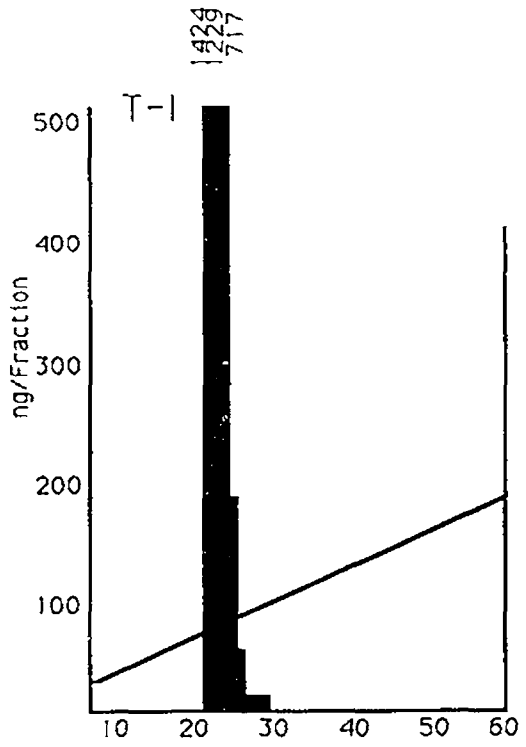
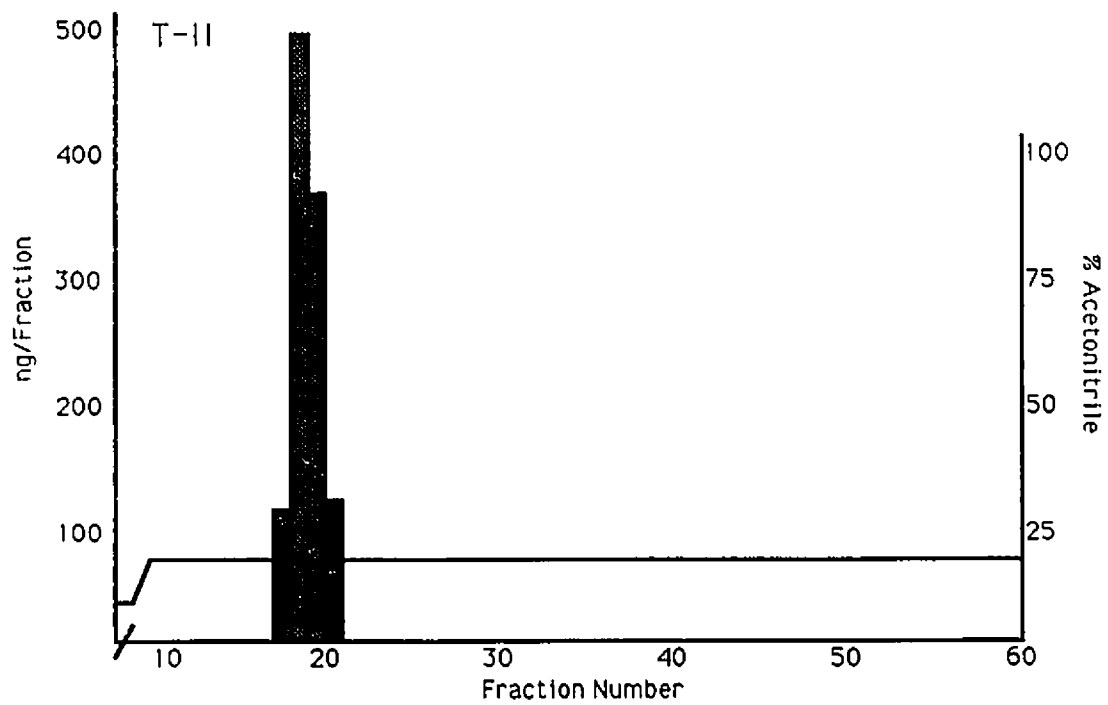
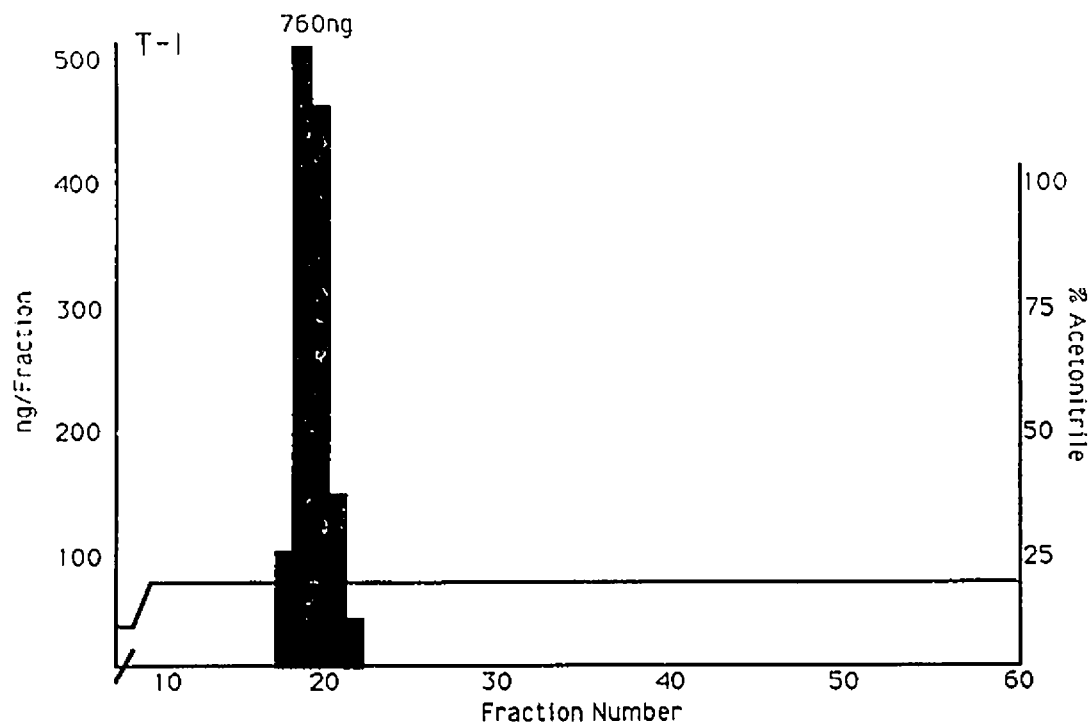


Figure 5.8. Purification of GnRH from tilapia *O. niloticus* brains using the phenyl column isocratic TFA method. Data of irGnRH for T-I (top) and T-II (bottom) were detected by antiserum GF-4. The number above fraction 19 of T-I HPLC elution indicates the amount of irGnRH detected. Solid lines indicate percent acetonitrile.



of irGnRH eluted in the positions that corresponded to synthetic sbGnRH, cGnRH-II and sGnRH. sbGnRH was approximately 700 fold more abundant than either sGnRH or cGnRH-II as detected by antiserum GF-4 (Fig. 5.9).

#### **Characterization of the primary structure**

Partially purified tilapia GnRH areas (T-I, T-II, T-III and T-IV) were individually further purified and subjected to Edman degradation as described in Chapter 2. Sequence data from the irGnRH areas were as follows:

T-I pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly,

T-II pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly,

T-IV pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly.

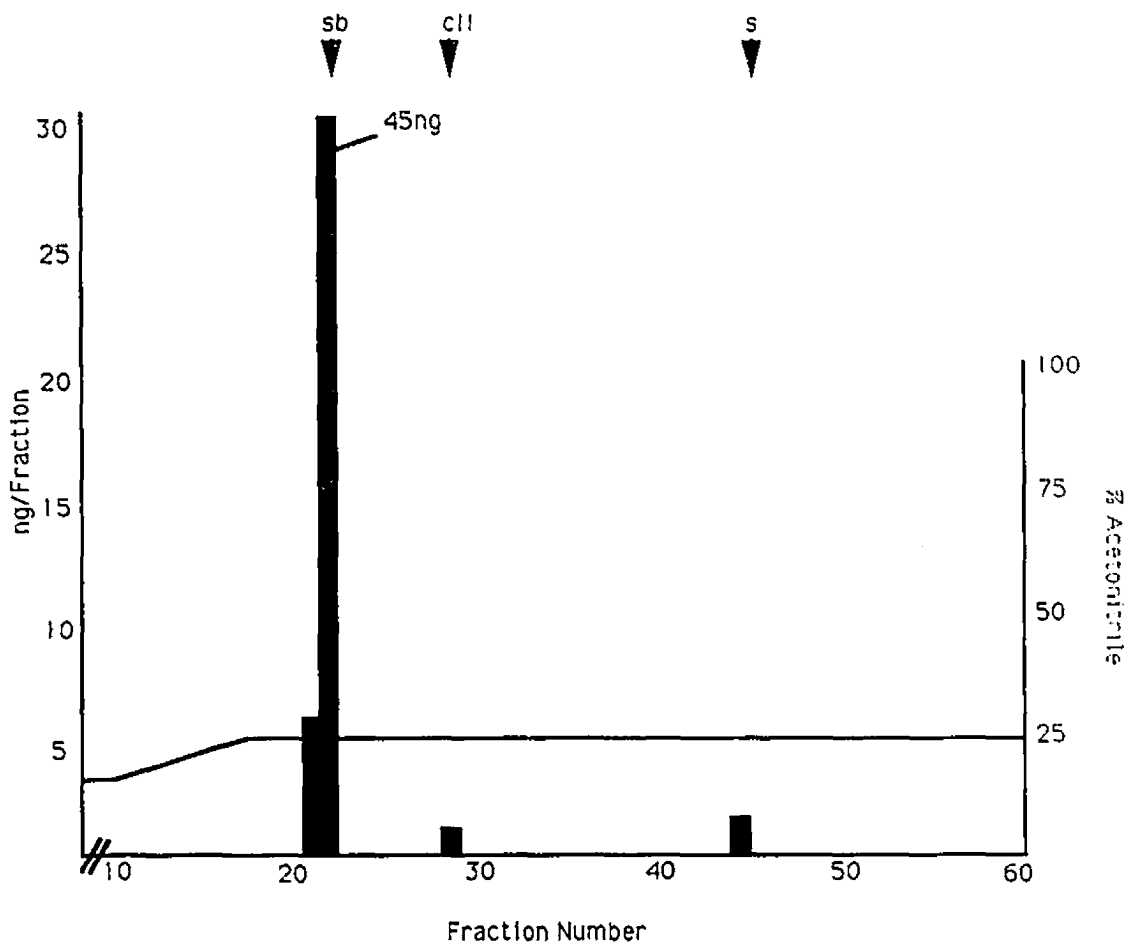
These sequences correspond to the known GnRH peptides sbGnRH, cGnRH-II and sGnRH for T-I, T-II and T-IV, respectively. T-III, although low in amount, was shown to be sGnRH.

#### **Release of GTH-II by GnRH in sea bream**

All three native GnRH peptides were found to release GTH-II in female seabream. Plasma GTH-II levels were observed to be elevated at all sampling times post-injection of the peptides. The potency of GTH-II release was found to be cGnRH-II > sGnRH > sbGnRH.

Figure 5.9. HPLC analysis of GnRH from tilapia *O. mossambicus* pituitaries. irGnRH was eluted from a C<sub>18</sub> column using the isocratic TEAF method and acetonitrile as mobile phase. Percent acetonitrile is shown by a solid line. The elution positions of synthetic standards are indicate by arrows (sb=sbGnRH, cII=cGnRH-II, s=sGnRH).

## Antiserum GF-4



### Release of prolactin by GnRH in *O. mossambicus*

Both forms of PRL were found to be released from pituitary cells after incubation with three native GnRH peptides. The potency of the GnRH native peptides on PRL release was cGnRH-II > sGnRH > sbGnRH. mGnRH analogue was also found to increase serum levels of PRL *in vivo* within 1h after injection, but the PRL levels returned to basal levels by 11h after injection.

### DISCUSSION

Sea bream brains contain three distinct forms of GnRH, one of which has a novel structure, whereas the other two are known forms, cGnRH-II and sGnRH. Two forms are identified here by primary structure and the third form, sGnRH by chromatographic evidence that supports the deduced structure obtained from cDNA (Dr. Y. Zohar, unpublished data). The early-eluting novel form (Fig. 5.1B) is an amidated decapeptide with the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH<sub>2</sub>, hereafter referred to as sea bream GnRH (sbGnRH). The primary structure of the second form of GnRH (Fig. 5.1C) in sea bream brains is identical to cGnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH<sub>2</sub>). The third, late-eluting form of GnRH is chromatographically characterized as sGnRH by elution position throughout the purification process (Fig. 5.1C), and by comparison with the synthetic standard in four preliminary experiments using sea bream brains collected at different times (data not shown

and Fig. 5.2). Proof of the structural identity of sbGnRH and cGnRH-II included Edman degradation to determine the sequence of the GnRH-(2-10) fragment. The amino-terminal pyroglutamic acid was identified by specific cleavage with pyroglutamyl aminopeptidase for all purified peptides. The amidated carboxy terminus was established by mass spectrometry for sbGnRH and cGnRH-II.

*Tilapia, O niloticus*, brains also contained three distinct forms of GnRH. All three forms of GnRH were identified by primary structure: sbGnRH, cGnRH-II and sGnRH. Mass and the establishment of the amino-terminal pyroglutamic acid and carboxy-terminus suggests that these peptides are bioactive. The proof that both sea bream and tilapia have three forms of GnRH suggests this pattern may be widespread among perciform fishes.

The identification of the primary structure of sbGnRH increases the known structures in the GnRH family to 10 forms (Fig. 5.10). All of these forms are decapeptides and have conserved amino acids in positions 1,2,4,9 and 10. The highest variability is observed at position 8; seven different amino acids are present in position 8. Other positions of variability (3 and 5-7) show fewer amino acid substitutions. Presumably, multiple forms of GnRH arise from nucleotide substitutions in GnRH genes that have either recently duplicated or existed for a long time. The presence of a serine in position 8 of sbGnRH represents only

Figure 5.10. Amino acid sequence of ten identified GnRH peptides. Boxes indicate amino acids that differ from the sbGnRH peptide. The two novel forms identified in the research for this thesis are shown by an asterisk (\*).

	1	2	3	4	5	6	7	8	9	10												
SEA BREAM *	p	GLU	-	HIS	-	TRP	-	SER	-	TYR	-	GLY	-	LEU	-	SER	-	PRO	-	GLY	-	NH <sub>2</sub>
MAMMAL	p	GLU	-	HIS	-	TRP	-	SER	-	TYR	-	GLY	-	LEU	ARG	PRO	-	GLY	-	NH <sub>2</sub>		
CHICKEN-I	p	GLU	-	HIS	-	TRP	-	SER	-	TYR	-	GLY	-	LEU	GLN	PRO	-	GLY	-	NH <sub>2</sub>		
CATFISH	p	GLU	-	HIS	-	TRP	-	SER	HIS	GLY	-	LEU	ASN	PRO	-	GLY	-	NH <sub>2</sub>				
HERRING *	p	GLU	-	HIS	-	TRP	-	SER	HIS	GLY	-	LEU	-	SER	-	PRO	-	GLY	-	NH <sub>2</sub>		
SALMON	p	GLU	-	HIS	-	TRP	-	SER	-	TYR	-	GLY	TRP	LEU	PRO	-	GLY	-	NH <sub>2</sub>			
CHICKEN-II	p	GLU	-	HIS	-	TRP	-	SER	HIS	GLY	TRP	TYR	PRO	-	GLY	-	NH <sub>2</sub>					
DOGFISH	p	GLU	-	HIS	-	TRP	-	SER	HIS	GLY	TRP	LEU	PRO	-	GLY	-	NH <sub>2</sub>					
LAMPREY-III	p	GLU	-	HIS	-	TRP	-	SER	HIS	ASP	TRP	LYS	PRO	-	GLY	-	NH <sub>2</sub>					
LAMPREY-I	p	GLU	-	HIS	TYR	SER	LEU	GLU	TRP	LYS	PRO	-	GLY	-	NH <sub>2</sub>							

a single amino acid substitution compared with mGnRH or cGnRH-I.

