Structure and Function of Gonadotropin-Releasing Hormone in the Thai Catfish, *Clarias macrocephalus*.

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ABSTRACT

Two forms of gonadotropin-releasing hormone (GnRH) were extracted from brain-pituitary tissues of two species of Thai catfish, *Clarias macrocephalus* and *C. batrachus*. The peptides were detected using high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA). The amino acid sequences of both forms were determined using Edman degradation. One form of GnRH in the brain-pituitary tissues of the Thai catfish was novel, whereas the second form of GnRH was identical to chicken GnRH-II. The presence of the N-terminal pGlu residue in both peptides was established by digestion with pyroglutamyl aminopeptidase. In addition, catfish GnRH-I was studied by mass spectrometry.

The localization of these two peptides was determined to be in the discrete brain areas and in the pituitary of female and male catfish, *C. macrocephalus*, using heterologous and homologous radioimmunoassays. Initially a heterologous RIA was used with mammalian GnRH as iodinated tracer and standard, and an antiserum made against salmon GnRH. Catfish GnRH-I (novel form) was found in most areas of the female and male brain with the

highest content and concentration in the female pituitary and in the male hypothalamus. Catfish GnRH-II (chicken GnRH-II) was found with the highest content in the female cerebellum and highest concentration in the pituitary. Catfish GnRH-II (chicken GnRH-II) was found with the highest content and concentration for males in the same area, hypothalamus. Additionally, a homologous RIA was used with catfish GnRH-II (chicken GnRH-II) as iodinated tracer and standard, and an antiserum prepared against chicken GnRH-II. Catfish GnRH-II was detected with the highest content and concentration in the cerebellum of both sexes. These values are higher than the results obtained in the heterologous assay. The location of catfish GnRH-I suggests that it plays a role in regulating the release of gonadotropin from the pituitary since the high content and concentration of this immunoreactive GnRH are detected in the hypothalamus and pituitary gland. In contrast, catfish GnRH-II may act as a neurotransmitter in the catfish brain, in particular in the cerebellum where a high content and concentration of immunoreactive GnRH are detected.

Physiological *in vivo* studies indicate that catfish GnRH-II is more effective than catfish GnRH-I and other forms of GnRH such as mammalian and dogfish GnRH for induction of ovulation in catfish, *C. macrocephalus*.

Eight GnRH analogs had varying potencies for the induction of ovulation, but the most effective forms were two forms of catfish GnRH-II (chicken GnRH-II) modified in positions six and ten. *In vitro* studies found that catfish GnRH-I not only causes the release of gonadotropin but also the release of growth hormone in a dose-dependent manner.

The primary structures of the two catfish GnRH peptides are important for understanding the evolution of this family peptide. The novel catfish GnRH shows that only positions 5, 7 and 8 vary in the GnRH molecule in jawed vertebrates, whereas catfish GnRH-II provides direct evidence that the structure of this GnRH is conserved in teleosts.

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LIST OF ABBREVIATIONS

GnRH:	Gonadotropin-releasing hormone
irGnRH:	Immunoreactive gonadotropin-releasing hormone
GtH:	Gonadotropin
LH:	Luteinizing hormone
FSH:	Follicle-stimulating hormone
mGnRH:	Mammalian gonadotropin-releasing hormone
sGnRH:	Salmon gonadotropin-releasing hormone
lGnRH:	Lamprey gonadotropin-releasing hormone
cGnRH-I:	Chicken gonadotropin-releasing hormone, first form isolated
cGnRH-II:	Chicken gonadotropin-releasing hormone, second form isolated
a:	Analog form of gonadotropin-releasing hormone
n:	Native form of gonadotropin-releasing hormone
RIA:	Radioimmunoassay
HPLC:	High performance liquid chromatography

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TO MY FATHER WHO TAUGHT ME PATIENCE

CHAPTER 1

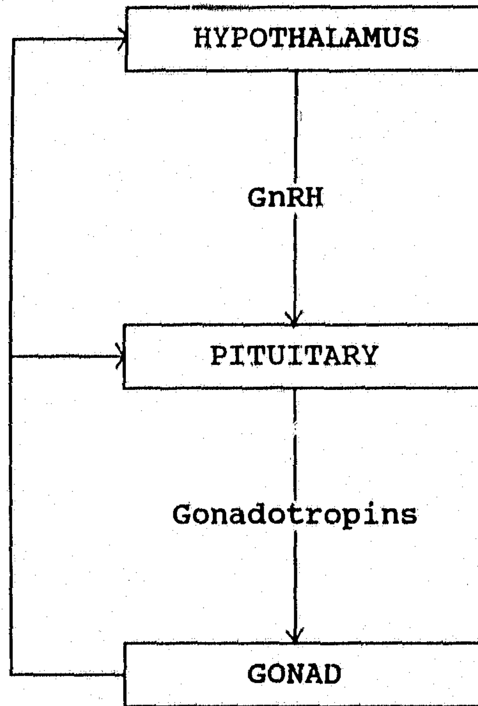
GENERAL INTRODUCTION

AN OVERVIEW

In teleosts, like most vertebrates, the regulation of reproductive processes includes cooperation between brain, pituitary and gonads. Exogenous and endogenous signals are integrated in the brain, which in turn releases a neurohormone near the gonadotropic cells in the pituitary gland. This hormone regulates the secretion of gonadotropins (GtH). Subsequently, GtH controls the release of gonadal steroids, and influences gametogenesis, oocyte maturation, ovulation and spermiation (Fig. 1.1).

The fundamental pattern of neural control of reproduction in vertebrates is present in early-evolving fish (fish that are phylogenetically ancient). This concept includes the use of brain peptides as mediators between the central nervous system (CNS) and the pituitary. Amongst the brain peptides that affect reproduction, gonadotropin-releasing hormone (GnRH) is the best known.

Figure 1.1. Brain-pituitary-gonad relationships.



OVULATION IN FEMALE
SPERMIATION IN MALE

Gonadotropin-releasing hormone was originally named for its ability to cause the release of gonadotropic hormones from the pituitary in mammals. These pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in turn activate gonadal maturation and lead to ovulation. The GnRH isolated from mammals is a peptide with 10 amino acids. This decapeptide has a pyroglutamyl residue at the amino-terminus (N-terminus) and an amidated glycine at the carboxy-terminus (C-terminus).

It is thought that GnRH exists as a folded peptide with a β-turn at residues 6 and 7 (Karten and Rivier, 1986). It is proposed that GnRH binds to the receptor in a folded form that allows covalent bonding of the N- and -C termini. Positions 1 and 10 are conserved in all known forms. It is suggested that this conformation is probably important for effective receptor binding and as a protection against enzymatic degradation. The side chains of histidine (His), tyrosine (Tyr) and arginine (Arg) appear to play an active role in hormone actions (Hazum *et al.*, 1986). In addition, residues 1, 2 and 3 are essential for release of gonadotropins (Conn, 1986; Conn *et al.*, 1987a).

In addition to the structure of mammalian GnRH, the primary structure of GnRH has been determined for five other molecules (Table 1.1): mammalian GnRH (Matsuo *et al.*, 1971; Burgus *et al.*, 1972), chicken I and chicken II GnRH (King and Millar, 1982a, b; Miyamoto *et al.*, 1982, 1983, 1984), salmon GnRH (Sherwood *et al.*, 1983), lamprey GnRH (Sherwood *et al.*, 1986) and dogfish GnRH (Lovejoy *et al.*, 1992). It is suggested that other forms of GnRH also exist (see Sherwood, 1987b). All these known forms of GnRH to date, isolated from five different species, show the greatest variability at position 8 (Table 1.1). However, all positions except 5, 7 and 8 are conserved in five peptides excluding lamprey. Lamprey GnRH has tyrosine (Tyr) at position 3 instead of tryptophan (Trp), but both Tyr and Trp are aromatic amino acids.

Over 2,000 GnRH analogs have been prepared from mGnRH and about 20 analogs for nonmammalian GnRHs. These analogs are used for analysis of the structural requirements for receptor binding, activation, and GnRH metabolic stability (see Crim *et al.*, 1987a). The terminal residues, pyro-Glu^{1} and Gly^{10}-amide, are believed to be involved in the formation of a region which binds to the receptor in all GnRH molecules. The change of either residue rapidly decreases the affinity of the peptide for the receptor (see Conn *et al.*, 1987a).

Table 1.1. Primary structure of six known forms of GnRH

	1 2 3 4 5 6 7 8 9 10
Chicken GnRH-I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2
Mammalian GnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
Chicken GnRH-II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
Dogfish GnRH	pGlu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly-NH_2
Salmon GnRH	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2
Lamprey GnRH	pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2

Substitutions in the first, second and third positions with hydrophobic D-amino acids result in antagonists which retain receptor affinity, but have reduced agonist activity (Conn, 1986).

There is an association between the termination of the action of GnRH and the degradation of the molecule. Enzymatic activities that cleave the peptide bond adjacent to the sixth position nave been reported. Conn *et al.* (1987b) reported that the enzymes cleaving residue 1, pyroglutaminase, and residue 10, deaminase, are also considered to be important in this process.

In contrast, enhancement of activity results from the replacement of the sixth residue with a D-amino acid containing bulky, hydrophobic substitutions, which can keep the conformational stability of GnRH analogs. These substitutions also stabilize the β-turn in the molecule needed for the biological activity. Furthermore, combining the substitution of a D-amino acid at the sixth position and ethylamide at the tenth position enhances receptor binding and biological potency of GnRH analogs (Fujino *et al.*, 1974). For example, the replacement of Gly^6 with the hydrophobic group 3-(2-naptnyl)Ala^6 produces an agonist (Nafarelin) with biological activity 200 times greater than GnRH (see Conn *et al.*, 1987a, b). The biological half-life of Nafarelin in humans is about

2 hours compared to 5-10 minutes for the naturally occurring releasing hormone. It is suggested that Nafarelin binds to a hydrophobic carrier site on serum albumin, which protects the bound peptide from proteolysis. Thus, the analog appears to have a long biological half-life and to act as a circulation source for the drug. A variety of GnRH analogs have been proven to be useful compounds in medicine, agriculture and aquaculture.

In some cultured fish, evidence suggests that the lack of final oocyte maturation, ovulation and spawning is the result of a failure to release gonadotropic hormones (Zohar, 1989). Therefore, the use of the neuropeptide, GnRH, for the induction of the GtH ovulatory surge and ovulation seems to represent the most efficient therapy.

The following sections review the literature concerning the physiology of the GnRH neuronal system and its regulation of the pituitary in teleosts. This provides a background for the present study on the control of reproduction in the Thai catfish, *Clarias macrocephalus*.

NEURAL CONTROL OF REPRODUCTION IN TELEOSTS

In the control of reproduction, the pituitary gland plays a crucial role. This gland consists of an

adenohypophysis and a neurohypophysis. According to the distribution of the different hormone-producing cells, the adenohypophysis can be divided into three parts: the rostral pars distalis, the proximal pars distalis and the neurointermediate lobe (see Van Oordt and Peute, 1983). The gonadotropic cells are located in the proximal pars distalis.

Teleosts appear to have two types of gonadotropin (GtH). Originally, it was thought that these two types were carbohydrate rich and carbohydrate poor (Idler *et al.*, 1975; Idler and Ng, 1979; see review by Idler and Ng, 1983). Both of these types of GtH were isolated from the pituitary of the American plaice, *Hippoglossoides platessoids*, the winter flounder, *Pseudopleuronectes americanus*, and the chum salmon, *Oncorhynchus keta*. One of them was relatively low in carbohydrate content (Con A-I) and had the ability to stimulate the uptake of vitellogenin into oocytes to a greater or lesser extent depending on the species. The other GtH was high in carbohydrate content (Con A-II) and had the ability to stimulate cyclic AMP in gonads, steroidogenesis, oocyte maturation, ovulation and spermiation.

However, Idler *et al.* (1991) have reported recently that the carbohydrate poor GtH is a fragment of the proopiomelanocortin (POMC) molecule. Hence, its status

as a gonadotropin is not certain. Meanwhile, Suzuki *et al.* (1988a) have isolated two distinct GtHs in fish, GtH I and GtH II. Both have sequence similarity with mammalian FSH and LH, but are distinct from the carbohydrate poor molecule. It was suggested that GtH I is involved in vitellogenesis, whereas GtH II is associated with final oocyte maturation and ovulation. The evidence was based on radioimmunoassay measurements of GtH I and GtH II levels in plasma and pituitary, during oocyte development (Suzuki *et al.*, 1988b), and on their potency in stimulating the oocyte maturation (Suzuki *et al.*,1988c).

In mammals, the release of gonadotropins depends on a neurohormone that stimulates not only LH, but also the release of FSH. It is, therefore, now referred to as gonadotropin-releasing hormone (GnRH) rather than luteinizing hormone-releasing hormone (LHRH). It is generally accepted that in all vertebrates, the release of GtHs is stimulated by releasing hormones. These structures resemble that of mammalian GnRH (mGnRH). Thus, GnRH plays a key role in the release of GtHs in mammals, and other vertebrates.

The presence of GnRH activity in the hypothalamus of teleosts such as goldfish, *Carassius auratus* (Crim *et al.*, 1978), winter flounder, *Pseudopleuronectes*

americanus, rainbow trout, *Oncorhynchus mykiss* (Crim and Evans, 1979; 1980), and cichlid, *Sarotherodon mossambicus* (King and Millar, 1980) was demonstrated more than ten years ago (see Peter, 1982; 1983). The evidence was that hypothalamic extracts from carp, *Cyprinus carpio*, had GnRH activity in a fraction with a molecular weight of less than 5000 (Breton *et al.*, 1975). Furthermore, hypothalamic extracts from a wide range of vertebrates cross-reacted with antisera made against mammalian GnRH (King and Millar, 1979; 1980).

Gonadotropin-releasing hormone isolated from mammals (mGnRH) and some of its analogs stimulate GtH secretion and can be used to induce ovulation in teleosts (Crim *et al.*, 1978; Peter and Crim, 1979; Peter, 1980; Weil *et al.*, 1980; Lam, 1982; Donaldson and Hunter, 1983; for review see Zohar, 1989). A combination of the analog, [D-Ala^6]-des-Gly^{10}-GnRH, and crude pituitary extract was used. Peter (1980) showed that multiple injections of GnRH or its analog at low or high doses can cause potentiation or suppression of GtH release in mature male goldfish. He also found that the main difference in the response to GnRH and its analog was that the latter caused a more prolonged release of GtH.

The evidence that hypothalamic GnRH activity may vary with the reproductive state has been investigated in some

teleost species. Greater GnRH immunological cross-reactivity was found in crude hypothalamic extract from carp in February compared to October, which correlates positively with changes in pituitary GtH content (see Peter, 1983). According to De Vlaming and Vodicnik (1975) there was less GnRH activity in the hypothalamic extract from pinealectomized shiners, *Notemigonus crysoleucas*, undergoing gonadal regression (long photoperiod conditions), and more in those in which pinealectomy caused acceleration of gonadal recrudescence (short photoperiod conditions).

LOCALIZATION OF GnRH IN THE TELEOST BRAIN

The main method used to locate GnRH cell bodies in the fish brain is immunocytochemistry. This method is based on using an antibody that is directed against the native form of the GnRH molecule from fish. Also, studies in fish have used antisera made against non-fish forms of GnRH (see Sherwood, 1987). These antisera detect fish GnRH under two categories: (1) the antiserum is directed against a sequential epitope on the GnRH molecule and this epitope is similar in fish and mammals, or (2) the antiserum is detected against a discontinuous or assembled epitope that results from the folding of the

GnRH molecules.

Location of GnRH in Early-Evolving Osteichthyes

In primitive bony fish, *Polypterus* and *Amia*, GnRH cell bodies have been found in the preoptic area. Fibers extend to the optic tectum, cerebellum, median eminence, and down the optic nerve to the retina where a plexus of fibers is present (Crim, 1983).

Location of GnRH in Late-Evolving Osteichthyes

In teleosts, immunoreactive GnRH neurons exhibit a similar distribution in all species studied (Münz and Claas, 1987). Firstly, the rostral GnRH cells and fibers are found in the ventral parts of the olfactory bulb, olfactory nerve and terminal nerve, in the ventral telencephalon and the preoptic regions (Halpern-Sebold and Schreibman, 1983; Münz *et al.*, 1981; Nozaki *et al.*, 1984a, b; Schreibman *et al.*, 1979).

In certain teleosts such as platyfish (*Xiphophorus maculatus*), and poecilid fish (*X. helleri*; hybrid, *X. spp*), irGnRH neurons were located in the olfactory-telencephalic area (Münz *et al.*, 1981; 1982). In other teleosts such as cichlids (*Cichlasoma biocellatum*); and

centrarchids (*Lepomis machrochirus*), the GnRH neurons were in the caudal part of the olfactory bulb. In goldfish (*Carassius auratus*) and catfish (*Ictalulus punctatus; Kryptopterus bicirrhi*) the irGnRH neurons were detected in a similar location (see Sherwood, 1987). Moreover, the presence of GnRH in the rostral and caudal olfactory bulb and in the ventral telencephalon was reported for eel, *Anguilla japonica* (Nozaki *et al.*, 1984a, b) and for *A. anguilla* (Dufour *et al.*, 1982). Based on their developmental appearance, the olfactory-telencephalic neurons are hypothesized to be the first of the GnRH cells to develop in the brain (Halpern-Sebold and Schreibman, 1983).

Later, the olfactory-related GnRH neurons were shown to be identical with the terminal nerve (Stell *et al.*, 1984). These GnRH neurons project to the retina, olfactory bulb, telencephalon and optic tectum (Münz *et al.*, 1982; Stell *et al.*, 1984). The function of the terminal nerve is still unclear. Early evidence suggested that sex-related olfactory stimuli, possibly pheromonal, acted through the GnRH-containing terminal nerve to affect the retina and also reproductive behavior. More recent evidence, however, showed that pheromonal olfactory stimuli were transmitted in the medial olfactory nerve rather that the terminal nerve

(Sorensen *et al.*, 1991).

Secondly, GnRH cell bodies were located in the diencephalon, lateral to the preoptic nucleus (Jackson, 1980; Münz *et al.*, 1981). These axons extended toward the hypothalamus and pituitary. In the African catfish, irGnRH was localized in the nucleus preopticus and in the neurosecretory fibers reaching the GtH producing cells in the proximal pars distalis of the pituitary gland (Goos *et al.*, 1985). Thirdly, GnRH cells were present in the hypothalamus, in the posterior part of the lateral tuberal nucleus (Halpern-Sebold and Schreibman, 1983); Kah *et al.*, 1982; Schreibman *et al.*, 1979). Fourthly, GnRH neurons were in the dorsal midbrain. Their fibers extend toward the third cranial nerve (Münz *et al.*, 1981).