Reproduction in sea bream is of interest not only because individual fish spawn daily over a period of several months, but also because this species undergoes sexual inversion. In captivity female sea bream mature sexually, but do not undergo final ovarian maturation, ovulation and spawning; this is thought to be due to low GTH-II release. Exogenous GnRH causes these fish to ovulate (Zohar, 1989). To address the question of which of the three forms of GnRH releases the gonadotropins, pituitaries were examined for irGnRH content. Clearly, sbGnRH was the dominant GnRH form (500 fold as measured by GF-4) in the axons terminating in the pituitary. This provides strong evidence that this form of GnRH is the releaser of GTH-II during ovulation. A similar story is true for tilapia where sbGnRH is the most abundant form of GnRH in the pituitary (Fig. 5.9). The similarity of gonadotropin release between mammals and fish is striking as both types of vertebrates appear to have only a single form of GnRH that releases gonadotropins. The proof that sbGnRH is the peptide in the pituitary gland may explain why both an antiserum raised against mGnRH and antiserum GF-4 cross-reacted with preoptic cells in sea bream and cichlid; sbGnRH and mGnRH differ by one amino acid (Garcia-Ayala et al., 1989; Davis and Fernald, 1990). The novel sbGnRH structure may also explain the earlier observations that a specific antiserum raised against sGnRH did not cross-react with

cells of the preoptic area in sea bream (Garcia-Ayala et al., 1989). Further, sGnRH cRNA probes used in the cichlid *H. burtoni* did not detect GnRH-producing cell bodies in the preoptic nucleus, the origin of the axons that terminate in the pituitary (White et al., 1994).

The identification of 3 forms of GnRH within the brain of one species raises the possibility that each form has a distinct function and is related to a specific phase of the reproductive cycle. We sent peptides to our collaborator Dr. Y. Zohar to study the release of one of the pituitary gonadotropins (GTH-II) in sea bream. Injection of each of the native GnRH peptides (5 or 25 $\mu$ g/kg body weight) into mature female sea bream resulted in elevated plasma GTH-II levels. However, the order of potency for the peptides after injection was cGnRH-II > sGnRH > sbGnRH. These data clearly demonstrate that each form of GnRH from sea bream brains elicits a significant increase in the release of GTH-II from the pituitary gland. However, this does not explain why sbGnRH, the dominant form of GnRH in the pituitaries of mature sea bream, is less potent than sGnRH or cGnRH-II in inducing GTH-II release. This may indicate that sbGnRH is degraded faster than the two other native GnRH peptides by specific peptidases present in the pituitary, kidney and liver of the female sea bream (Goren et al., 1990; Zohar et al., 1990a). Another possibility is that sbGnRH, the mostly recently evolved form, may use one of the existing receptors that have higher affinities for mGnRH, sGnRH or cGnRH-II,

the established forms of GnRH in the more primitive teleosts. Similarly, in the catfish, *Clarias gariepinus*, the more recently-evolved catfish GnRH is less potent than cGnRH-II for induction of GTH-II secretion and has a lesser binding affinity than cGnRH-II to the pituitary GnRH receptor (Schulz et al., 1993).

A similar result was found when our collaborator, G. Weber, applied the three native GnRH peptides to pituitary cell cultures of tilapia *O. mossambicus* and measured the release of PRL<sub>177</sub> and PRL<sub>188</sub>. The releasing potency of the GnRH peptides was the same for PRL release in *O. mossambicus* as it was for GTH-II release in sea bream: cGnRH-II > sGnRH > sbGnRH. Again, the most abundant form of GnRH in the pituitary had the lowest effect on pituitary cells. The *in vivo* application of mGnRH analogue to *O. mossambicus* increased serum PRL, indicating that the effect of GnRH on PRL release is not restricted to cell cultures.

The releasing ability of PRL by GnRH suggests that GnRH axon terminals in the pituitary are capable of regulating functions other than reproduction. Marchant and coworkers (1989) have demonstrated that GnRH can release growth hormone from isolated goldfish pituitary cells. Thus, GnRH may also initiate or regulate associative functions secondarily important to reproduction. For example, PRL may be involved with the behaviour in brooding eggs and caring for offspring or in behavioural changes in breeding pairs. Also, PRL has a profound effect on gill ion permeability and

ion regulation, which may be needed in spawning environments.

Evidence presented in this chapter shows that the novel sbGnRH form is present in perciform fishes, a group of fish that originated approximately 100 million years ago. The sea bream form of GnRH is identified by primary structure in both sea bream and tilapia *O. niloticus*. However, a review of the immunological and chromatographic data show that a third form of GnRH, which may be identical with sbGnRH, is also present in other perciform fishes including tilapia *O. mossambicus* (Fig. 5.3), snook (*Centropomis unidecimalis*, Sherwood et al., 1993b), cichlid (*Haplochromis burtoni*) (see Chapter 6) and mullet, (*Mugil cephalus*; Sherwood et al., 1984). In all cases, an early-eluting irGnRH is in a similar position to that of the purified sbGnRH. A third form of GnRH was also indirectly identified in orders that are closely related to Perciformes: Pleuronectiformes (flounder; Idler and Everard, 1987), Scorpaeniformes (grass rockfish; see Chapter 6) and Gasterosteiformes (stickleback; Andersson et al., 1992). By comparing the published reports of the elution of GnRH in these fish to the data presented here, it is probable that this form of GnRH is sbGnRH.

An additional three orders of fish that evolved earlier than the perch-like fish also have three forms of GnRH. These fish are: herring (*Clupea harengus pallasii*; Chapter 3), sabalo (*Prochilodus lineatus*; Somoza et al., 1994) and

milkfish, (*Chanos chanos*; Sherwood et al., 1984). The novel form of GnRH in herring was shown to be distinct from sbGnRH in Chapter 3. However, in the sabalo and milkfish, the three forms of GnRH in the brain follow a similar HPLC elution pattern to that of the sea bream based on HPLC and RIA studies. HPLC evidence shows that the early-eluting form of irGnRH elutes in a similar position compared to sbGnRH. The second form is cGnRH-II, and the third form is sGnRH. Although one form of GnRH in fish such as the sabalo and milkfish is similar to sbGnRH based on chromatographic and immunological evidence, it is unresolved whether this unidentified form is sbGnRH, hGnRH or another GnRH. Only the sequence of the peptide or cDNA in each species will establish the identity of the first form. It is certainly possible that these forms have a distinct identity from sbGnRH, especially in the more phylogenetically ancient fish.

Multiple forms of a peptide could have arisen from multiple copies of genes as a result of genome duplication, unequal sister chromatid exchange, duplication resulting in a redundant DNA sequence or homologue crossover during meiosis. After each of these events, it is thought that one copy of the gene is conserved, whereas the other copy that is not required for normal function diverges. Eventually, two distinct gene products may be produced.

Genome duplication has occurred in some fish species such as the salmon and sturgeon (see Allendorf and Thorgaard,

1984). For sea bream, however, the haploid number of chromosomes is 24 (Dr. Y. Zohar, unpublished data). This makes it unlikely that sbGnRH resulted from a genome duplication in sea bream.

Unlike genome duplication, exon duplication would result in a tandem organization of two GnRH coding regions within one gene. The cDNA for sbGnRH has not been reported, but two distinct forms of GnRH are not encoded in one precursor based on the cDNAs reported to date: mGnRH (Seeburg and Adelman, 1984), sGnRH (Bond et al., 1991), cfGnRH and cGnRH-II (Bogerd et al., 1994) and chicken GnRH-I (Dunn et al., 1993) in other species. The lack of evidence for polyploidy or exon duplication in the GnRH gene of the sea bream leaves the possibility that unequal cross-over or unequal transfer of genetic material may have occurred.

The distribution of GnRH among the teleosts suggests that sbGnRH must have arisen from sGnRH or cGnRH-II. cGnRH-II is present in all teleosts examined (Sherwood et al., 1993a). In contrast, sGnRH appears to have arisen from mGnRH in species that evolved near the origin of the teleosts (see Chapter 2). The sGnRH form continued to be expressed in all teleosts that subsequently evolved with the exception of three species of catfish, in which cfGnRH replaces sGnRH.

It is more likely that sbGnRH arose from a modification of sGnRH rather than cGnRH-II. Evidence to support this hypothesis is that sbGnRH differs from sGnRH by two amino

acid substitutions, but differs from cGnRH-II by three. Also the minimum number of nucleotide substitutions that would be required for the change from sGnRH to sbGnRH is two, whereas the number of substitutions from cGnRH-II to sbGnRH is three.

The final possibility is that a third form of GnRH arose early in the teleosts and is the ancestral molecule for hGnRH and sbGnRH. This third molecule may have emerged due to a gene duplication of the mGnRH or sGnRH gene. The presence of hGnRH is proof that such an event occurred early in teleost evolution. However, further studies are needed to determine if a third form of GnRH is found in other teleosts that evolved after herring and before fish that contain sbGnRH.

## Chapter 6 The origin of sea bream GnRH.

A version of this chapter has been accepted for publication and is reworked here:

Powell, J.F.F., Fischer, W.H., Park, M., Craig, A.G., Rivier, J.E., White, S.A., Fernald, R.D., Francis, R., Licht, P., Warby, C. and Sherwood, N.M. Primary structure of solitary form of gonadotropin-releasing hormone (GnRH) in cichlid pituitary; three forms of GnRH in brain of cichlid and pumpkinseed fish. *Regulatory Peptides*.

### INTRODUCTION

In Chapter 5, I reported the identification of three forms of GnRH from the brains of perciform fishes, the sea bream, *Sparus aurata*, and tilapia *Oreochromis niloticus*. These three forms, sGnRH, cGnRH-II and sea bream GnRH (sbGnRH), raise the question of the functional significance of multiple forms in the teleost brain. Three distinct locations of GnRH neurons were reported for another cichlid fish, *Haplochromis burtoni*, but the identity of the irGnRH located in the preoptic nucleus and pituitary was unknown (Davis and Fernald, 1990; White et al., 1994).

Immunological and *in situ* hybridization studies in *H. burtoni* show that neither sGnRH nor cGnRH-II are the gonadotropin releasing hormone (Davis and Fernald, 1990; White et al., 1994). Further, Francis and coworkers (1994) observed that the sGnRH-producing cells of the anterior

forebrain are not involved in pituitary regulation. This situation is distinct from salmonids in that sGnRH is probably the primary releasing hormone of the gonadotropins among salmonids (Okuzawa et al., 1990; Amano et al., 1992).

The identification of the form of GnRH that acts as releaser of the gonadotropins is an important concept. Although the structure of a pituitary GnRH peptide has been described for herring (Chapter 3), the identical elution position of chicken GnRH-I and sbGnRH (see below) preclude direct identification of sbGnRH in the pituitary of tilapia or sea bream. Therefore, in this chapter I examine the form of GnRH in the pituitary of *H. burtoni* and determine the primary structure. In addition, this chapter describes chromatographical and immunological evidence for the presence of three forms of GnRH in the brains of the cichlid (*H. burtoni*), the pumpkinseed (*Lepomis gibbosus*), and the rockfish (*Sebastes rastrelliger*). To elucidate where the third form arose in evolution, I selected and examined the forms of irGnRH in two other fish that are phylogenetically distant from the above fish, the zebrafish (*Brachydanio rerio*) and the Japanese medaka (*Oryzias latipes*).

## **MATERIALS AND METHODS**

### **Tissue collection and peptide extraction**

Thirty-five brains and 69 pituitaries of cichlid fish (*H. burtoni*) captured in Africa were separately shipped from Stanford University and received frozen on dry ice at the

University of Victoria. A total of 28 brains and pituitaries from adult and juvenile pumpkinseed fish (*L. gibbosus*) captured by sporting methods in Blenkinsop Lake, Victoria, B.C. were removed and immediately frozen on dry ice. Brains together with pituitaries (7.4g) were removed from 25 grass rockfish, *Sebastes rastrelliger*, captured off the central California coast in October 1993. In addition, a separate collection of 16 pituitaries (0.7g) was made in August 1994. Brains together with pituitaries (0.43g) from aquaria stock of 51 Japanese medaka, *Oryzias latipes*, were also collected at the University of Santa Barbara. The medaka were maintained at conditions of 16h L, 26C and were in breeding condition. Tissues were frozen and shipped on dry ice from the University of California at Santa Barbara to the University of Victoria. In addition, 37 brains together with pituitaries (0.25g) were removed from adult zebrafish, *Brachydanio rerio*, maintained at conditions of 14h L, 23C at the University of Victoria and immediately frozen on dry ice in Victoria. All tissues were stored at -80°C.

#### **Peptide extraction**

Each of the groups of tissues was powdered separately using a cold mortar and pestle, then extracted with HCl and acetone as described. Lipid extractions were done to remove soluble lipids as described (see Chapter 2). The extracts were then reduced in volume in a vacuum centrifuge and

filtered using a 0.22 $\mu$ m filter (Waters) before application to HPLC. Preparation of the extract and subsequent HPLC and RIA analysis was done for cichlid brain by C. Warby.

#### **HPLC of cichlid, rockfish, zebrafish and medaka brain extracts**

A blank sample (800 $\mu$ l water) was applied to HPLC and the eluted fractions assayed for GnRH. Thereafter, the brain extract was applied to the same C<sub>18</sub> Supelco column connected to a Varian Model 5000 HPLC (cichlid) or Beckman Model 166 HPLC (other fish). The TEAF isocratic HPLC method (Chapter 2) was used to separate peptides.

#### **HPLC of pumpkinseed brain extract**

A blank sample (800 $\mu$ l water) was applied to HPLC and the 1ml eluted fractions were dried and assayed. Brain extract was applied to the same C<sub>18</sub> Supelco column connected to a Beckman Model 166 HPLC and the HFBA-HPLC method (Chapter 2) was employed to separate peptides. In both cichlid and pumpkinseed HPLC methods, fractions of 1ml were collected for 60 min. Aliquots of 100 $\mu$ l from each fraction were dried in a vacuum centrifuge prior to assay for GnRH.

#### **Standards**

After the HPLC samples of fish extracts were eluted, synthetic standards were applied. For the cichlid study, five GnRH forms (lamprey GnRH, cGnRH-I, cGnRH-II, sGnRH and

mGnRH) were chromatographed as described for the cichlid extract. The combined standards were injected at a concentration of 250ng each in a total of 800 $\mu$ l.

Seven standards for comparison to the pumpkinseed brain extract were applied to HPLC using the conditions described. The combined standards not only contained the five forms mentioned above, but also synthetic catfish GnRH and dogfish GnRH. In addition, sbGnRH alone, cGnRH-I alone and the two peptides together were applied to the HPLC using the HFBA method. For comparison of standards to the rockfish, zebrafish and medaka brain extracts, a solution of the seven standards cited above and sbGnRH were applied to the column and the isocratic TEAF-HPLC method used. For rockfish pituitary extract, 200ng each of synthetic sbGnRH, cGnRH-II and sGnRH were applied to the column using the TEAF isocratic HPLC method. The elution positions of the standards on the chromatograph were confirmed by RIA for GnRH using 10 $\mu$ l aliquots of the fractions.

### **Radioimmunoassay**

HPLC fractions from the extracts and standards were assayed as previously described (Chapter 2). Synthetic mGnRH was used as the standard and as iodinated tracer. Antiserum GF-4 (raised against sGnRH) was used at a final dilution of 1:25,000 and had an average binding of 24% with a sensitivity ( $B/B_0 = 80\%$ ) of 11pg. Antisera BLA-4 or BLA-5 (raised against lamprey GnRH) was also used in the RIA to

detect irGnRH for both pumpkinseed and cichlid HPLC fractions. BLA-4 and BLA-5 were used in a 1:5,000 dilution with a binding of 15% and a sensitivity of 36pg.