FACTORS AFFECTING THE RELEASE OF GnRH

GnRH, with its 10 amino acids, is a small molecule and easily degraded by enzymes. It is difficult to measure GnRH directly because it is released into the portal vessels or near the gonadotropes in the pituitary. Many studies in mammals propose that the fluctuations of serum LH are a consequence of pulsatile release of LH from the pituitary in response to the pulsatile release of GnRH

(Sarkar *et al.*, 1976; Lincoln, 1988). Thus, the measurement of the pulsatile release of LH may indicate the level and pattern of GnRH release. The GnRH pulsatile generator and the GnRH neurons in turn, are influenced by a large number of gonadal hormones, neuroamines and neurotransmitters (Matsumoto and Arai, 1978; Palkovits, 1982; Kiss and Halasz, 1985; Pelletier, 1987).

Effects of Gonadal Steroids on the Release of GnRH

The gonads produce sex steroid hormones which regulate gonadal processes such as spermiation, oocyte maturation and ovulation. Based on the findings of Suzuki *et al.*(1988b), GtH I is primarily involved in stimulating steroidogenesis during vitellogenesis, whereas GtH II is associated with final maturation and ovulation. Dickhoff and Swanson (1989) proposed that GtH I is responsible for the production of estrogens and androgens, which in turn stimulate the synthesis of GtH II in the pituitary. This hypothesis suggests a positive feedback of gonadal steroids on the production of GtH II. This positive feedback was also suggested by Gielen *et al.* (1982) for the rainbow trout (*Oncorhynchus mykiss*), and by Borg *et al.* (1985) for the three-spined stickleback. In

contrast, a negative feedback on the release of GtH was reported for gonadal steroid hormones in rainbow trout (Billard *et al.*, 1977; Bommelaer *et al.*, 1981; Van Putten *et al.*, 1981) and African catfish (De Leeuw *et al.*, 1986b). Gonadectomy caused a rise in plasma GtH levels, whereas certain steroid replacements resulted in a drop of plasma GtH levels.

Several possibilities have been proposed regarding a steroidal negative feedback on GtH secretion (Goos, 1987). There is a mechanism that links the inhibitory action of gonadal steroids and that of dopamine. Steroids have been shown to stimulate dopaminergic inhibition of GtH release in the brain and/or pituitary of teleosts (Lambert *et al.*, 1984; De Leeuw *et al.*, 1985b; Timmers and Lambert, 1989). There is evidence to support this in the African catfish (De Leeuw *et al.*, 1987a), in goldfish (Billard and Peter, 1977), carp (Breton *et al.*, 1975) and Indian catfish (*Heteropneutes fossilis*) (Singh and Singh, 1979). In addition, other mechanisms might be involved in the feedback of gonadal steroids on GtH release. In mammals, it has been shown that gonadal steroids affect GnRH receptor capacity although the effect varies among species (Clayton and Catt, 1981; Conn *et al.*, 1982). In rats, there is an increase in GnRH receptor number and gonadotropin

secretion after castration. Treatment with testosterone prevents this castration-induced increase in GnRH receptor number. This observation suggests that gonadal steroids can exert a negative feedback on GtH release by regulating GnRH receptors.

In vitro studies with rat pituitaries show that cells cultured in the presence of either testosterone or dihydrotestosterone have decreased GnRH receptors. This is accompanied by reduced sensitivity to GnRH (Giguere and Labrie, 1981). However, estradiol under some conditions can also sensitize gonadotropes to GnRH *in vitro* and a 25-50% increase in GnRH receptors follows (Loumaye and Forni, 1982; Tang *et al.*, 1982). These studies suggest that steroidal regulation of GnRH receptors is complex and varies among species.

In teleosts such as African catfish, castration resulted in a rise in pituitary GnRH receptor number. Steroid implantation reversed this effect. These events parallel those in mammals. They confirm a negative feedback of gonadal sex steroids mediated via GnRH receptors, on GtH release (Habibi *et al.*, 1989).

Influence of Pheromones on the Release of GnRH

Pheromones play a role in teleost reproduction by regulating spawning behavior and endocrine events (Colombo *et al.*, 1982; Stacey *et al.*, 1987; Stacey, 1989). In the zebra fish (*Brachydanio rerio*), pheromones released from ovulated females attract male conspecifics (Van Den Hurk and Lambert, 1983). Meanwhile pheromones released by males attract females in the goby *Gobius jozo* (Colombo *et al.*, 1980) and African catfish *Clarias gariepinus* (Resink, 1988). The exposure of male goldfish to females that have ovulated spontaneously induces a marked increase in serum GtH levels during spawning in male goldfish *Carassius auratus* (Sorensen and Stacey, 1989).

Effects of Neuroamines and Neurotransmitters on the Release of GnRH.

In goldfish and other teleosts, dopamine (DA) plays an inhibitory role on basal GnRH-induced pituitary GtH secretion (see Peter *et al.*, 1986; De Leeuw *et al.*, 1987a; Yu and Peter, 1992). In carp (Billard *et al.*, 1983), eel (Dufour *et al.*, 1984), and the African catfish (De Leeuw *et al.*, 1985a, 1986a) the action of dopamine is

restricted to an inhibition of the GnRH-stimulated GtH release, whereas in goldfish dopamine was found to inhibit not only the GnRH-stimulated, but also the spontaneous GtH release (Chang *et al.*, 1984). On the other hand, a dopaminergic inhibition appeared to be completely absent in the Atlantic croaker, *Micropogonias undulatus* (Copeland and Thomas, 1989), and the gilthead seabream, *Sparus auratus* (Zohar *et al.*, 1987). The involvement of norepinephrine (NE), and serotonin (5-HT) on the pituitary GtH secretion has been studied in goldfish. It was found that NE and 5-HT stimulated the secretion of pituitary GtH (Chang *et al.*, 1984; Chang and Peter, 1984; Somoza *et al.*, 1988). In addition, 5-HT has an inhibitory effect on growth hormone release in goldfish (Somoza and Peter, 1991).

GnRH ACTION AND FUNCTION

There are three basic components for the operation of an endocrine system (Csaba, 1990):

(1) the hormone as a signal molecule,

(2) the receptor as a signal carrier, and

(3) a signal mediator mechanism

The concept of receptors on the surface of the cell membrane, as a site of action of peptide hormones,

derived from many years of studying hormone effects (Haynes *et al.*, 1960). Several studies have been done on the GnRH receptors in mammals and in some fish, such as goldfish. Goldfish pituitary contains two types of GnRH binding sites. One is a high affinity, low capacity site and the other is a low affinity, high capacity site (Habibi *et al.*, 1989). In mammals and fish, the location and mode of action of GnRH receptors are probably the same in that receptors are located on the cell surface, will form microaggregations once bound to the hormone, and are internalized into coated pits (Hazum *et al.*, 1986). The hormone and receptor trigger the intracellular events that result in biological activity.

Receptors for peptide hormones and neurotransmitters, in general, are usually high molecular weight proteins on the plasma membrane of the cells. The first step in the action of a peptide hormone is the binding to its receptor, which can be studied by measuring the binding of radiolabelled peptides to a particular tissue. This process is rapid, reversible, saturable and of high affinity and specificity. The success of this reaction depends on the availability of labelled ligands of very high specific activity, which allows the use of concentrations low enough to minimize nonspecific binding. Rapid washing procedures may further reduce the

nonspecific binding. The radiolabelled ligand studies define only binding sites that may or may not represent the physiologically functional receptors of interest.

Some of the general biological responses that may follow the peptide-receptor interaction are:

(1) an increase or decrease of hormone or transmitter secretion,

(2) stimulation or inhibition of muscle contraction, or

(3) depolarization or hyperpolarization of neuronal membranes.

The effector event leading to such responses might be calcium mobilization or a change in membrane permeability to sodium, potassium or chloride ions. Hormone action was shown to be mediated usually by stimulation of adenylate cyclase with formation of cyclic AMP (cAMP), which acted as a second messenger (see Csaba, 1990).

The receptors play a central role in reproduction. The interaction of GnRH with specific plasma membrane receptors in pituitary gonadotropes is the first event in stimulation of GtH secretion (Conn *et al.*, 1981). GnRH receptors have been found in several other tissues including gonadal tissue (see Hsueh and Jones, 1981), adrenal cortex (Eidne *et al.*, 1985) and the central nervous system (Millan *et al.*, 1986).

The complete structure of the mammalian GnRH receptor

has not yet been published, but it is believed to be a membrane bound glycoprotein with sialic acid residues. The binding site of the receptor is known to contain a sugar moiety, tryptophan, and tyrosine residues, and possibly 2 carboxylic groups (Hazum *et al.*, 1986). The structure of the GnRH receptor has not been published for non-mammalian vertebrtaes, but evidence has been presented that GnRH receptors are present in the winter flounder (Crim *et al.*, 1987b), catfish and goldfish (Habibi *et al.*, 1989; Habibi *et al.*, 1990).

There is a significant increase in GnRH receptors at low concentrations of GnRH agonists and a decrease at higher concentrations (Loumaye and Catt, 1981). In contrast, GnRH antagonists do not cause down regulation of GnRH receptors. They prevent the regulatory effects of the agonist ligands on both LH secretion and GnRH receptor numbers.

Pituitary GnRH receptors have been characterized as specific binding sites with high affinity for GnRH and its agonists or antagonists. In rats, similar receptors are present in gonads (testis and ovary) where GnRH exerts direct actions that are predominately inhibitory in nature (Hsueh and Erickson, 1979a, b; Birnbaumer *et al.*, 1985; Rivier and Vale, 1989). Meanwhile, human placenta contains low-affinity GnRH binding sites that

interact with GnRH agonistic and antagonistic analogs (see Catt *et al.*, 1985).

The functions that result from the interaction of GnRH with its receptor are varied. In vertebrates (except hagfish), GnRH is thought to modulate both pituitary function and the neural systems that stimulate reproductive behavior (Demski, 1984). In many animals, GnRH enhances sexual behavior and probably is important in modulation of behavior (Shivers et al., 1983). Moreover, GnRH is known to act as a neuromodulator and/or neurotransmitter in the sympathetic ganglia of the bullfrog (Jan *et al.*, 1979; Jan *et al.*, 1980; Jan and Jan, 1982; 1983; Jones, 1987). The presence of GnRH in the retina of teleosts, birds and amphibians suggests that GnRH also plays a role in modulating visual responses (Münz *et al.*, 1982; Stell *et al.*, 1984).

RELATIONSHIP OF GnRH AND THIS STUDY

GnRH has been used in aquaculture where the main use of this hormone is in fish farming in the field of reproduction, especially the induction or synchronization of ovulation and the stimulation of spermiation. Prior to GnRH, a widely-used hormone preparation was pituitary extract in the form of a dry powder or acetone-dried

pituitary. The technique called hypophysation started in the early 1930's in Brazil and was aimed at inducing ovulation in fish which did not spawn in captivity (Pickford and Atz, 1957; Lam, 1982; Donaldson and Hunter, 1983).

There are several problems with the use of crude pituitary preparations. The biological activity of crude pituitary is not known. Also, there is a decline in biological activity of the crude preparation with time. For example, dry carp pituitary loses half its activity within five years (Bieniarz *et al.*, 1980). A decline in activity of 25% in 18 months has been reported from partially purified salmon pituitary stored at 35°C in comparison with samples stored at -40°C (Donaldson *et al.*, 1978).

The use of gonadotropin-releasing hormone for the induction of a GtH ovulatory surge, ovulation and spawning seems to be more efficient than dried pituitary preparations. GnRH and its analogs stimulate the secretion of endogenous gonadotropins. GnRH peptides can be synthesized and obtained in a pure form and they have a low degree of biological species specificity. GnRH and its analogs are currently applied in a variety of commercially important fish. The potency varies according to the structure of the analog and the

recipient species (Crim *et al*., 1987a). Fishes tested with GnRH include salmonids, cyprinids, milkfish, rabbitfish, seabass, loach, walleye, sea bream (Zohar *et al*., 1990; Glubokov *et al*., 1991), catfish (Ngamvongchon *et al*., 1986, 1988; Zonneveld *et al*., 1988), sablefish (Solar *et al*., 1990), and sea bass (Garcia, 1990).

The development of GnRH-based spawning induction technology depends on several efforts in three major fields:

(1) the species-specific interaction between GnRH and the release of GtH,

(2) structure-activity relationship of GnRH and its analogs,

(3) the mode of GnRH administration to the fish.

Sherwood (1987a) reviewed the role of GnRH in stimulating gonadotropin secretion in vertebrates from primitive fish to humans. Of these vertebrates, there are about 20,000 species of bony fish of which catfish represent about 2,000 species (Nelson, 1984).

The response of a particular fish species depends on the endogenous forms of GnRH, receptor specificity, and the form of GnRH that is administered. Indeed, the presence of two or more forms of GnRHs in one species is the common pattern with only a few exceptions (Table 1.2). Thus, alterations in the GnRH molecule may change

its potency for releasing GtH and its binding affinity to the pituitary receptor.

The native forms of five GnRHs have been tested for potency in only a few species. For example, lamprey GnRH advanced ovulation in lamprey (Sower *et al.*, 1987). Mammalian and salmon GnRH were equally effective *in vivo* and *in vitro* in the seabream, *Sparus aurata* (Zohar *et al.*, 1989) and *in vivo* in salmon (Van Der Kraak *et al.*, 1987). In Thai carp, salmon GnRH induced ovulation in 11.1% of the fish but demonstrated higher percentage of spawning when giving either the higher dose or combine with dopamine antagonist (Sukumasavin, 1992). In the sterlet fish, mammalian and chicken I GnRH did not cause ovulation, whereas salmon GnRH induced ovulation in 30% of the fish (Horvath *et al.*, 1986). These data suggest that the potencies of molecular forms of GnRH in particular species are due to their affinity for the receptor and their rate of degradation in a species. The relationship between the structure of the native form of GnRH and function can be altered considerably by synthesizing GnRH analogs.

A number of GnRH analogs made with the D form of amino acids, unlike the native L form, are potent in fish for release of gonadotropins or induction of ovulation and spermiation. To date, two of the most active and

Table 1.2. GnRH in teleost fish. The table is adapted from Sherwood, 1988. L-I=lamprey GnRH-I, L-II=lamprey GnRH-II, S=salmon GnRH, C-I=chicken GnRH-I, C-II=chicken GnRH-II, M=mammalian GnRH, New=novel form GnRH.

Species	GnRH form						
	L-I	L-II	S	C-II	C-I	M	New
Hagfish (Eptatretus stouti)	-	-	-	-	-	-	-
Lamprey (Petromyzon marinus)	*	*					
Ratfish (Hydrolagus colliei)				*			
Dogfish (Squalus acanthias)				*			*
Dogfish (Poroderma africanum)			*	*			
Reedfish (Calamoichthys calabaricus)						*	
Sturgeon (Acipenser transmontanus)				*		*	
Alligator Gar (Lepisosteus spatula)				*		*	
Bowfin (Amia calva)						*	
Moray Eel (Gymnothorax fimbriatus)			*			*	*
Herring (Clupea harengus pallasi)			*	*			
Milkfish (Chanos chanos)			*	*			
Goldfish (Carassius auratus)			*	*			
Catfish (Clarias gariepinus)				*			*
Salmon (Oncorhynchus keta)			*	*			
Trout (Salmo gairdneri)			*	*			
Hake (Merluccius capensis)			*		*		
Codfish (Ganus morhua morhua)			*				
Molly (Poecilia latipinna)			*	*			
Snook (Centropomus undecimalis)			*	*			*
Sea Bass (Centropristis striatus)			*	*			
Mullet (Mugil cephalus)			*	*			
Tilapia (Tilapia sparrmanii)			*		*		
Wrasse (Coris julis)			*				
Winter Flounder (Pseudopleuronectes americanus)			*		*	*	

commonly used analogs for fish are

	1 2 3 4 5 6 7 8 9 10
mGnRH	pGlu-His-Trp-Ser-Tyr- Gly -Leu-Arg-Pro-Gly.NH2
mGnRH-a	pGlu-His-Trp-Ser-Tyr-**D-Ala**-Leu-Arg-Pro.**NEt**
sGnRH	pGlu-His-Trp-Ser-Tyr- Gly -Trp-Leu-Pro-Gly.NH2
sGnRH-a	pGlu-His-Trp-Ser-Tyr-**D-Arg**-Trp-Leu-Pro.**NEt**

Recently, other analogs have been shown to be more active than the native GnRH peptides in fish (Zohar, 1989; see Crim *et al.*, 1987a; Van Der Kraak *et al.*, 1987; Horvath *et al.*, 1986). Zohar *et al.* (1990) found that analogs appear to be more resistant to enzymatic breakdown and last longer than the native GnRH peptides.

The use of GnRH for inducing breeding has now been tested in a variety of freshwater and marine fishes. The form of GnRH, time, dose and method of administration are important.

With these concerns in mind, I conducted my research with these objectives:

(1) **A preliminary study of GnRH in catfish brain**

The six known forms of GnRH as shown in Table 1.1 are from mammals, birds, reptiles, bony fish, cartilaginous and jawless fish. The question is whether other species

of teleosts also contain GnRH material in the brain. As indicated, the catfish family appears to have the largest number of species amongst the teleosts. Therefore, this research is the first study to provide information on a novel form of GnRH in the brains of two species of catfish. My research on this topic is presented in Chapter 2.

(2) **Characterization of the primary structure of catfish GnRH**

Knowledge of GnRH structures can be used to prepare synthetic forms of native GnRH and its analogs. The latter may give higher potency for induced spawning from the aquacultural point of view. Once the structure is determined it can help us to synthesize the peptide and its analogs as well as answer one of the mysterious questions of how the neuropeptide evolved in the vertebrate. Besides, it might also help to determine the importance of specific amino acids. This information is provided in Chapter 3.

(3) **Localization of GnRH in the catfish brain**

From the current literature comes the idea that GnRH not only acts as a gonadotropin stimulator but also plays a role as a neurotransmitter in the brain. The function of GnRH is elucidated by studying the distribution of GnRH in the brain. Instead of using the technique of

immunocytochemistry to answer this question, I used the combined methods of high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) so that different forms of GnRH could be identified in dissected brain areas. This study is in Chapter 4.

(4) **Physiological studies of catfish GnRH peptides in the induction of ovulation in the catfish**

The story of GnRH is not complete until the functions of different forms have been tested in fish. In Chapter 5, several native forms and analogs of GnRH have been tested for their effect on the induction of ovulation in the Thai catfish, *Clarias macrocephalus*. In addition, a range of doses has been administered to find the optimal dose of GnRH for the induction of the ovulation in catfish. Finally, *in vitro* experiments have been undertaken to study the release of gonadotropins and growth hormone from the pituitary.