Antiserum R-42 was used in a dilution of 1:250,000 and had a sensitivity of 3pg and an average binding of 14%. Fractions with high immunoreactive GnRH (tracer binding,  $B/B_0$ , of 20% or less) were serially diluted 1:2 and reassayed. The value closest to 50% tracer binding is reported.

For cichlid HPLC fractions, antiserum B-6 (1:5000, raised against mGnRH) was used also (Kelsall et al., 1990). Binding and sensitivity were 37% and 15pg, respectively.

#### **Purification of GnRH from cichlid pituitary extract**

In the first HPLC step for the purification of GnRH from the cichlid pituitary extract, a  $C_{18}$  Supelco column that had not been exposed to standards was connected to a Beckman Model 166 HPLC. Fractions 23-25 contained immunoreactive GnRH (irGnRH) as measured with antisera GF-4 and BLA-5. To ensure that the various forms of irGnRH were detected, I assayed samples of 10 $\mu$ l, 25 $\mu$ l and 100 $\mu$ l from all 60 fractions irGnRH using antisera GF-4, BLA-5 and B-6. Antiserum GF-4 was used in all subsequent steps of purification, each of which was the same as detailed for tilapia (Chapter 5).

### Characterization of the primary structure

Partially purified cichlid pituitary GnRH (fractions 21-23) was further purified by narrow-bore HPLC. The mass of the intact molecule was determined (using a 1% aliquot) with matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). The purified peptides were sequenced and the pyroglutamic residue was confirmed as described (Chapter 2). To determine whether the C-terminus of cichlid GnRH was amidated, researchers at the Salk Institute used an esterification reaction that was monitored with MALDI-MS. An aliquot (5 $\mu$ l) of 6M HCL in ethanol/ethylacetate (formed by reacting acetylchloride with ethanol) was added to approximately 0.3 pmol bombesin-OH (free acid form of bombesin) as an internal control. The reaction was carried out for one hour at room temperature in the presence of 2mM  $\beta$ -mercaptoethanol. External control reactions of 1pmol of sbGnRH standard, antide (a GnRH antagonist; Ljungvist et al., 1987), antide-OH, bombesin and bombesin-OH were also carried out. The reaction mixtures were analysed by MALDI-MS. The formation of an ethyl ester resulted in a mass shift of 28 Da. The absence of mass shift indicated the lack of any free carboxylic function.

## RESULTS

### Identification of GnRH in brains extracts

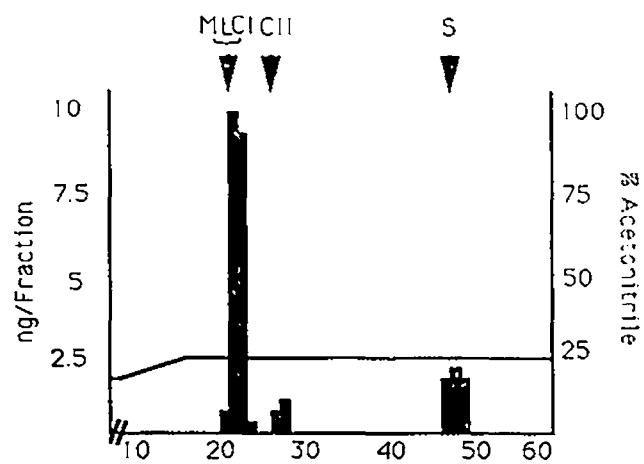
From cichlid extract prepared from the combination of brains and pituitaries, three distinct areas of irGnRH

Table 6.1: Amounts of irGnRH detected in brain and or pituitary extracts from *H. burtoni*, pumpkinseed, rockfish, medaka and zebrafish. Amount of irGnRH detected in the RIA of HPLC eluates is recorded below the elution position of the corresponding synthetic standard.

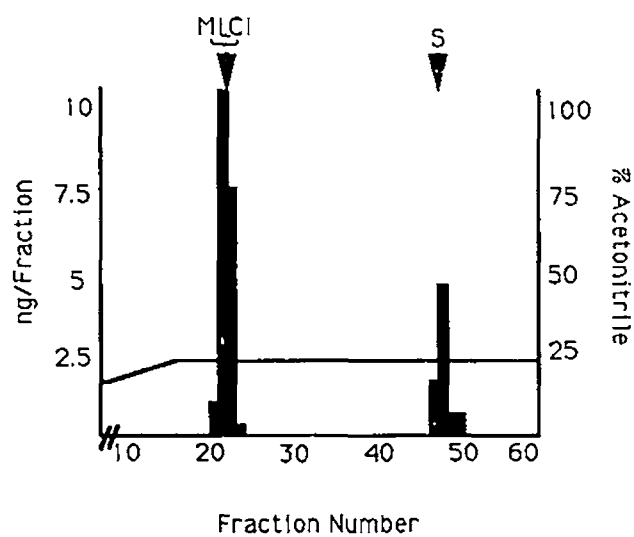
	No.	Antisera	sb	cII	s
Cichlid brain/pit.	35	GF-4	17.7ng	0.9ng	3.8ng
		BLA-4	19.2	-	6.2
Cichlid pituitary	69	GF-4	246	-	-
Pumpkinseed brain/pit.	28	GF-4	0.65	0.05	0.14
		BLA-4	0.3	-	0.9
Rockfish brain/pit.	25	GF-4	1.5	13	38
		BLA-5	10	-	286
Rockfish pituitary	16	GF-4	23	-	0.6
		BLA-5	54	-	1.2
		B-6	-	-	-
Medaka brain/pit.	51	GF-4	-	5	5
		BLA-5	-	2.5	11
Zebrafish brain/pit.	37	GF-4	-	0.9	3.5
		BLA-5	-	-	1.5

Figure 6.1. HPLC analysis of GnRH from cichlid *H. burtoni* combined brain-pituitary extract. irGnRH was eluted from a C<sub>18</sub> column using the isocratic TEAF method. The elution positions of synthetic standards are indicated by arrows: M=mammalian GnRH, L=lamprey GnRH-I and CI=chicken GnRH-I; CII=cGnRH-II, S=sGnRH. The first three standards (M, L, CI) coelute. Percent acetonitrile is shown by solid lines.

Antiserum GF-4  
Cichlid



Antiserum BLA-4  
Cichlid



eluted from the HPLC column and were detected using antiserum GF-4 (Table 6.1, Fig 6.1). In contrast, antiserum BLA-4 detected only 2 areas of irGnRH (Fig 6.1). In the cichlid HPLC method (isocratic TEAF), the synthetic standards eluted in distinct positions except for three standards (cGnRH-I, mGnRH and lamprey GnRH) that eluted together. These are referred to here as the cGnRH-I group of standards. Elution positions of irGnRH detected with GF-4 corresponded to the synthetic standards: the first immunoreactive material eluted with the cGnRH-I group of standards; the second immunoreactive material eluted with the cGnRH-II standard; and the third immunoreactive material eluted with sGnRH (Fig. 6.1). Elution positions of the two areas detected by antiserum BLA-4 corresponded to the first and third immunoreactive material detected with antiserum GF-4 (Fig. 6.1). HPLC fractions of the cichlid brain and pituitary extract, assayed with antiserum B-6, showed only 0.41ng of irGnRH in a fraction (no. 23) that eluted 1min prior to the mGnRH synthetic standard.

In extracts from rockfish brains and pituitaries, antisera GF-4 and BLA-5 detected three areas of irGnRH that corresponded to the elution positions of synthetic sbGnRH/cGnRH-I, cGnRH-II and sGnRH, respectively (Table 6.1, Figs 6.2, 6.3). Rockfish pituitary extracts contained two forms of irGnRH that were detected by antisera GF-4 and BLA-5. Antiserum B-6 (specific for mGnRH) failed to recognize any form of GnRH in the extracts of rockfish pituitaries.

The amounts of irGnRH detected in rockfish pituitaries are shown in Table 6.1.

Immunoreactive GnRH from medaka extract was detected in fractions that corresponded to the elution positions of synthetic cGnRH-II and sGnRH (Table 6.1, Fig. 6.2). Likewise, antisera GF-4 and BLA-5 detected two forms of irGnRH in HPLC eluates of zebrafish brain extract (Table 6.1, Fig. 6.2). In each case, only two forms of irGnRH were detected.

HPLC samples from pumpkinseed brain extract contained irGnRH in 3 elution positions as detected by antiserum GF-4 (Table 6.1, Fig. 6.4), but two areas as detected by BLA-4. The HPLC program for the pumpkinseed extract and standards was different compared to the cichlid program and resulted in distinct elution positions for each of the seven standards. The irGnRH that cross-reacted with GF-4 showed similar elution times from the HPLC column compared with the synthetic standards: the first eluted with cGnRH-I; the second eluted with cGnRH-II; and the last eluted with sGnRH (Fig. 6.4). Synthetic sbGnRH coeluted with synthetic cGnRH-I and hence the first irGnRH material to elute in both cichlid and pumpkinseed HPLCs elutes in the same position as sbGnRH (Fig. 6.5).

#### **Standards for rockfish, medaka and zebrafish tissues**

The elution positions of synthetic GnRH standards have been determined by absorbance and by antisera cross-

Figure 6.2. HPLC analysis of GnRH from brain extracts of rockfish (top), medaka (middle) and zebrafish (bottom). Percent acetonitrile is shown by solid lines. irGnRH was eluted from a C<sub>18</sub> column using the isocratic TEAF method and detected using antisera GF-4 and BLA-5. Numbers above fractions indicate amount of irGnRH detected. The elution positions of synthetic standards are indicated by arrows: SB=sbGnRH, CII=cGnRH-II, S=sGnRH.

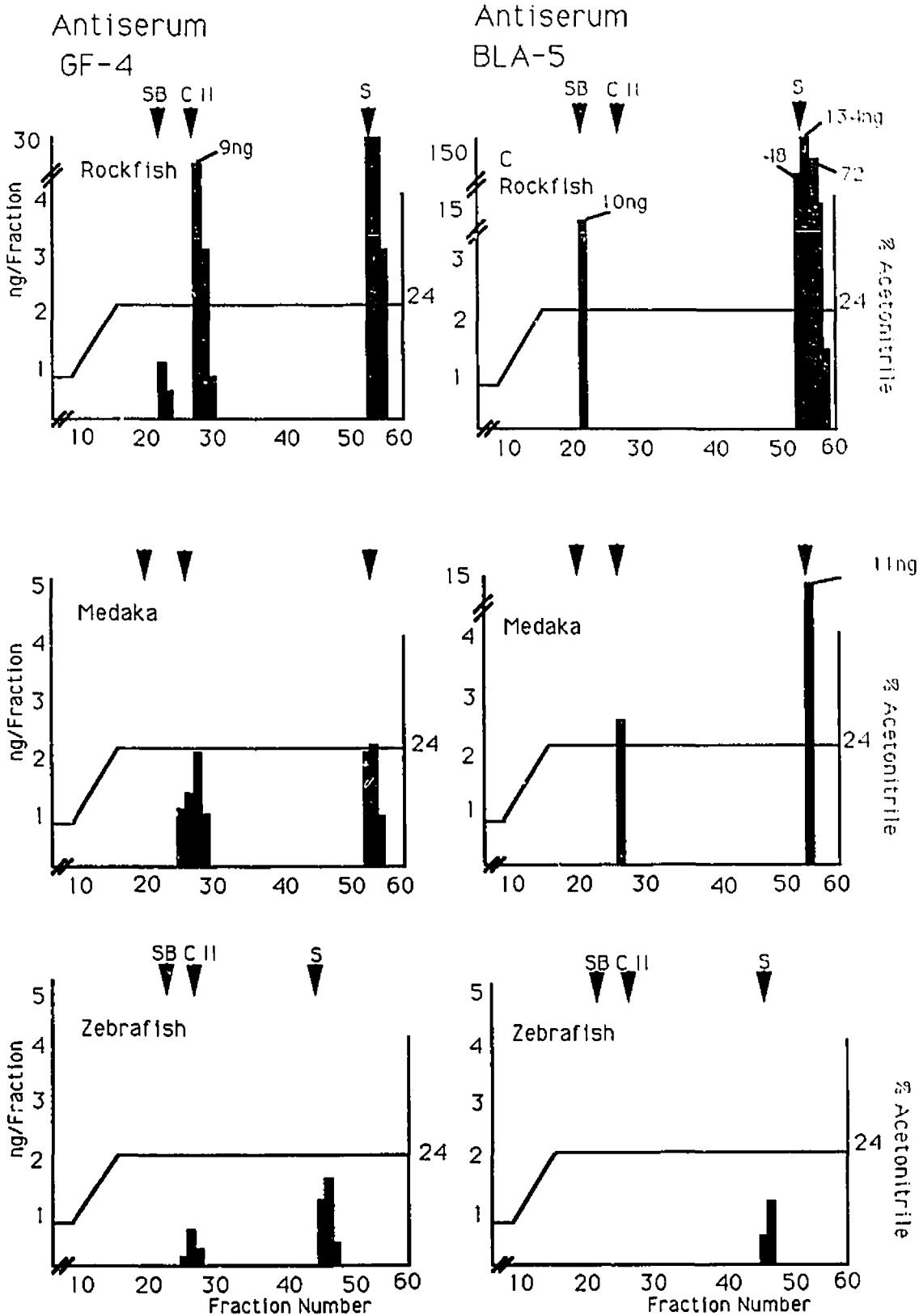
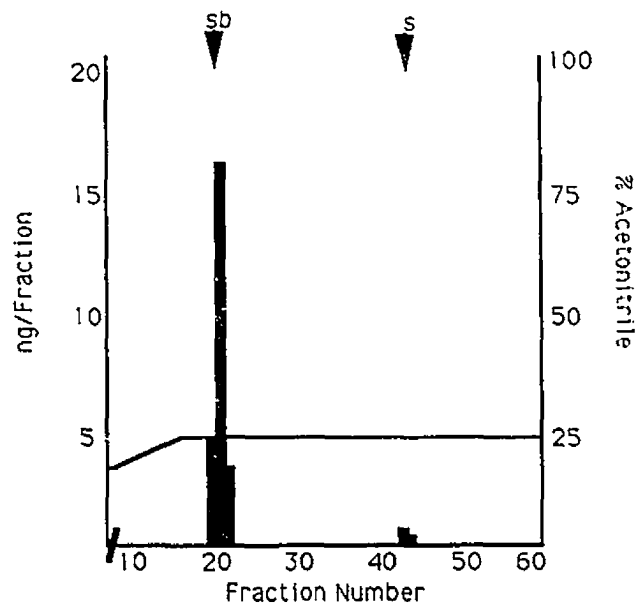


Figure 6.3. HPLC and RIA analysis of irGnRH from rockfish pituitary. GnRH was eluted from the HPLC column using the isocratic TEAF method and detected with antisera GF-4 (left) and BLA-5 (right). Solid lines indicate acetonitrile.