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CHAPTER 2

CHROMATOGRAPHIC AND IMMUNOLOGICAL STUDIES OF GONADOTROPIN-RELEASING HORMONE FROM THE BRAIN OF TWO SPECIES OF CATFISH, *Clarias macrocephalus* and *C. batrachus*

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INTRODUCTION

In catfish the neurohormone gonadotropin-releasing hormone (GnRH) is important in controlling the reproductive system in mature fish (Goos *et al.*, 1987; Ngamvongchon *et al.*, 1988). Several studies have demonstrated the location of GnRH in catfish brains. Immunoreactive GnRH (irGnRH) in the African catfish, *Clarias gariepinus*, is located in the nerve cells of the nucleus preopticus and in their axons and secretory granules near the gonadotropic cells in the proximal pars

distalis of the pituitary (Goos *et al.*, 1985; Peute *et al.*, 1987). In *Clarias batrachus*, the irGnRH is present in the olfactory system, the base of the forebrain, the nucleus preopticus, nucleus lateralis tuberis pars posterior, the pituitary, the optic chiasm, inner layer of the retina, midbrain and caudal hypothalamus (Subhedar and Rama Krishna, 1988). This distribution supports the idea that GnRH may synchronize reproductive processes with environmental cues.

However, neither the number of distinct forms of GnRH in Thai catfish brains nor their primary structures are known. Thus the physiological effects of GnRH in catfish have not been tested with the native forms of catfish GnRH. Rather, analogs of GnRH or chorionic gonadotropin have been used to induce ovulation in catfish (Ngamvongchon *et al.*, 1986; 1988; De Leeuw *et al.*, 1985).

In the present experiment, I studied whether GnRH-like molecules are present in the brains of 2 species of catfish (*Clarias macrocephalus* and *Clarias batrachus*) and whether the structures are novel or the same compared with the five identified GnRHs. Identification of the different forms of GnRH in catfish is important for studies on the evolution of the molecule in teleosts belonging to different taxonomic groups and useful for aquaculture.

MATERIALS AND METHODS

Fish brains. Whole brains and pituitaries of mature catfish, *Clarias macrocephalus* and *Clarias batrachus*, were collected during the spawning season in Thailand (July, 1988). Tissues from both males and females were included in the 25 samples for each species. The tissues were immediately placed in liquid nitrogen, then in dry ice for transportation to University of Victoria, Canada. They were stored at -80°C thereafter.

Extraction. Twelve frozen brains of each species (6 females and 6 males) were powdered in a Waring blender with liquid nitrogen. The total weights of the brains and pituitaries of *C. macrocephalus* and *C. batrachus* were 4.06 g and 3.18 g, respectively. The tissue from each species was treated separately throughout the experiment. The powdered tissue was treated as described (Sherwood *et al.*, 1989) The final aqueous phase was evaporated in a vacuum concentrator to 2.5 ml. An aliquot of 100 μl from each extract was removed for radioimmunoassay (RIA).

Radioimmunoassay. Samples (100 μl each) were dried in a vacuum concentrator and rehydrated in 600 μl phosphate-buffered saline, pH 7.0. The pH of the rehydrated samples was then adjusted to pH 7.0 with 1M NaOH. RIA was done as previously described (Sherwood *et al.*, 1983,

1986). The samples were assayed with synthetic mammalian GnRH as iodinated tracer and standard. Each HPLC fraction was assayed with four different antisera. Antiserum R-42 was made against mammalian GnRH and used at a final dilution of 1:250,000. RIA sensitivity (ED_{80} or B/Bo = 80%) was 3.5 pg. Three other antisera were also used to assay each HPLC fraction: B-6 (made against mammalian GnRH, 1:5,000, 80% B/Bo = 8.2 pg), GF-4 (made against salmon GnRH, 1:25,000, 80% B/Bo = 3.5 - 6.6 pg), and Bla-4 (made against lamprey GnRH, 1:5,000, 80% B/Bo = 28 pg).

High Performance Liquid Chromatography (HPLC). The extracts of 2.4 ml from each species were divided and injected onto the HPLC in two different programs. The extracts were injected through a 1-ml injection loop onto a guard column (0.46 x 2 cm, 5 μm particles) and analytical column of C-18 material (0.46 x 25 cm, 5 μm paricles, Supelco, Oakville, Ontario). A Varian 5000 liquid chromatograph was programmed for a flow rate of 1 ml/min.

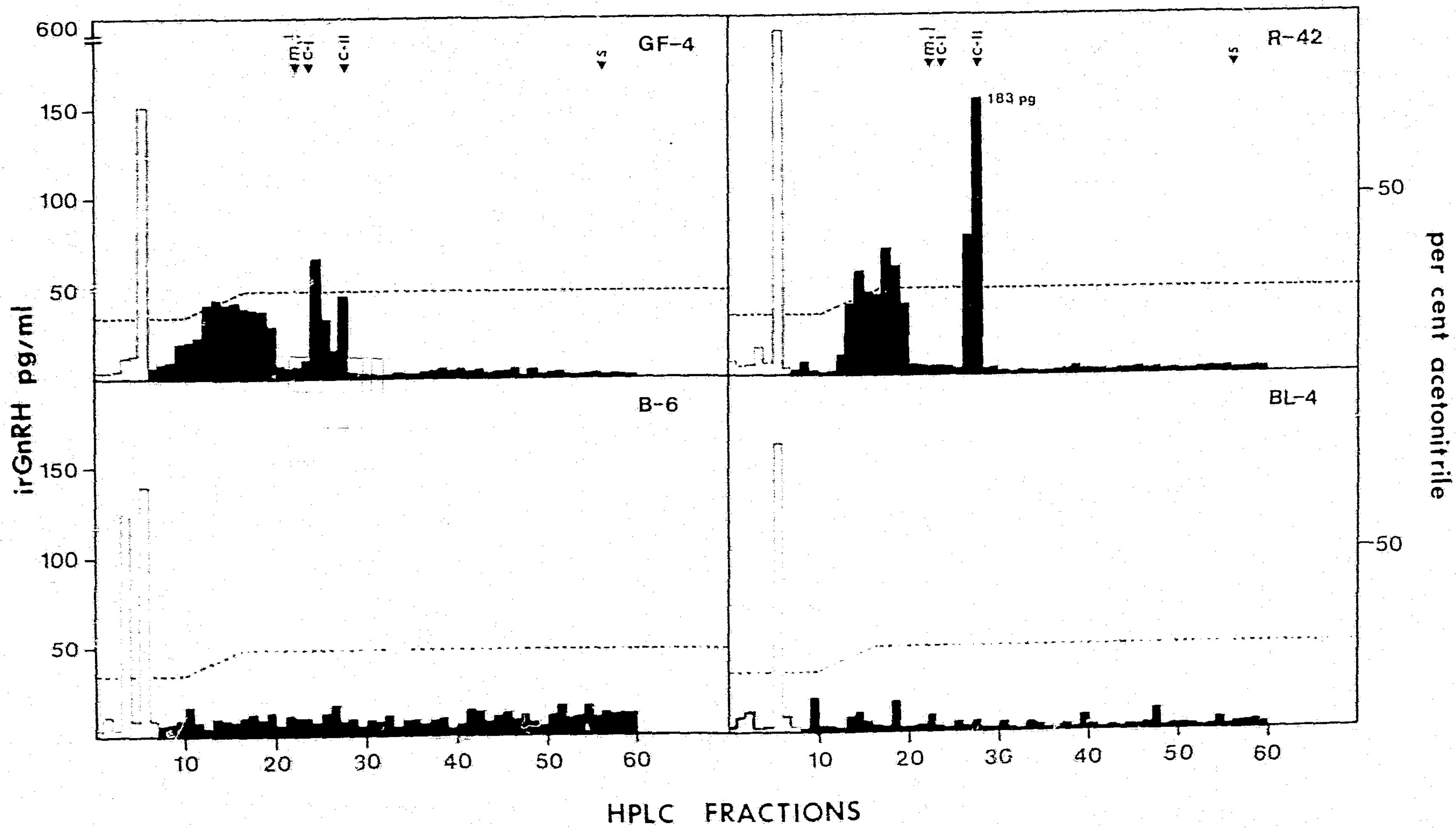
In program 1, the filtrate was applied as two injections (800 and 400 μl) at the beginning of a 10-min period of the mobile phase consisting of 17% acetonitrile (CH_3CN) in 0.25M TEAF, pH 6.5 (0.2M formic acid, pH adjusted to 6.5 with triethylamine). Acetonitrile was

then increased to 24% over a 7-min period. GnRH was eluted during the isocratic phase of 24% acetonitrile (Figs. 2.1, 2.2). In program 2, the filtrate was injected (800 and 400 μl) at the beginning of a 10-min period of 5% CH_3CN in triethylammonium formate (TEAF). The percentage of acetonitrile was increased as a linear gradient from 5 to 60% for 50 min (Figs. 2.3, 2.4). One-ml fractions were collected from both programs (60 fractions each) for each species. Aliquots (100 μl) of each fraction were assayed for immunoreactive GnRH (irGnRH).

The same procedure was used for a blank in which TEAF was injected in place of the brain-pituitary extract. Prior to each procedure under the same conditions, 1-ml fractions were collected after injection of the blank. All blank fractions were assayed with GF-4 in order to determine whether previously injected peptides were being carried over to a subsequent run.

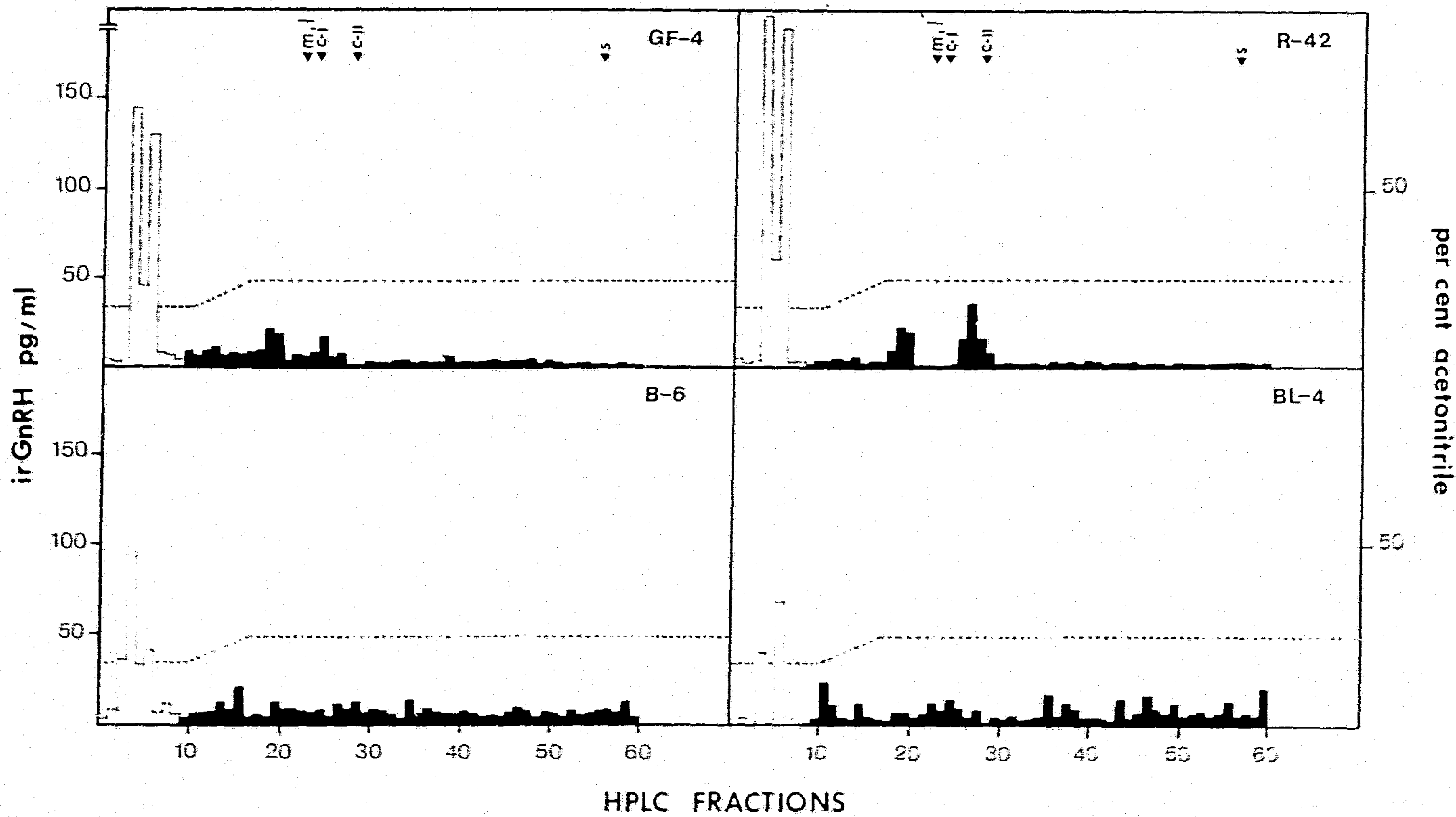
The synthetic standards of five known forms of GnRH (mammal, salmon, chicken I, chicken II and lamprey; 200 ng each) were mixed, diluted with TEAF to a final volume of 800 μl and injected onto the HPLC column in both programs. Fractions from the standards were also assayed with antiserum GF-4.

Figure. 2.1. Reverse-phase HPLC of brain-pituiary extracts from catfish, *Clarias macrocephalus*. A novel form of GnRH eluted as a broad peak before the five synthetic GnRHs, which are shown by arrows (m=mammal; l=lamprey; C-I=chicken I; C-II=chicken II; S=salmon GnRH). A second peak eluted with the chicken GnRH-II standard as shown most clearly by cross-reactivity with antiserum R-42. The amount of irGnRH in 1-ml HPLC fractions is represented by black bars. The open bars show nonspecific cross-reactivity. Fractions were assayed by RIA with four antisera: GF-4 (anti-sGnRH), R-42 (anti-mGnRH), B-6 (anti-mGnRH), and Bla-4 (anti-lGnRH). The antisera were made against the GnRH forms noted, but GF-4, and R-42 also cross-react with other forms. Antisera B-6 and Bla-4 did not cross-react with any GnRH forms. The dotted line represents the percentage of acetonitrile (AN) in the mobile phase. The program was isocratic at 24% AN from 17 to 60 min.



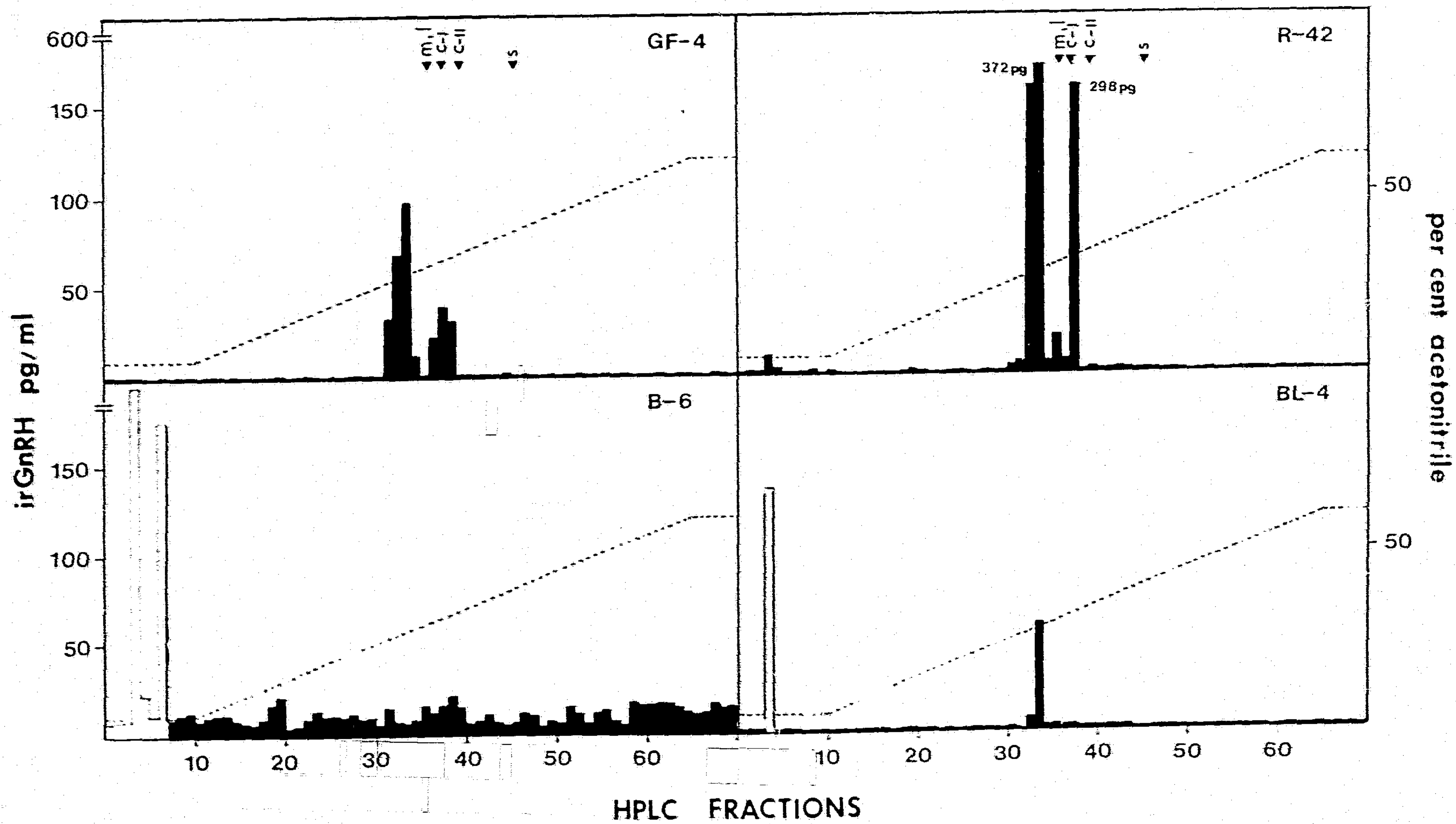
GF-4
R-42
B-6
BL-4
183 pg
600
150
100
50
irGnRH pg/ml
per cent acetonitrile
10
20
30
40
50
60
HPLC FRACTIONS

Figure. 2.2. Reverse-phase HPLC of brain-pituitary extracts from catfish, *Clarias batrachus*. A novel form of GnRH eluted before the five synthetic GnRHs which are shown by arrows. A peak also eluted close to chicken GnRH-II as seen by R-42. The amounts of irGnRH in 1-ml HPLC fractions are indicated as black bars. Nonspecific cross-reactivity is shown by open bars. Fractions were assayed with antisera GF-4, R-42, B-6, and Bla-4. The peaks were most clearly detected with antisera R-42 and GF-4 but not with B-6 and Bla-4. The dotted line represents the percentage of AN in the mobile phase. The program was isocratic at 24% AN from 17 to 60 min.