## Antiserum GF-4



## Antiserum BLA-5

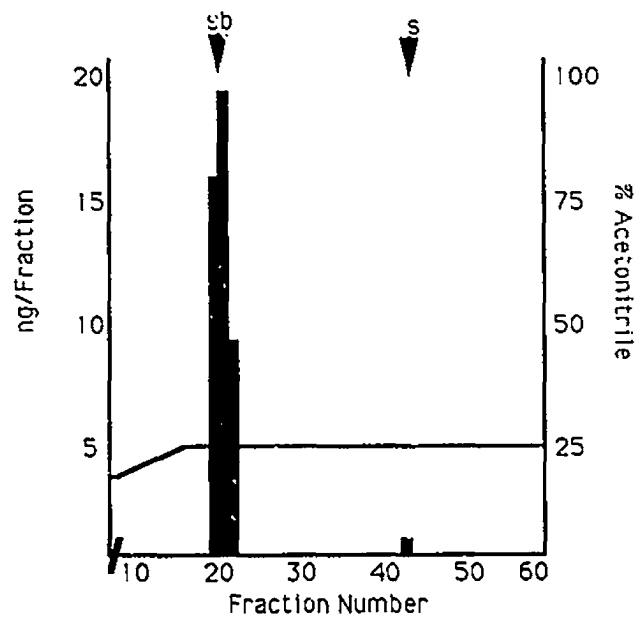
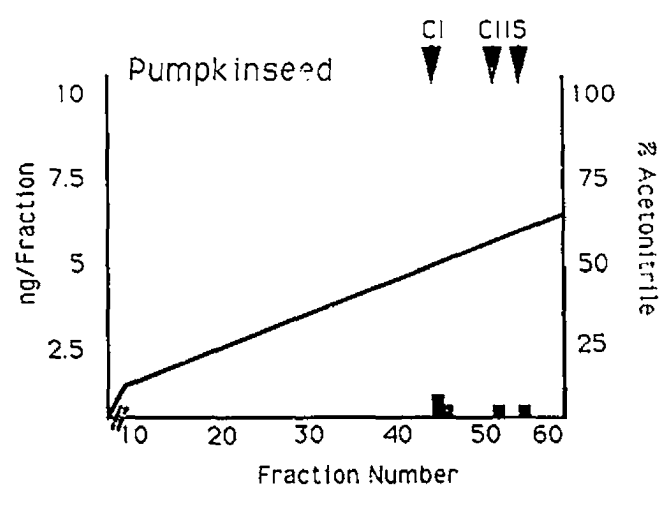


Figure 6.4. HPLC analysis of irGnRH from brain extracts of pumpkinseed fish. Percent acetonitrile is shown by solid lines. irGnRH was eluted from a C<sub>18</sub> column using the HFBA method and detected using antisera GF-4 and BLA-4. The elution positions of three of the seven synthetic standards are indicated by arrows: CI=cGnRH-I, CII=cGnRH-II, S=sGnRH.

### Antiserum GF-4



### Antiserum BLA-4

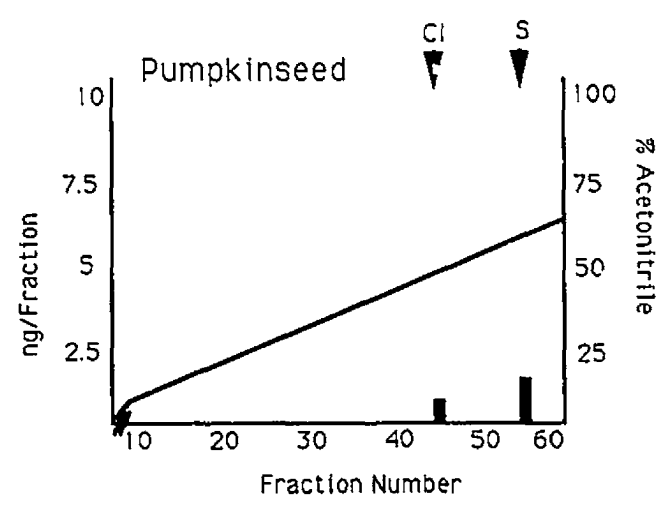
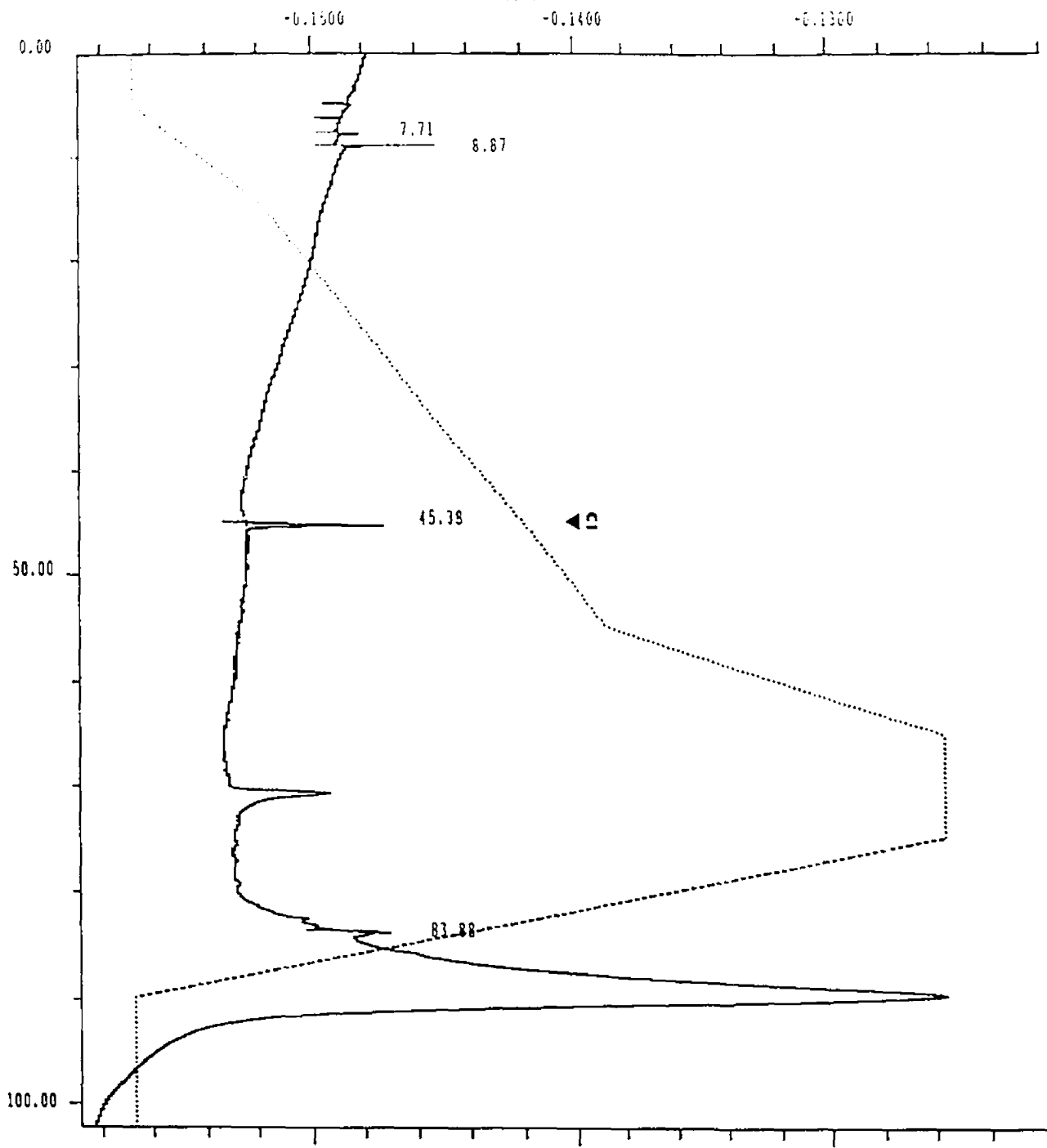


Figure 6.5. HPLC chromatograph of two synthetic GnRH standards for comparison of elution times. The standards were injected onto the column at zero min and the HFBA method was used. Top: 300ng of sea bream GnRH (sb), Middle: 400ng of chicken GnRH-I (cI), Bottom: 300ng of sea bream GnRH and 400ng of chicken GnRH-I. Elution position is indicated by arrows. The thin line represents the gradient of solution B (0.1M HFBA in 75% acetonitrile).

Absorbance



Absorbance

-0.1400

-0.1200

-0.1000

0.00

50.00

100.00

4.77

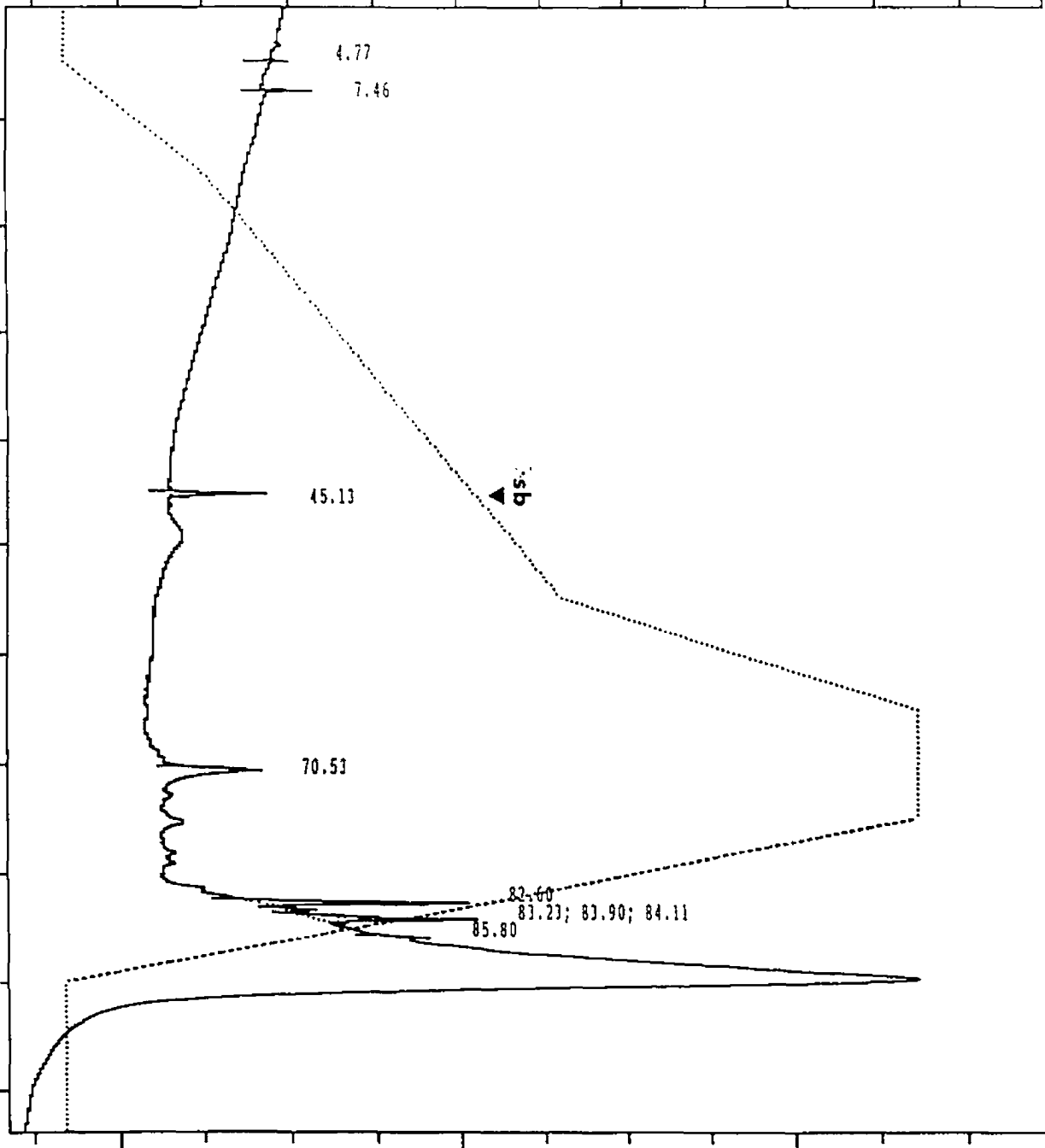
7.46

45.13

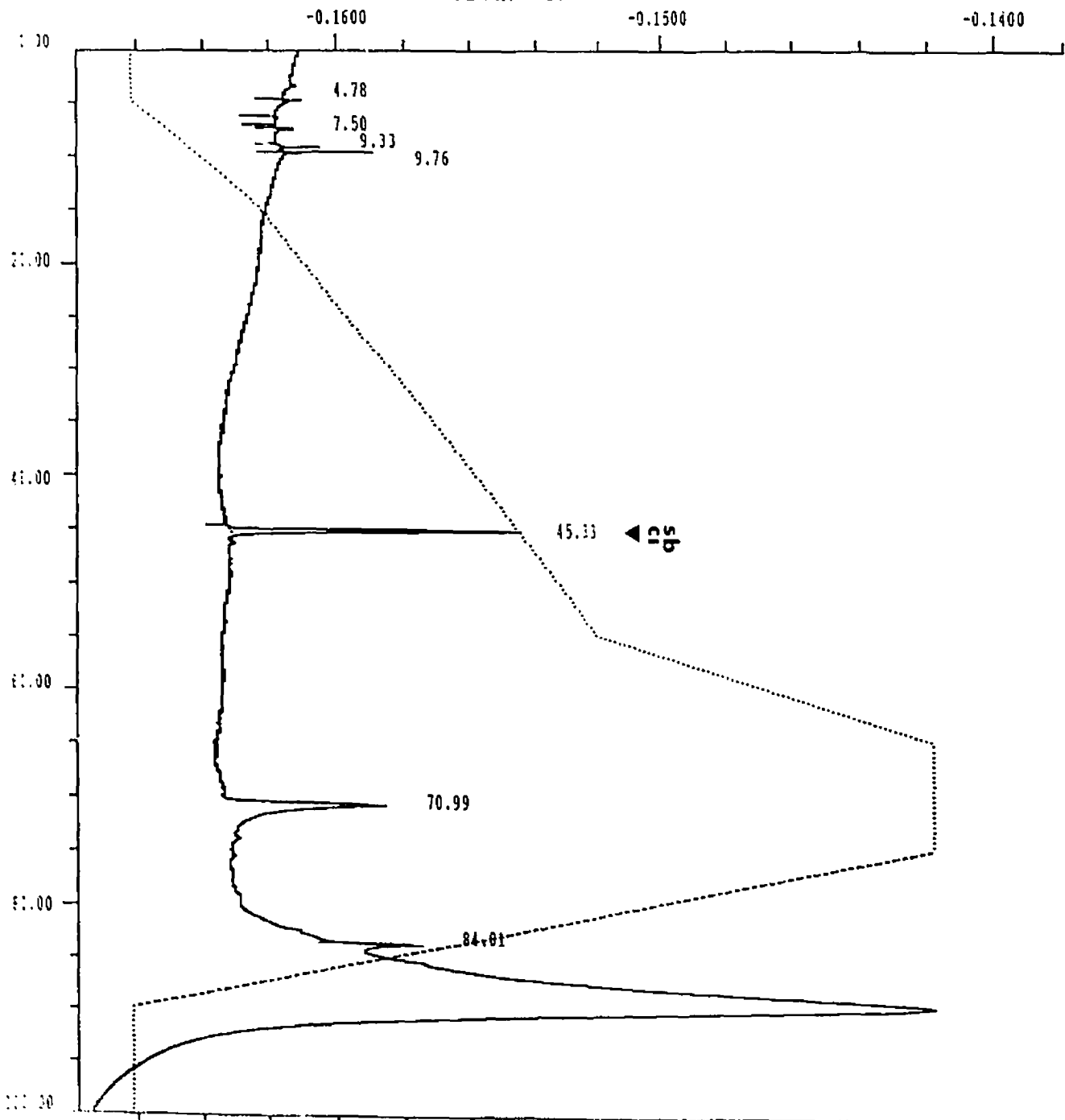
70.53

82.60  
83.23; 83.90; 84.11  
85.80

qs



Absorbance

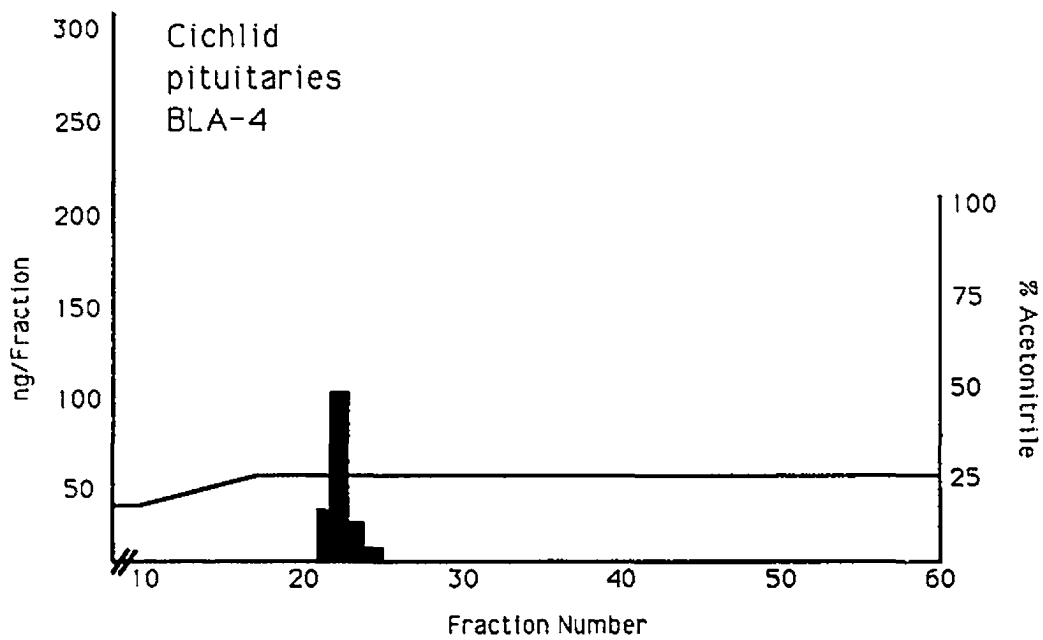
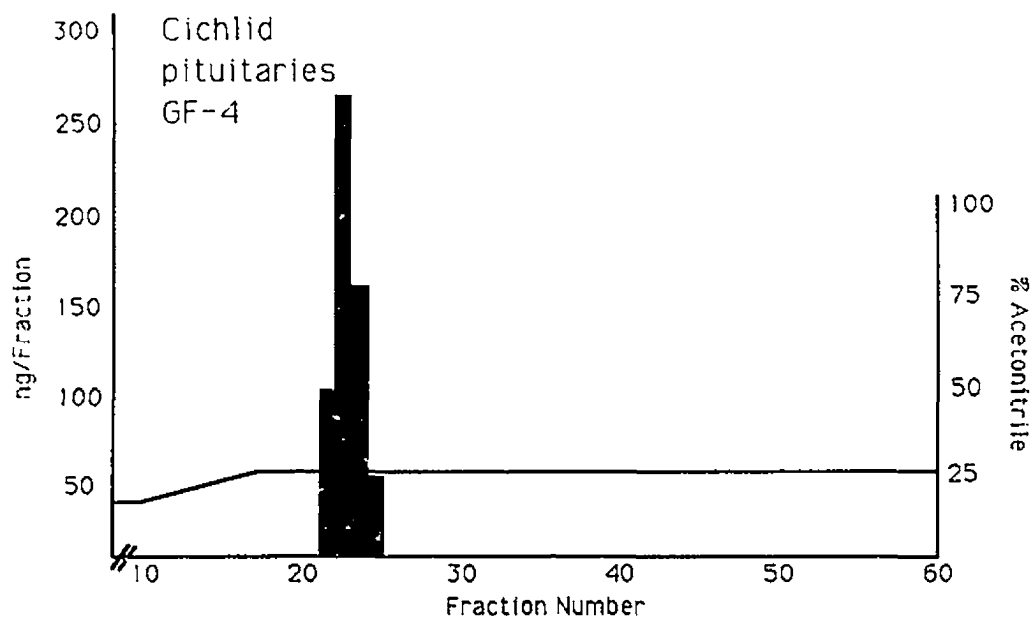


reactivity (Kelsall, et al. 1990; Sherwood, et al. 1991; Ngamvongchon, et al 1992a). In the present study, the elution positions of six standards (mGnRH, cGnRH-I, cGnRH-II, sbGnRH, lGnRH-I and sGnRH) were tested to determine if synthetic sbGnRH eluted with cGnRH-I and to determine if any standards shifted with the column conditions used for individual brain samples. Antisera GF-4 and BLA-5 detected synthetic sbGnRH and chicken GnRH-I in the same elution position (Fig. 6.5); cGnRH-I also elutes with mGnRH and lGnRH-I (Fig. 6.1). The elution position of synthetic sGnRH differed between the rockfish/medaka and zebrafish standards. For rockfish and medaka, synthetic sGnRH eluted in fractions 55-59. The elution position of sGnRH after HPLC of zebrafish brain was fraction 42-45 (Fig. 6.2). For the cichlid brain and pituitary study, antiserum BLA-5 detected synthetic lamprey GnRH (Fig. 6.1). This elution position is determined by subtracting the amount of irGnRH detected by antiserum GF-4 from the amount detected by BLA-5 for that fraction. Antisera GF-4 and BLA-5 cross-react equally with cGnRH-I and mGnRH, but GF-4 does not cross-react with lGnRH.

#### **Primary structure of GnRH isolated from H. burtoni pituitary**

One area of irGnRH material was detected by antisera GF-4 and BLA-4, but not by B-6, from the cichlid pituitary extract after the first HPLC step (Table 6.1, Fig. 6.6). This material eluted in the same position as sbGnRH/cGnRH-I.

Figure 6.6. HPLC and RIA analysis of the single form of cichlid pituitary GnRH. The irGnRH was eluted from the HPLC column using the isocratic TEAF method and was detected with antisera GF-4 (top) and BLA-5 (bottom). Solid lines indicate acetonitrile.



As well, only one area of irGnRH was detected in each successive HPLC application. Final yield after purification was 246ng as detected by antiserum GF-4.

MALDI mass spectrometry of the intact peptide isolated from the pituitary showed that a species was observed at  $m/z$  1113.9. After the peptide was digested with pyroglutamate aminopeptidase and subjected to Edman degradation on the automated protein sequencer, the following sequence was obtained: (His)-Xaa-Ser-Tyr-(Gly)-Leu-Ser-Pro-(Gly) (residues in parenthesis were assigned tentatively with less than 80% confidence and Xaa denotes that no assignment was made). MALDI-MS analysis of the pyroglutamate aminopeptidase treated material showed that a species was present at  $m/z$  1002.6. The mass difference between the intact and enzyme treated measurements is consistent with removal of an N-terminal pyroglutamate residue.

Amidation of the C-terminus of the peptide was determined by an esterification reaction monitored with MALDI-MS. The formation of an ethyl ester with a mass shift of 28 Da was observed only for the positive control reactions (the non acidic residue containing peptides of antide-OH and bombesin-OH). No mass shift was observed for the negative controls (sbGnRH, antide and bombesin). The mass of the intact cichlid GnRH  $[M+H]^+$  species was unaffected by the esterification reaction. This absence of mass shift indicated the lack of any free carboxylic functions. When bombesin-OH was included in the esterification as an

internal positive control, the bombesin ethyl ester could be detected, whereas the cichlid GnRH was present in its unmodified form as expected for the amidated peptide.

Based on the partial sequence information and the mass measurement, we concluded that the residue Xaa in the partial amino acid sequence information had a mass of 186 Da, which is consistent with a tryptophan residue (186.2 Da.). These measurements suggest that the sequence is: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH<sub>2</sub>. This sequence is identical to that of sbGnRH. The calculated monoisotopic mass for the [M=H]<sup>+</sup> for this sequence is 1113.51 Da compared with an observed value of 1113.9.

#### DISCUSSION

The only GnRH form detected in the cichlid pituitary was sbGnRH, although three forms were present in the brain (sbGnRH, cGnRH-II and sGnRH) at the time of collection. This is due to the storage of GnRH in axon terminals in the anterior pituitary of teleosts such as cichlids. The large amount of GnRH that accumulated in this storage site made it possible to purify and determine the primary structure of the stored GnRH peptide.

The presence of only one form of GnRH in the pituitary suggests that this form is involved in gonadotropin (GTH) release. It is not known if both GTH-I and GTH-II are released by sbGnRH as neither GTH-I nor GTH-II have been isolated from cichlids. However, in a closely-related fish,

the sea bream, sbGnRH released GTH-II (see Chapter 5); GTH-I was not measured because this assay has not been developed. It is not uncommon for one form of GnRH to release two gonadotropins as shown by most mammals where mGnRH is the only form present, but releases both LH and FSH (see Fink, 1988).

From the cichlid, rockfish, and pumpkinseed brains, there is chromatographical evidence of three forms of GnRH (Figs 6.1, 6.2 and 6.4). Antiserum GF-4 detects all three of these forms, whereas antiserum BLA-4/5 detects mainly two forms, although cGnRH-II-like immunoreactivity is detected in small amounts in some tissues. The pattern of elution for the three forms of irGnRH appears to be identical for all three species of fish. The first irGnRH form to elute from the HPLC column is the most prevalent form in most fish at the time of brain collection and appears to be sbGnRH. This early-eluting material from brain extract is in the same position as the GnRH peptide in the pituitary, which was identified by primary structure as sbGnRH. The HPLC-RIA evidence suggests that the two additional forms detected from cichlid, rockfish and pumpkinseed brains represent cGnRH-II and sGnRH.

The HPLC method used for pumpkinseed brains gives support for the presence of sbGnRH in the first-eluting peak. In this method, seven different GnRH standards eluted in different positions. Later, the sbGnRH standard was shown to coelute with the cGnRH-I standard (Fig. 6.5). The first

HPLC peak from pumpkinseed brain eluted in the same position as the cGnRH-I and sbGnRH standards (Fig. 6.4). This eliminates the other six standards from consideration. Therefore, the first peak from pumpkinseed HPLC is probably sbGnRH because sbGnRH and not cGnRH-I was identified by primary structure in closely-related perciform fishes: cichlid (*H. burtoni*), tilapia (*Oreochromis niloticus*), and gilthead seabream (*Sparus aurata*).

In *H. burtoni* and rockfish, the early eluting material also appears to be sbGnRH although the HPLC method differed from the pumpkinseed HPLC method. In the cichlid and rockfish studies, three standards (mGnRH, lamprey GnRH and cGnRH-I) eluted from HPLC in the same position as the first peak. The early-eluting material was detected by antiserum GF-4 and therefore cannot be lamprey GnRH. This peptide is unlikely to be mGnRH as GF-4 detected 35 fold the amount detected by B-6; the latter detects only mGnRH and has the same cross-reactivity as GF-4 for mGnRH (Sherwood et al., 1991). The first-eluting irGnRH is not likely to be cGnRH-I because the pituitary GnRH from cichlid was also early-eluting and was identified as sbGnRH.

The second-eluting irGnRH material in cichlid, rockfish and pumpkinseed samples corresponds to cGnRH-II by elution time and cross-reactivity; it is detected by GF-4, but not (or poorly) by BLA-4/5. This agrees with cross-reactivity data in that GF-4 detects 4% and BLA-4 only 0.6% of synthetic cGnRH-II (Sherwood et al., 1991). This evidence

confirms a recent report in which the cGnRH-II cDNA was identified from the same species of cichlid, *H. burtoni* (White et al., 1994).

In the three fishes, the third irGnRH material to elute from the column corresponds to the elution position of the sGnRH standard (Figs 6.1, 6.2 and 6.4). Both antisera GF-4 and BLA-4 cross-react with this material as predicted with synthetic sGnRH (GF-4: 68%, BLA-4: 88%). Thus, both antisera detect sGnRH, but BLA-4 detects a greater amount. Again, the salmon GnRH cDNA for the cichlid (*H. burtoni*) has been reported (Bond et al., 1991) and supports the conclusion that the third irGnRH is sGnRH.

There is also an argument based on evolutionary principles suggesting that the early-eluting material in cichlid, rockfish and pumpkinseed brain extracts is not cGnRH-I. A review of the phylogenetic distribution of the GnRH family of peptides shows that cGnRH-I is present only in reptiles and birds (see Sherwood et al., 1993a). It can not be excluded that cGnRH-I arose independently in two distinct classes of vertebrates, but it is improbable among classes without a recent common ancestor.

In comparison to the cichlid, rockfish and pumpkinseed fishes, only two forms of GnRH, cGnRH-II and sGnRH were detected in brain extracts from medaka and zebrafish. The two forms detected by antisera GF-4 and BLA-5 are determined to be cGnRH-II and sGnRH based upon cross-reactivity and elution position compared to synthetic standards. The

identification of three forms of GnRH in the brains of rockfish suggests sbGnRH emerged in a fish ancestral to rockfish but not medaka. Nelson (1984) reports that 10 extant orders and 50 million years separate medaka and rockfish (see Carroll, 1988). Therefore, the sbGnRH gene could have arisen by gene duplication or another mechanism between the emergence of the Cyprinodontoformes (medaka) and Scorpaeniformes (rockfish).

The zebrafish had only two forms of GnRH, cGnRH-II and sGnRH detected in brain extracts. Other fish in the same order, such as goldfish (*Carassius auratus*) also have cGnRH-II and sGnRH in their brains (Sherwood and Harvey, 1986; Kah et al., 1988). One can not conclude that three forms of GnRH developed only in the perch-like fishes and neighbouring orders because three forms have been identified from the brains of herring (Chapter 3) and from an Ostariophysean fish, *Prochilodus lineatus* (Somoza et al., 1994) by HPLC-RIA. Clearly, a number of extant teleosts including herring, sabalo, rockfish, cichlids, sea bream, pumpkinseed and snook (Sherwood et al., 1993b) have three forms of GnRH in their brains. This demonstrates that a third form in addition to cGnRH-II and sGnRH may have evolved more than once. It is also possible that further studies will show that the third GnRH gene emerged in a fish ancestral to herring and was retained, but with nucleotide substitutions in the ancestral fish that led to the rockfish and perch-like fish.

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J. Powell.

## Chapter 7 The origin of vertebrate GnRH

### INTRODUCTION

In the preceding chapters, I have presented evidence to show that the number and structure of GnRH peptides in the brains of boney fishes change throughout the evolution of fish. The most conserved of the GnRH peptides is cGnRH-II, which is present from the early-evolved boney fishes to the most recently evolved groups. The identification of several different GnRH peptides in axons terminating in the pituitary gives direct evidence that the structural change in GnRH as a result of evolution of GnRH has not altered the central role of GnRH in reproduction. One clear concept concerning the structure and function of GnRH has emerged from this study: the pituitary form of GnRH that acts as the releaser of the gonadotropins may vary with respect to structure among groups of fish, whereas the sGnRH and cGnRH-II forms are tightly conserved. In the fish studied to date, all boney fish have cGnRH-II and all teleosts except the butterflyfish, eel and catfish have sGnRH.

Nine of the 10 forms of GnRH identified in vertebrates are present among the fishes; only chicken GnRH-I has not been detected. Evidence that the immediate ancestors of early boney fishes and tetrapods had mGnRH is supported by the identification of mGnRH in sturgeon (Chapter 2). The form of GnRH present in cartilaginous fishes is not mGnRH. This evidence opens the question of whether a hypothetical ancestral form of GnRH exists. In dogfish, *Squalus*

*acanthias*, the form of GnRH is dogfish GnRH. A second form of GnRH is present in dogfish and is cGnRH-II (Lovejoy et al., 1992a). As well, lamprey also have two forms of GnRH: lamprey GnRH-I and lamprey GnRH-III (Sherwood et al., 1986a; Sower et al., 1993). The presence of two forms in jawless and jawed groups of vertebrates does not clarify the time of gene duplication from an ancestral GnRH. The duplication event could have occurred after the emergence of each of the two groups from the stem line or prior to the emergence of jawless fishes. GnRH is clearly established near the emergence of the vertebrates as shown by GnRH in lamprey, although the structure of GnRH in the hagfish has not been reported. The puzzling question concerns the evolutionary origin of GnRH: is GnRH present in protochordates and does it have a reproductive function.

irGnRH was first identified in the ascidian, *Ciona intestinalis*, using an antiserum raised against mGnRH (Georges and DuBois, 1980). irGnRH was localized by immunocytochemistry to the area between the neural gland and neural ganglion. The posterior roots of the neural ganglia and the dorsal strand were associated with irGnRH cells or fibres. Subsequently, two groups of investigators identified irGnRH in chromatographic eluates. Using column chromatography, Dufour and coworkers (1988) described an irGnRH peptide of a molecular mass similar to that of mGnRH in *Ascidella aspersa*. Later, Kelsall and coworkers (1990) used HPLC and RIA to analyse extract from the tunicate

*Chelyosoma productum*. These workers showed that extracts of the tunicate neural complex contained irGnRH that eluted in a broad area not corresponding to any specific standard GnRH peptides. They also used immunocytochemistry to show that antiserum BLA-4 cross-reacted with irGnRH nerve fibres in at least one posterior and anterior root of the tunicate neural ganglion.