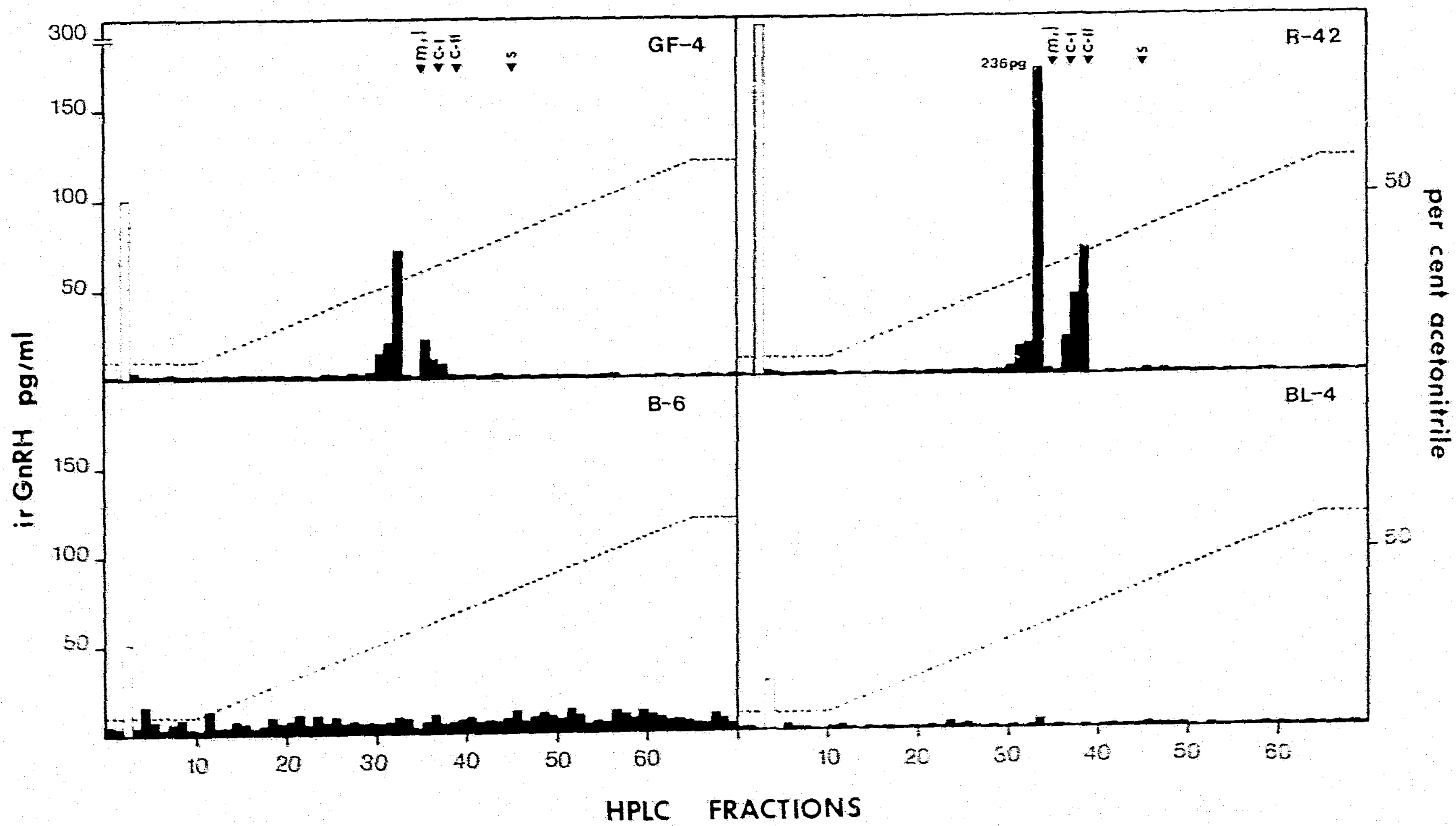
GF-4
R-42
B-6
BL-4
150
100
50
irGnRH pg/ml
50
per cent acetonitrile
10
20
30
40
50
60
HPLC FRACTIONS

Figure. 2.3. Reverse-phase HPLC of brain-pituitary extracts from catfish, *Clarias macrocephalus*. A novel form of GnRH was eluted in a high concentration with an increasing gradient of acetonitrile (AN). A second peak eluting near chicken GnRH-II was also detected with GF-4 and R-42. Five synthetic GnRHs are indicated as arrows. Each HPLC fraction was assayed with four antisera: GF-4, R-42, B-6, and Bla-4. The doc̈ted line indicates the percentage of AN. The program was a gradient of AN from 5 to 60%.



GF-4
R-42
B-6
BL-4
372pg
298pg
irGnRH pg/ml
per cent acetonitrile
HPLC FRACTIONS
600
150
100
50
10
20
30
40
50
60

Figure. 2.4. Reverse-phase HPLC of brain-pituitary extracts from catfish, *Clarias batrachus*. A novel form of GnRH was eluted in a high concentration with an increasing gradient of acetonitrile (AN); it eluted before the five synthetic GnRHs indicated as arrows. A second peak eluted near chicken GnRH-II. Each fraction was assayed with four antisera: GF-4, R-42, B-6, and Bla-4. The percentage of AN is represented by the dotted line. The program was a gradient of AN from 5 to 60%.


GF-4
B-42
B-6
BL-4
236pg
m.I
c-I
c-II
s
300
150
100
50
ir GnRH pg/ml
per cent acetonitrile
10
20
30
40
50
60
HPLC FRACTIONS

RESULTS

The results show that catfish GnRH has two forms in each species, *Clarias macrocephalus* and *C. batrachus*. The dominant form of catfish GnRH is a novel form (catfish GnRH-I) and the minor form (catfish GnRH-II) is not distinguishable from chicken GnRH-II.

Figure 2.1 shows that the two HPLC peaks of irGnRH for *C. macrocephalus* can be clearly separated on an isocratic program. The elution of the first HPLC peak before the 5 synthetic standards supports the idea that catfish GnRH-I is a novel form. The broad peak of catfish GnRH-I in the isocratic program also suggests that the early eluting peak is distinct from identified GnRH forms. The five synthetic forms eluted from the HPLC after catfish GnRH-I in the same order in both programs : mammalian and lamprey GnRH eluted together followed by chicken I, chicken II and the hydrophobic salmon GnRH. The two peaks of irGnRH were sharper in the gradient program (Fig. 2.3). Antisera R-42 and GF-4 clearly detected both forms of GnRH. Cross-reactive material eluting in the first 10 fractions is assumed to be nonspecific because (1) the quantity changes with the pH of the sample, (2) ions elute in the early fractions and could alter the RIA, (3) the cross-reactivity remains regardless of

whether GnRH-like material is detected by the antiserum in fractions within the elution range of the standards, and (4) antisera R-42, GF-4 and Bla-4 do not detect GnRH as part of a larger precursor molecule. No attempt was made to purify the early-eluting material.

In *C. batrachus*, catfish GnRH-I also eluted about 2-3 fractions earlier than mammalian and lamprey GnRH, whereas catfish GnRH-II eluted close to chicken GnRH-II (Fig. 2.2, 2.4). The elution pattern was almost identical to that for *C. macrocephalus*.

The cross-reactivity profile of the two GnRHs was similar in both species of catfish. Antiserum B-6, which recognizes the mammalian GnRH, did not cross-react with any material in either species. Likewise, antiserum Bla-4 made against lamprey GnRH did not cross-react with either peak. Bla-4 was made against lamprey GnRH, but also cross-reacts with mammalian, salmon and chicken I GnRHs. It does not cross react (0.38%) with chicken GnRH-II, which supports the evidence that the second eluting peak is chicken GnRH-II. Antisera R-42 and GF-4 cross-react with both HPLC peaks in both species. Both antisera are known to cross-react with chicken GnRH-II and with several other forms of GnRH (Kelsall *et al.*, 1990).

DISCUSSION

The novel form of catfish GnRH is clearly different from other known forms of vertebrate GnRH. The evidence is based on the elution time, the broad peak and the cross-reactivity. Firstly, catfish GnRH-I eluted earlier from two HPLC programs than mammalian, lamprey, chicken I, chicken II and salmon GnRH. Secondly, catfish GnRH-I is not recognized by antiserum Bla-4, which does cross-react with mammalian, salmon, chicken I and lamprey GnRH. Thirdly, catfish GnRH-I does not cross-react with antiserum B-6, known to detect residues 5-10 of mGnRH. Fourthly, catfish GnRH-I does cross-react with antiserum GF-4, which does not detect lamprey GnRH (Kelsall *et al.*, 1990). It should be noted that a heterologous RIA was used to measure the GnRH-like molecules. This assay has been tested for percentage cross-reactivity with the known forms of GnRH (Sherwood *et al.*, 1991) and hence the actual quantity of cGnRH-II can be calculated. The percentage cross-reactivity is shown below. However, the quantity of the novel form may vary from that reported here when the structure is known and a homologous assay is developed.

In this experiment, there is no evidence of salmon-like GnRH. Sherwood and Lovejoy (1989) reported the presence

of sGnRH in the other teleosts (trout, milkfish, mullet, goldfish and herring). In some teleosts, for example winter flounder, there are three GnRH-like substances. Their HPLC elution times are similar to those of mammalian, chicken I and sGnRH (Idler and Crim, 1985; Idler and Everard, 1987). However, the relationship between catfish GnRHs and flounder GnRHs is not known. The early-eluting peak of flounder GnRH may be mGnRH or a novel form.