Tunicata is an interesting group in which to study GnRH. The neural ganglion lies dorsal to the neural gland; the latter has been suggested to have a pituitary-like function. Irrespective of the vertebrate pituitary homology, involvement of neural and other tissues that contain irGnRH are of interest with respect to the evolution of GnRH structure and function. In collaboration with S. Reska-Skinner, I investigated the vertebrate origin of GnRH in the tunicate *Chelyosoma productum* by identifying the primary structure of the irGnRH observed by Kelsall and coworkers (1990).

## **MATERIALS AND METHODS**

### **Extraction of peptides from tissues**

Animals (n=1200) were gathered from the waters of Juan de Fuca Strait by SCUBA divers in October, 1993 and held at University of Victoria marine facilities. The dorsal portion of the animals including the neural ganglion, the neural gland and the dorsal strand was dissected after removal of the tunic, the covering for which the animal is

named. Frozen tissues (1300g) were stored at  $-80^{\circ}\text{C}$  until they were powdered and subjected to extraction of peptides as described in Chapter 2.

#### **Purification of GnRH peptides**

Extract containing tunicate peptides was divided into three equal portions and pumped onto three Sep-Pak cartridge HPLC columns as described (Chapter 2). The material was eluted as described for Sep-Pak HPLC and assayed for irGnRH. Further purification of GnRH peptides followed the procedures outlined for herring GnRH with the following exceptions. An area of irGnRH that eluted early in the Sep-Pak HPLC was detected by antiserum GF-4 and was called Tunicate GnRH-I. This material was further purified and assayed with antiserum GF-4 only. A later-eluting area of irGnRH from Sep-Pak HPLC was detected by antiserum BLA-5: this material was called Tunicate GnRH-II and purified.

In the  $\text{C}_{18}$  TFA step of purification, the Tunicate GnRH-II irGnRH material was applied to the column and could not be eluted using TFA. An additional step of isocratic TEAP using 30% acetonitrile was used to elute the material.

#### **Radioimmunoassay (RIA)**

RIA was done using the described methods (Chapter 2). Antisera GF-4 and BLA-5 were used during the purification procedure. In addition, antiserum R-42 was used in the last step of the purification for confirmation of intact GnRH

molecules. The binding ( $B/B_0$ ) and limit of detection was the same as previously reported (Chapter 3) for each antiserum.

#### **Peptide sequencing and determination of mass**

Methods for the determination of peptide sequence and mass were as described for herring (Chapter 3) with the following exception. irGnRH material purified by narrow-bore HPLC had a mass of  $>2000\text{Da}$ . This material was subjected to a reduction reaction which resulted in two identical molecules that were then sequenced and the mass determined.

### **RESULTS**

#### **Purification and sequence identification of Tunicate GnRH-I**

irGnRH detected by antiserum GF-4 in Sep-Pak HPLC eluates was further purified by four more steps before narrow-bore HPLC (Figs 7.1 and 7.2). Antiserum BLA-5 did not detect Tun-1 throughout the purification procedure. However, antiserum R-42 detected 1.7ng of irGnRH in eluates from the phenyl column TFA HPLC indicating that an intact form of GnRH was present. After this step, GF-4 detected 0.4ng of irGnRH. Yield of purified Tunicate GnRH-I from narrow-bore HPLC was estimated at  $1\mu\text{g}$ , indicating that antisera cross-reactivity with Tunicate GnRH-I was less than 0.1%. This material was applied to narrow-bore HPLC and subjected to

Figure 7.1. irGnRH in HPLC fractions from Sep-Pak cartridge columns. The irGnRH was detected by antisera GF-4 (dark bars) and BLA-5 (stippled bars). Fractions designated Tunicate GnRH-I and Tunicate GnRH-II were individually combined, reduced in volume and further purified. Solid line indicates percent acetonitrile.

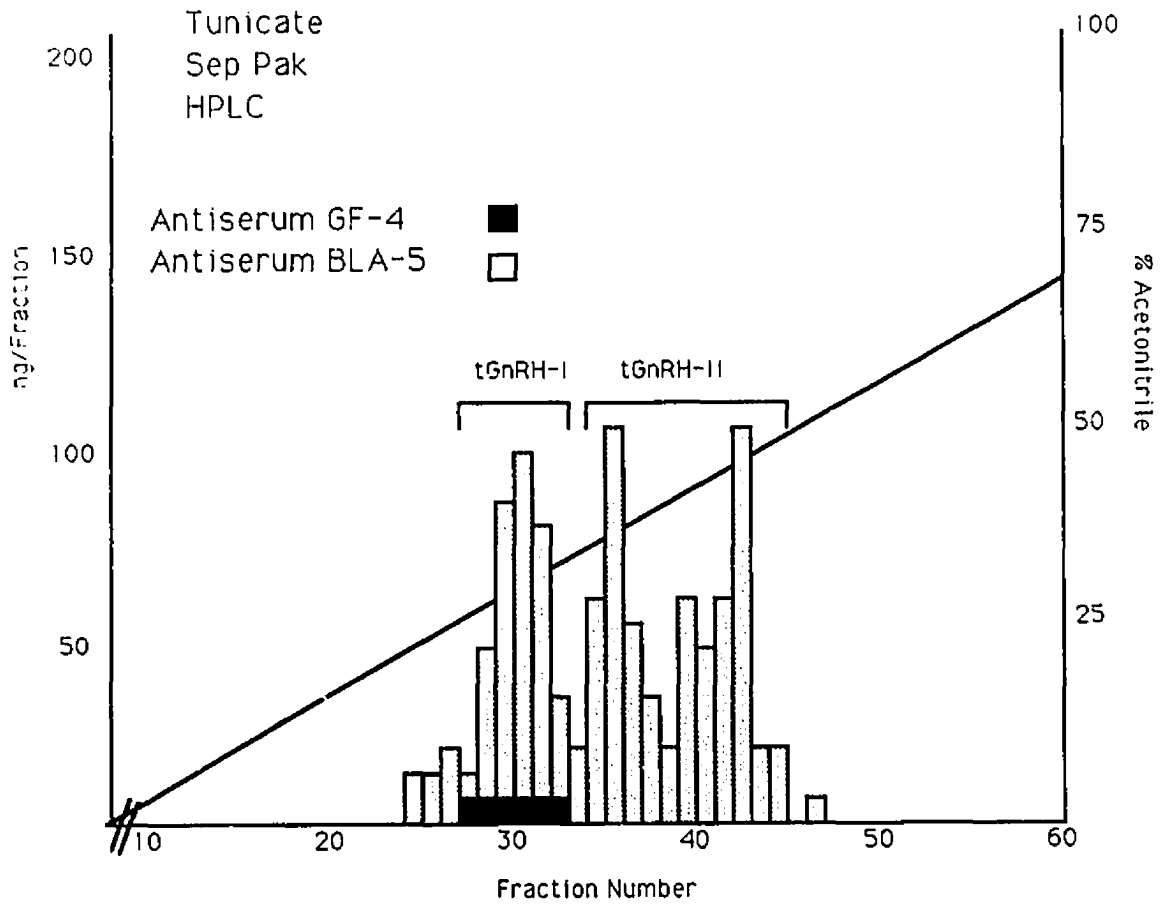
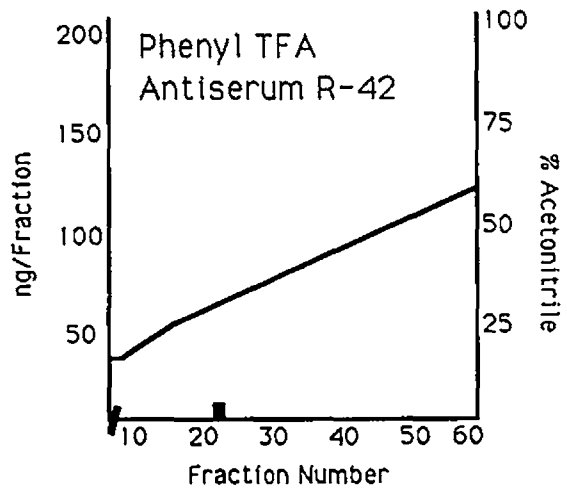
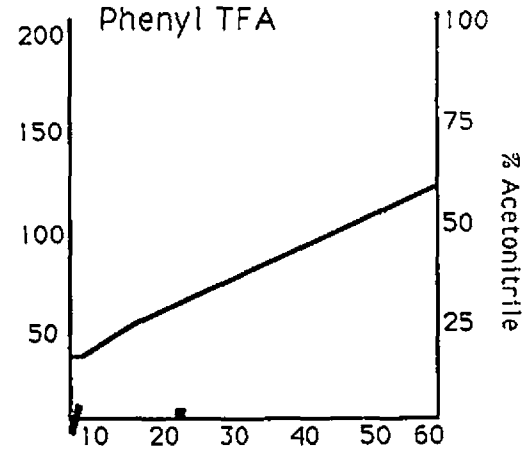
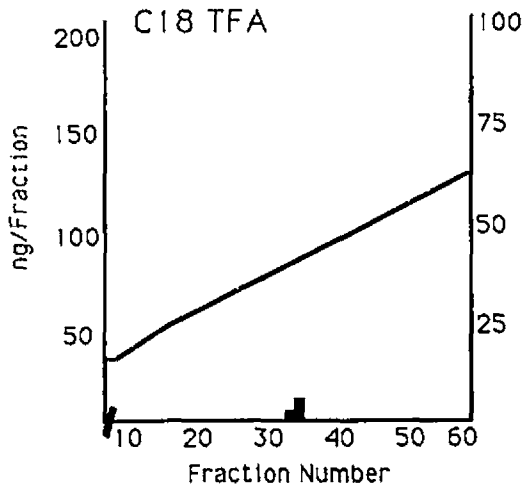
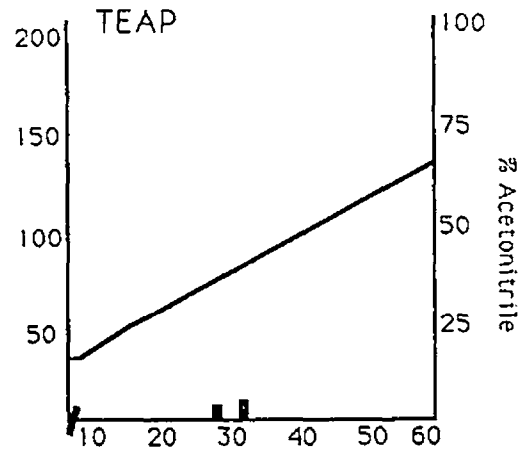
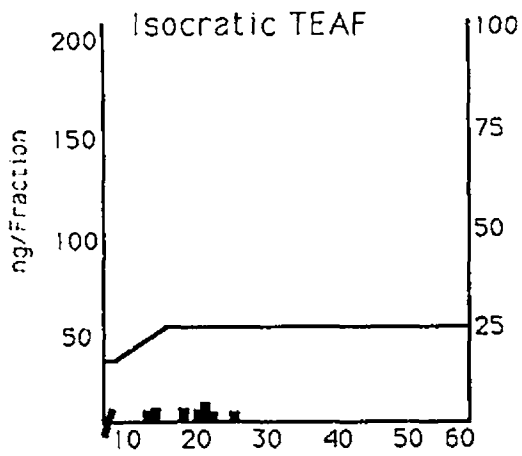


Figure 7.2. irGnRH detected in the eluates of HPLC steps in the purification of Tunicate GnRH-I. Fractions with irGnRH were combined and further purified by successive steps: isocratic TEAF, gradient TEAP, C<sub>18</sub> TFA and phenyl TFA. Antiserum GF-4 was used in each step of the purification. Antiserum R-42 was used in the last step to confirm an intact GnRH peptide. irGnRH from the last step was further purified by narrow-bore HPLC, digested with pyroglutamyl aminopeptidase and sequenced for amino acids. Solid lines indicate percent acetonitrile.

Antiserum GF-4 Tunicate GnRH-I



Edman degradation sequencing. Failure to produce a signal during Edman degradation indicated a blocked N-terminus. The material was digested with pyroglutamyl aminopeptidase as described (Chapter 2) and sequenced. The sequence of Tunicate GnRH-I was determined to be:  
pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly.

#### **Purification and primary structure of tunicate GnRH-II**

Tunicate GnRH-II purified from irGnRH material that eluted late from Sep-Pak HPLC (Figs 7.1 and 7.3) was further purified using narrow-bore HPLC. Edman degradation sequencing was attempted, but failure to produce a signal indicated a blocked N-terminus. As well, mass determination indicated a mass >2000Da. After the reduction reaction and digestion with pyroglutamyl aminopeptidase, Edman degradation yielded the sequence that was determined to be:  
pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly.

#### **DISCUSSION**

The identification of tunicate GnRH-I (tGnRH-I) and tunicate GnRH-II (tGnRH-II) extends the known forms of GnRH to 12 peptides (Fig. 7.4) As well, this is the first report of the primary structure for GnRH peptides in an invertebrate. The identification of GnRH in a tunicate opens the question of whether GnRH existed before the pituitary evolved. A tunicate pituitary can not be ruled out yet, but it is known that the neural gland previously

Figure 7.3. irGnRH detected in the eluates of HPLC steps in the purification of Tunicate GnRH-II. Fractions with irGnRH were combined and further purified by successive methods: isocratic TEAF, TEAP, C<sub>18</sub> TFA, isocratic TEAP and phenyl TFA. Antiserum BLA-5 was used in each step of the purification, antiserum R-42 was used in the last step to confirm an intact GnRH peptide. irGnRH from the last step was further purified by narrow-bore HPLC, digested with pyroglutamyl aminopeptidase and sequenced for amino acids. Solid line indicate percent acetonitrile.

Antiserum BLA-5 Tunicate GnRH-II

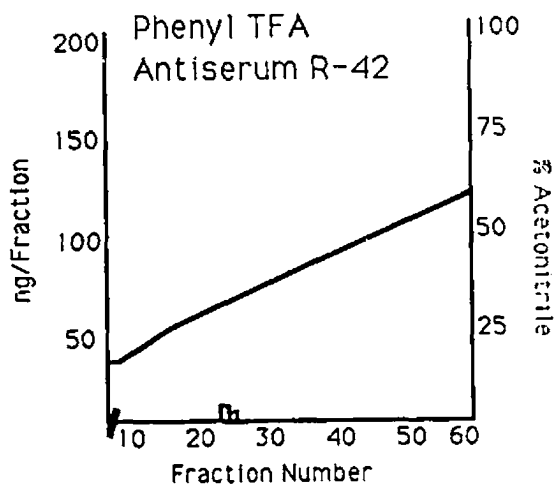
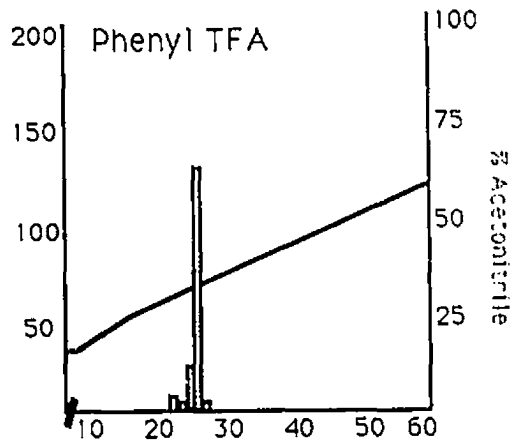
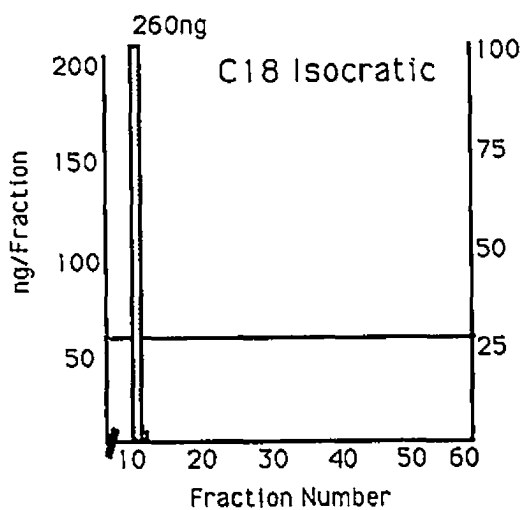
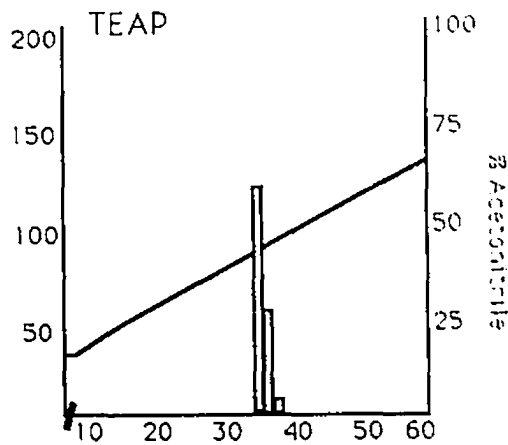
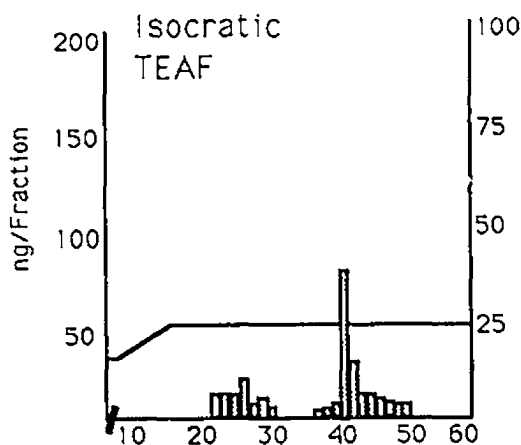


Figure 7.4. Amino acid sequence of the tunicate GnRH peptides. Amino acids 5 to 8 of tunicate GnRH peptides are shown in bold to emphasize the main area of difference.

	1	2	3	4	5	6	7	8	9	10
tGnRH-I	pGLU	-HIS	-TRP	-SER	- <b>ASP</b>	- <b>TYR</b>	- <b>PHE</b>	- <b>LYS</b>	-PRO	-GLY-NH <sub>2</sub>
tGnRH-II	pGLU	-HIS	-TRP	-SER	- <b>LEU</b>	- <b>CYS</b>	- <b>HIS</b>	- <b>ALA</b>	-PRO	-GLY-NH <sub>2</sub>

thought to be homologous to the vertebrate pituitary, is derived from the neural tube and not the oral cavity as in vertebrates (Elwyn, 1937).

The amino acid sequence of both tunicate GnRH peptides is strikingly similar to vertebrate GnRH peptides in that the peptides are decamers with N-termini modified to form pyrroglutamyl residues. Amino acid identity is high with vertebrate GnRH peptides, ranging from 50% with lamprey GnRH-I to 60% for other forms. Identical residues at positions 1-3 in tunicate GnRH peptides compared with other vertebrate GnRH peptides indicate that the peptide region responsible for gonadotropin release has been unchanged for over 500 million years.

tGnRH-I resembles most closely the phylogenetically ancient lGnRH-III with identical amino acids in positions 1-4 and 8-10. As well, tGnRH-I is predicted to form a salt bridge between the negatively charged Asp<sup>5</sup> and positively charged Lys<sup>8</sup>. The salt bridge phenomenon is shared by both lamprey GnRH peptides which have bridges between residues 6 and 8. This structure is thought to stabilize the U-conformation of the beta-turn configuration of the molecule. Potent synthetic analogues containing such a bridge are thought to reinforce the U-conformation associated with receptor binding (Karten and Rivier, 1978).

tGnRH-II is the first GnRH peptide to incorporate a cysteine residue. This amino acid is the basis of the formation of a homodimer and it was the dimer that was

purified from tunicate tissues. Dimerization of receptors is thought to occur in mammals before receptor activation is complete. After aggregation of occupied receptors, LH is released from porcine pituitary cells (Gregory et al., 1982). tGnRH-II may provide an ancestral link to this mechanism. In addition, dimerization may reduce degradation if the enzymes are inhibited from making contact with the peptide due to spatial constraints.

There are at least two distinct GnRH peptides identified by primary structure in the tunicate *Chelyosoma productum*. Therefore, the paired presence of GnRH forms in the vertebrate brain can be phylogenetically extended to include the tunicates. This observation does not fit the idea that the entire genome duplicated close to the appearance of the vertebrates. If large segments of DNA, such as the *Hox* gene clusters duplicated, then the GnRH genes do not appear to have been included in the duplication. Garcia-Fernandez and Holland (1994) note that a single cluster of *Hox* genes is present in amphioxus, which emerged after tunicates, but that at least three more *Hox* gene clusters are present in zebrafish and four clusters in mammals. It might be predicted that a single copy of the GnRH gene would be present in tunicates and multiple forms would be present in vertebrates. However, it can not be discounted that a duplication of a GnRH gene has occurred in *C. productum* after its emergence from the stem line.

The unresolved aspect of two distinct GnRH structures in a tunicate is the role of each GnRH peptide. The tunicate has no clearly defined homologue to the vertebrate pituitary. Indeed, the origin of the pituitary is vigorously debated (Ruppert, 1990; Nozaki and Gorbman, 1992). Kelsall and coworkers (1990) noted a concentration of irGnRH fibres in the anterior and posterior roots of the neural ganglion of *C. productum*. A later study by Mackie (1995) shows that irGnRH in whole mount preparations of *Ciona intestinalis* is in neurons that form a dorsal strand plexus that stretches from the neural gland/neural ganglion complex to the gonads and sends branches to other tissues. This implies that GnRH may act as a neurosecretory hormone, but the action may be directly on target tissues without the involvement of a pituitary gland. Further research is required to determine the site of GnRH receptors in the tunicate. Identification of the primary structure of tunicate GnRH peptides is the first step toward investigation of the possible roles of GnRH in tunicates.

## Chapter 8 General Conclusions

### Identification of GnRH peptides

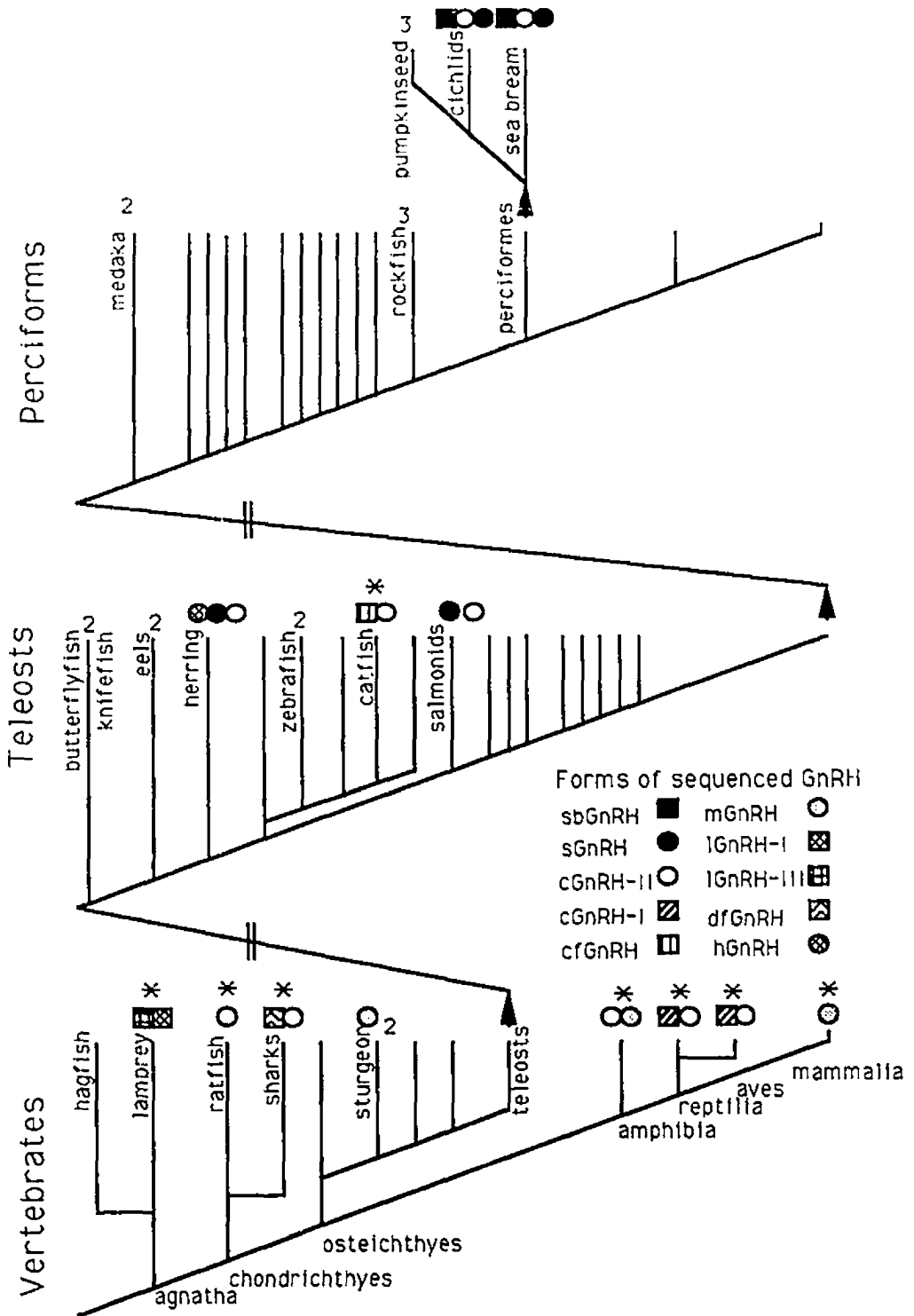
The determination of GnRH primary structures leads to insight concerning the evolution of neuropeptides in boney fishes. At the beginning of my thesis research, the early-evolved boney fishes were thought to have two forms of GnRH in their brains: mGnRH and cGnRH-II based on indirect methods (Sherwood et al., 1991). However, in a euteleost such as the salmon, the mGnRH form had disappeared, the cGnRH-II form was shown indirectly to be retained and a novel form, sGnRH appeared as shown by primary structure (Sherwood et al., 1983; Sherwood, 1986). The explanation of the transition from one form of GnRH to another required the direct identification by primary structure of mGnRH in an early-evolved boney fish such as sturgeon. Thereafter, the disappearance of mGnRH in boney fishes and the appearance of sGnRH could be determined in finer detail by examining the fish assemblages that emerged in evolution between sturgeon and salmon and also after salmon.

In this thesis, the primary structures of GnRH peptides were established in representatives of a primitive boney fish (sturgeon), an early-evolved teleost (herring), an early-evolved euteleost (salmon) and in three species of the recently-evolved perciforms (sea bream and two species of cichlids). In all, thirteen GnRH primary structures from these fish are described including two novel forms.

The identification of the primary structure of mGnRH in sturgeon establishes this peptide as phylogenetically ancient and common to two groups of divergent vertebrates: boney fishes and tetrapods (Fig. 8.1). mGnRH is shown also to be present in primitive boney fishes including the butterflyfish by using indirect HPLC-RIA methods. Between the emergence of the knifefish and the herring, the mGnRH form disappears and two forms, sGnRH and hGnRH appear (Fig. 8.1). Herring are the first living boney fish to have three forms of GnRH in the brain of one species detected by primary structure. Interestingly, mGnRH and sGnRH are never located together in the brains of any fish examined. As well, hGnRH is not seen in any of the other fish examined.

Three forms of GnRH were also identified in the brains of the sea bream, pumpkinseed, two species of tilapia and another cichlid, *H. burtoni*. The three forms were determined by primary structure to be sGnRH, cGnRH-II and a novel form, sbGnRH. The determination of GnRH primary structures from pituitaries of herring (hGnRH) and *H. burtoni* (sbGnRH) and the relative abundance of sbGnRH in the pituitaries of other perciforms, suggests the role of gonadotropin releaser. As well, the detection of sbGnRH in many of the perciform fishes indicates that the distribution of sbGnRH is widespread among species.

Figure 8.1. Phylogenetic distribution of the forms of GnRH among the vertebrates. Where a sequence of the peptide has been determined, a symbol represents the form(s) present. An asterisk (\*) appears over peptides that were not determined in animals used in this thesis. The number of GnRH forms in representatives of a given order is above individual vertical lines. These later data are based on HPLC and RIA studies. In orders of fishes where forms of GnRH have not been investigated in this study, the vertical bar is not identified. sbGnRH= sea bream GnRH; sGnRH= salmon GnRH; cGnRH-II= chicken GnRH-II; cGnRH-I= chicken GnRH-I; cfGnRH= catfish GnRH; mGnRH= mammalian GnRH; lGnRH-I= lamprey GnRH-I; lGnRH-III= lamprey GnRH-III; dfGnRH= dogfish GnRH; hGnRH= herring GnRH.



### Mechanism of GnRH Evolution

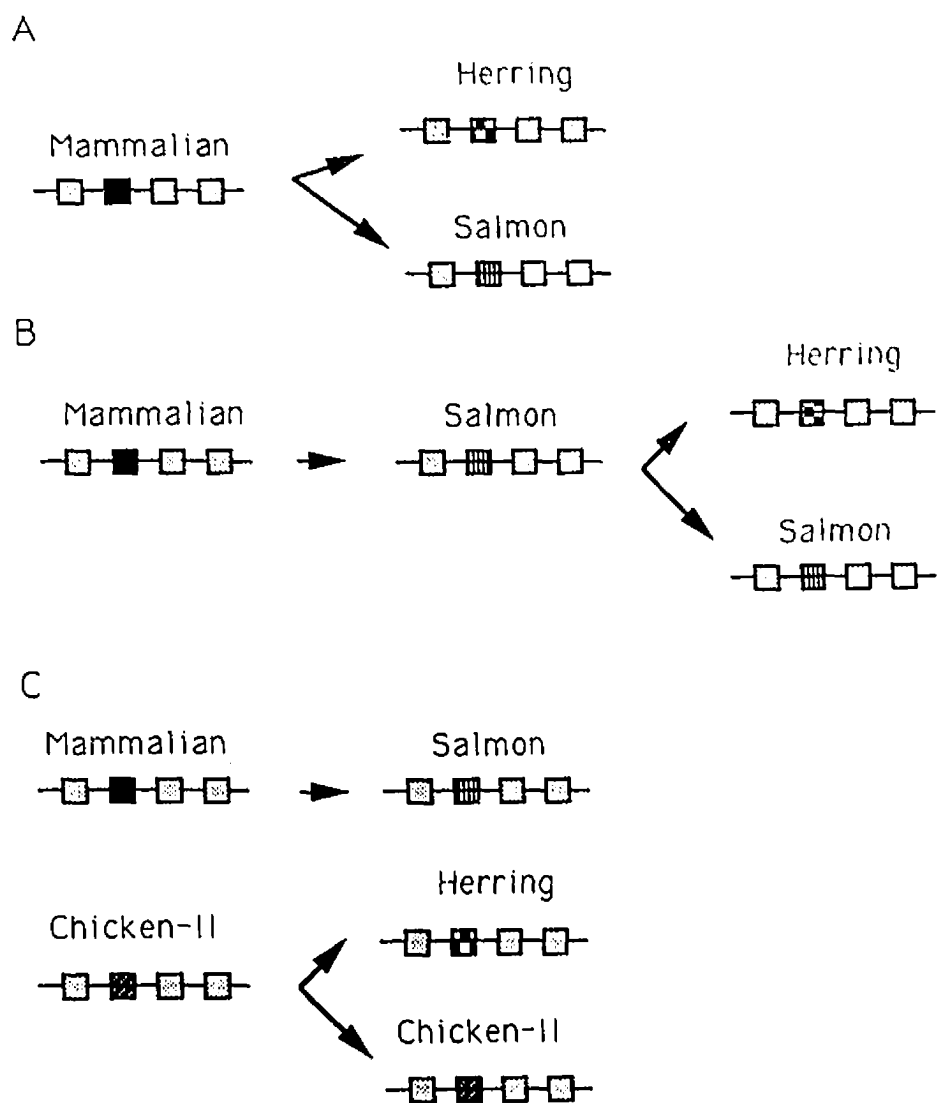
A gene duplication followed by nucleotide substitutions is the most likely explanation of the emergence of the salmon and herring GnRH peptides (Fig. 8.2). In this hypothesis, one possibility is that the mGnRH gene was duplicated and simultaneously had nucleotide substitutions within the peptide coding region (exon 2) that produced two new GnRH peptides: hGnRH and sGnRH (Fig. 8.2A).

Alternatively, a less parsimonious explanation is that the mGnRH gene is altered to code for sGnRH (Fig. 8.2B).

Thereafter, a duplication event resulted in two GnRH genes, one that retained the coding for sGnRH and the other gene that was altered to become hGnRH. This is the more likely explanation because only sGnRH and cGnRH-II are detected in knifefish, whilst sGnRH, cGnRH-II and hGnRH are detected in herring. A third scenario of gene evolution (Fig. 8.2C) involves the duplication and nucleotide substitution of the cGnRH-II gene to produce the hGnRH form. However, this is unlikely because hGnRH was isolated from the herring pituitary where it was the most abundant form. It has been established (see Northcutt and Muske, 1994) that cGnRH-II is not directly involved in the release of pituitary gonadotropins. Therefore, it is unlikely that hGnRH arose from the cGnRH-II gene.

A similar duplication and nucleotide substitution scenario is plausible for the emergence of sbGnRH. sGnRH is present in perciform fishes, but hGnRH is not. It is

Figure 8.2. Possible mechanism of GnRH gene evolution. A) Mammalian GnRH undergoes duplication followed by nucleotide substitution in exon 2 of both genes to give rise to herring GnRH and salmon GnRH. B) Mammalian GnRH undergoes nucleotide substitution to give rise to salmon GnRH, which duplicates followed by nucleotide substitutions in only one gene copy giving herring GnRH. C) Mammalian GnRH undergoes nucleotide substitution to give rise to salmon GnRH. Chicken GnRH-II duplicates and one copy undergoes nucleotide substitution to give rise to herring GnRH. Boxes indicate the four exons of the GnRH gene. The second box indicates the GnRH form; solid: mammalian GnRH, checkered: herring GnRH, vertical lines: salmon GnRH, oblique lines: chicken GnRH-II.



equally likely that sbGnRH is a modification of the sGnRH gene as of the hGnRH gene because each shares 80% identity with the amino acid sequence of the sbGnRH peptide. If the origin of hGnRH and sbGnRH occurred close together in evolution, then sbGnRH would be present in species that evolved near the emergence of the herring. This would then indicate that sbGnRH is one of the ancestral forms of euteleosts. However, neither hGnRH nor sbGnRH was detected in any of the salmonids surveyed. Further, sbGnRH was not detected in the phylogenetically distinct zebrafish and medaka, but was detected in the rockfish. From these data one may infer that the appearance of sbGnRH occurred closer to the evolution of the perciform fishes and that sbGnRH is a derived rather than ancient form. However, stem-line species of the salmonids or other later-evolving orders of fish may prove to have sbGnRH in their brains.

#### **Physiological roles of GnRH**

In the present study, the identity of sGnRH is confirmed for salmon and the second form in salmon brains is shown by primary structure to be cGnRH-II. In this study, injection of a sGnRH analogue was demonstrated to advance the ovulation date prior to that of saline-injected controls. The mechanism by which the ovulation date was advanced is shown to be associated with a decrease in plasma GTH-I levels and increase in plasma GTH-II levels prior to ovulation. This implies that at least one function of GnRH

in the events leading to ovulation is to elicit a decrease in GTH-I levels concomitant with an increase in GTH-II levels. This has some distinct aspects compared to the mammalian system where mGnRH from the hypothalamus elicits an early release of follicle stimulating hormone, but triggers both follicle stimulating hormone and luteinizing hormone release to induce ovulation (see Fink, 1988).

In addition to a putative role in releasing gonadotropins in fish, sbGnRH may cause release of other pituitary hormones including prolactin. GnRH is also known to release growth hormone in fish (Marchant et al., 1989). The observation that GnRH releases pituitary hormones other than the gonadotropins is puzzling, but it is possible that these pituitary hormones have secondary functions, either physiological or behavioural and may not be related to reproduction. Equally likely is that reproduction is governed by a suite of pituitary hormones and that the primary releaser of the gonadotropins (GnRH) serves to coordinate these functions. The establishment of the native GnRH peptides by primary structure is critical to further investigation of other possible roles.

#### **Origin of vertebrate GnRH**

The origin of GnRH peptides predates the emergence of the chordates. The tunicate, *Chelyosoma productum*, has two distinct forms of GnRH as identified by primary structure (Fig. 8.3). The function of these two forms is

Figure 8.3. Amino acid sequence of the twelve known GnRH peptides. Amino acids 5 to 8 of tunicate GnRH peptides are shown in bold to emphasize the main area of difference compared to vertebrate GnRH peptides.

	1	2	3	4	5	6	7	8	9	10
TUNICATE-I	pGLU	HIS	TRP	SER	ASP	TYR	PHE	LYS	PRO	GLY-NH <sub>2</sub>
TUNICATE-II	pGLU	HIS	TRP	SER	LEU	CYS	HIS	ALA	PRO	GLY-NH <sub>2</sub>
	pGLU	HIS	TRP	SER	LEU	CYS	HIS	ALA	PRO	GLY-NH <sub>2</sub>
LAMPREY-III	pGLU	HIS	TRP	SER	HIS	ASP	TRP	LYS	PRO	GLY-NH <sub>2</sub>
LAMPREY-I	pGLU	HIS	TYR	SER	LEU	GLU	TRP	LYS	PRO	GLY-NH <sub>2</sub>
SEA BREAM	pGLU	HIS	TRP	SER	TYR	GLY	LEU	SER	PRO	GLY-NH <sub>2</sub>
MAMMAL	pGLU	HIS	TRP	SER	TYR	GLY	LEU	ARG	PRO	GLY-NH <sub>2</sub>
CHICKEN-I	pGLU	HIS	TRP	SER	TYR	GLY	LEU	GLN	PRO	GLY-NH <sub>2</sub>
CATFISH	pGLU	HIS	TRP	SER	HIS	GLY	LEU	ASN	PRO	GLY-NH <sub>2</sub>
HERRING	pGLU	HIS	TRP	SER	HIS	GLY	LEU	SER	PRO	GLY-NH <sub>2</sub>
SALMON	pGLU	HIS	TRP	SER	TYR	GLY	TRP	LEU	PRO	GLY-NH <sub>2</sub>
CHICKEN-II	pGLU	HIS	TRP	SER	HIS	GLY	TRP	TYR	PRO	GLY-NH <sub>2</sub>
DOGFISH	pGLU	HIS	TRP	SER	HIS	GLY	TRP	LEU	PRO	GLY-NH <sub>2</sub>

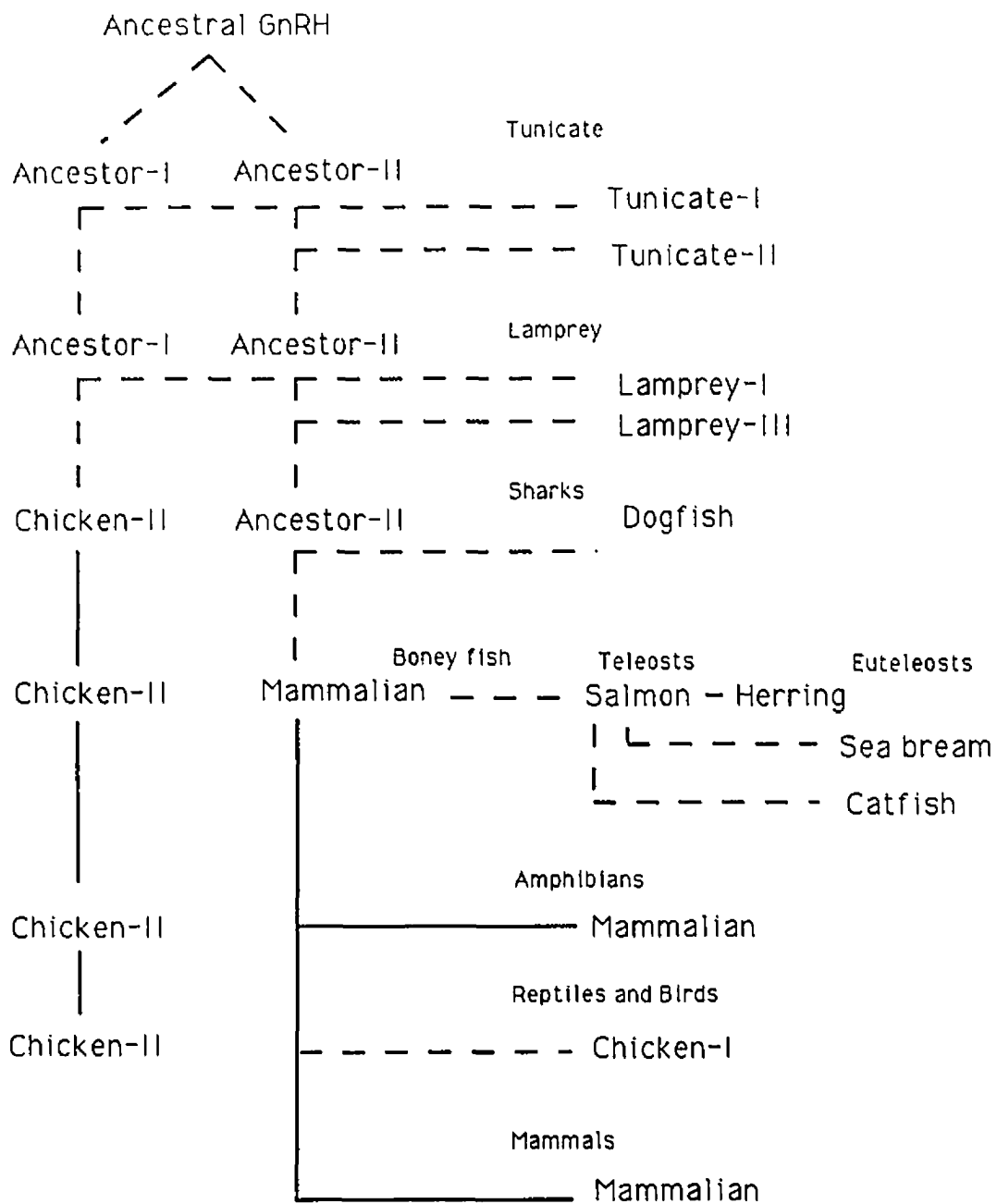
undetermined. However, they bear striking resemblance to vertebrate GnRH peptides in sequence identity: residues 1-4, 9 and 10 are conserved in both tunicate and vertebrate GnRH peptides. The only exception is lamprey GnRH-I in which amino acid 3 differs compared with the other eleven GnRH peptides (Fig. 8.3). Therefore, from a phylogenetic perspective, the tunicate represents the most ancient lineage from which GnRH peptides have been identified.

The presence of two GnRH peptides implies that a duplication of the GnRH gene occurred either prior to the emergence of the protochordates or during the evolutionary period between the stem and extant tunicates (Fig. 8.4).

This paired occurrence of GnRH peptides is continued into all classes of vertebrates including at least one placental mammal (see Sherwood et al., 1993a; Dellovade et al., 1993). The form of GnRH present among the classes varies. This indicates that portions of the peptide can be changed without altering the gonadotropin function. The conservation of amino acids 1, 2, 4, 9 and 10 indicates similar functions among vertebrates and implies homology with the tunicate GnRH peptide.

Essential to understanding GnRH function in tunicates will be the identification of the GnRH receptor. Firstly, this will enable the identification of target tissues and give insight into the functions of the two different GnRH peptides; binding affinity will also be important to

Figure 8.4. Hypothetical scheme for the evolution of known forms of GnRH from an ancestral GnRH peptide. Dotted lines indicate that the derivation of a peptide is not known with certainty, but is based on amino acid sequence and appearance or disappearance of GnRH peptides.



understanding function. Secondly, if the receptor protein sequence is conserved, function may also be conserved. One principle interest is the evolution of the chordate nervous system and its relationship with the development of the brain-pituitary-gonadal axis. Identification of tunicate GnRH peptides is an additional step into understanding the origins of neuropeptide function in reproduction. The possibility of a direct relationship between GnRH neurons and the gonad in tunicates remains to be investigated in detail. The conservation of GnRH structure in an animal that appears not to have a vertebrate-type pituitary may indicate an ancestral condition for direct neural control of reproduction.

### **Molecular investigations**

The identification of four additional novel GnRH peptides indicates that the evolution of this neuropeptide family is complex. The determination of the sequence and the organization of each GnRH gene will elucidate the evolution of this peptide because additional information is available to compare with peptide structure. Further, tissue expression for each GnRH mRNA can be determined, lending insight to novel functions. Finally, the ontogeny of GnRH neurons can be determined if the cells can be definitively identified by antisera or molecular probes.

The identification of sbGnRH among fishes establishes that six distinct forms of GnRH are present in boney fishes as

determined by primary structure. Future studies on the primary structure, mRNA and gene will determine whether additional novel forms are present and will refine the phylogenetic position and mechanisms by which these peptides evolved. This will provide a foundation on which to continue studies to determine the functions and control of expression for each GnRH form.

#### **Future directions**

Work in this thesis investigated GnRH in 22 species out of a possible 22,000 species of living boney fishes. The possibility that other novel forms exist in the brains of other assemblages is present. The distribution of shared or novel GnRH forms among the other fishes will give insight regarding the evolution of this neuropeptide.

There is a need to determine the GnRH structure in species that arose between the tunicates and lamprey such as amphioxus and the cephalochordate hagfish. Thereby, similarities in form and function can be determined and applied to vertebrate evolution. This juncture in evolution encompasses the appearance of Hatschek's pit and reminds us of the argument that Hatschek's pit is a predecessor of the vertebrate pituitary (Nozaki and Gorbman, 1992). Identification of GnRH in these groups would be valuable in determining the role of GnRH at the transition between invertebrates and vertebrates.

The identification of two GnRH peptides in tunicates indicates that GnRH evolution is likely to extend into the more ancient deuterostomes such as hemichordates and echinoderms. Further, the origin of GnRH peptides may extend to protostomes. Essential to this investigation is determination of GnRH primary structure for the design of antisera and molecular probes.

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### Publications

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