Catfish GnRH-II, the late-eluting peak of irGnRH, eluted in almost the same position as chicken GnRH-II in both HPLC systems. The relative cross-reactivity of catfish GnRH-II was similar to that of chicken GnRH-II with the 4 antisera. R-42 cross-reacts (54%) with chicken GnRH-II, GF-4 cross-reacts less (4%), and antisera B-6 and Bla-4 do not cross-react (less than 1%) (Sherwood *et al.* 1991). This is indirect evidence that catfish GnRH-II and chicken GnRH-II are identical in structure.

Based on chromatographic and immunological evidence, there are several forms of GnRH that may have the same structure as chicken GnRH-II: catfish GnRH-II (*C. macrocephalus, C. batrachus* and *C. gariepinus*) (Chapter 2; Sherwood *et al.*, 1989), salmon GnRH-II (Sherwood and Lovejoy, 1989), ratfish GnRH (Lovejoy and Sherwood, 1989)

and dogfish GnRH-II (Sherwood and Sower, 1985). Recently the primary structures for ratfish GnRH (Lovejoy *et al.*, 1991) and dogfish GnRH-II (Lovejoy *et al.*, 1992) were shown to be identical to chicken GnRH-II (Miyamoto *et al.*, 1984).

The information provided from the HPLC and RIA supports the idea that catfish GnRHs, like other GnRH family members, have 10 amino acids and an amide at the C-terminus. This is based on the recognition of GnRH by antiserum R-42. R-42 usually does not detect extended forms, fragments of GnRH or the free acid form lacking the amide group (Copeland *et al.*, 1979; Kelsall *et al.*, 1990). The non-recognition of catfish GnRH-I by antiserum B-6 is the difference between catfish GnRH-I and mGnRH. In addition, catfish GnRH-I is predicted to have an arginine, lysine or combination of hydrophilic residues, since the earlier elution time is about 1-3 minutes before mammalian and lamprey GnRH. The highly charged arginine is present in mGnRH and lysine in lamprey GnRH. Therefore, catfish GnRH-I appears to be the most hydrophilic of the family members.

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CHAPTER 3

PRIMARY STRUCTURE OF TWO FORMS OF GONADOTROPIN-RELEASING HORMONE IN THE THAI CATFISH BRAIN, (*Clarias macrocephalus*)

Parts of this chapter have been previously published and to be published:

Ngamvongchon, S., Lovejoy, D.A., Fischer, W.H., Craig, A.G., Nahorniak, C.S., Peter, R.E. and Sherwood, N.M. 1992. Primary structures of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. Molecular and Cellular Neurosciences 3:17-22.

Lovejoy, D.A., Fischer, W.H., Ngamvongchon, S., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E. and Sherwood, N.M. 1992. Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. Proceedings of the National Academy of Sciences (USA) 89 (in press).

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is important in the control of reproduction in vertebrates. Six structurally distinct forms of GnRH have been found and each, with the exception of dogfish and lamprey GnRH, is distributed in a number of species. Human GnRH, for example, occurs not only in mammals but also in primitive bony fish (Sherwood *et al.*, 1991) and amphibians (Rivier *et al.*, 1981). In the search for new natural forms of GnRH, freshwater fish are of special interest. It has been argued that the formation of new species or traits may be greater in freshwater compared with salt water because 39% of all fish species live in freshwater even though only 0.01% of the earth's water is fresh (Nelson, 1984). Catfish, which include over 2000 species of fish, illustrate this concept.

The structure of GnRH in a freshwater species of fish has not been previously reported. However, some form of GnRH is likely to be present because synthetic GnRHs and their analogs have been used to spawn cultured catfish (De Leeuw *et al.*, 1985; 1987; Ngamvongchon *et al.*, 1986; 1988) and there is material in the catfish brain that cross-reacts with antisera to GnRH (Goos *et al.*, 1985; Peute *et al.*, 1987; Subhedar and Rama Krishna, 1988;

Sherwood *et al.*, 1989; Chapter 2). In this study I determine the structural characterization of two forms of catfish GnRH.

MATERIALS AND METHODS

Collection of Specimens

Sexually mature Thai catfish, *Clarias macrocephalus*, were purchased from a fish farm near Bangkok, Thailand, in May 1989. Whole brains and pituitaries were removed and placed on dry ice. They were shipped to the University of Victoria, Canada, and stored at -80°C. For purification, 1448 brains and pituitaries weighing 529.4 g were used.

Extraction of GnRH

The frozen brain and pituitary tissues were immersed in liquid nitrogen and crushed to a fine powder with a mortar and pestle that had been prechilled with dry ice. The powdered tissue was extracted in 4 volumes of 0.05% trifluoroacetic acid (TFA), 19.95% water, and 80% acetonitrile, HPLC grade (percentage by volume). The material was stirred for 3 hours and then centrifuged at

20,000g to remove the solids. These solids were removed and discarded. In five successive extractions, hydrophobic substances were removed from the supernatant using a volume of petroleum ether (30-60°C bp) that was 20% of the supernatant volume.

Purification of GnRH

The supernatant was tranferred to a Savant Speed-Vac concentrator and the volume was reduced 90%. An equal volume of HPLC-grade water (Milli-Q) was added to the concentrate before it was pumped through C18 cartridges (Waters) connected in series. Material remaining on the column was eluted using a mixture of mobile phase A (0.05% TFA in water) and mobile phase B (0.05% TFA in 80% acetonitrile as above; pH 2.2). The acetonitrile gradient increased from 4 to 64% over 55 minutes. Sixty 1-ml fractions were collected. Immunoreactive GnRH was identified (Sherwood *et al.*, 1986) using a polyclonal antiserum (GF-4) directed against salmon GnRH ([Trp^7, Leu^8]GnRH). The cross-reactivity of GF-4, described previously (Kelsall *et al.*, 1990), was 100% for salmon GnRH, 220% for mammalian GnRH, 292% for chicken GnRH-I, 70% for chicken GnRH-II, and less than 0.02% for lamprey GnRH. This assay is not homologous and may be subject to

interference in crude extracts. However, the antiserum has been used successfully to detect GnRH from catfish extracts (Sherwood *et al.*, 1989; Chapter 2).

The purification procedure was modified slightly from one described in Lovejoy *et al.*(1991). The active fractions from the column (Sep-Pak) were combined and concentrated to 20% of their original volume. This material was initially separated on a C18 analytical column (Supelco) with an acetonitrile gradient of 1% /min in 0.1 M heptafluorobutyric acid (HFBA; pH 2.0). The active fractions representing catfish GnRH-I and -II from this stage were separated, concentrated 50%, and injected onto the same Supelco C18 column using a 0.5%/min acetonitrile gradient in 0.3 M triethylammonium formate (pH 2.5). The two forms of GnRH were purified individually. After concentration, the active fractions were run in 0.13 M triethylammonium phosphate (pH 6.5) using the same acetonitrile gradient. In the final purification stage, the immunoreactive GnRH fractions were resolved on a Vydac diphenyl analytical column using a 1%/min acetonitrile gradient in 0.05% TFA (pH 2.2).

Characterization of the Primary Structure

Initially, 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. Then, an aliquot representing 25% of the purified GnRH was dried in a Savant Speed-Vac concentrator and digested using calf liver pyroglutamyl amino-peptidase (Boehringer-Mannheim Biochemicals). The details of this procedure have been reported previously by Fischer and Spiess (1987) and Lovejoy *et al.* (1991). The digested sample of each form of catfish GnRH was sequenced using an Applied Biosystems Protein Sequencer (Model 470A). A sample representing about 40 ng of the purified catfish GnRH-I was added to a glycerol and 3-nitrobenzyl alcohol matrix in a ratio of 1:1. The mass spectra were measured using a Jeol JMS-HX110 double focusing mass spectrometer fitted with a Cs^+ gun. An accelerating voltage of 10 kV and a Cs^+ gun voltage of 25 kV were employed. An accelerating/electric field voltage scan from m/z 1100 to m/z 1500 (60-s scan slope) at a nominal resolution of 3000 was performed first with the sample and subsequently to measure CsI calibrant peaks. The mass accuracy of this scan was ±20ppm.

For capillary zone electrophoresis (CZE) analyses,

purified preparations were dissolved in 0.1% TFA at pH 2.5. Injection of the sample was accomplished by application of pressure for 4 s. A constant voltage (15 kV) was applied (current 84 μA for phosphate buffer) and separation was accomplished within 20 min. The capillary length to the detector was 50 cm.

Peptide Synthesis

Solid-phase synthesis of the peptides, based on the deduced structures, was carried out on a methylbenzhydrylamine (MBHA) resin using previously described methods (Merrifield, 1963). The peptides were deprotected and cleaved from the support using hydrofluoric acid. After purification of the synthetic peptides with reverse-phase high performance liquid chromatography (Rivier *et al.*, 1984), the structures were confirmed by mass spectral and amino acid composition analyses.

RESULTS

Assay of immunoreactive (ir)GnRH in fractions eluted from Sep-Pak columns indicated that two major forms were present. Immunoreactive GnRH in fractions 22-27 was

termed catfish GnRH-I and immunoreactive GnRH in fractions 28-32 was labeled catfish GnRH-II (Fig. 3.1). GnRH immunoreactivity in the post Sep-Pak fractions (Fig. 3.1) for catfish GnRH-I was 63 ng/g frozen brain tissue, or 24 ng/brain, and for catfish GnRH-II was 1.1 ng/g frozen tissue, or 0.4 ng/brain. At the final stage of purification (Fig. 3.2), 7100 ng catfish GnRH-I and 190 ng of catfish GnRH-II were measured in the fractions. This represented a recovery of 24 and 35% for catfish GnRH-I and -II, respectively, compared with the Sep-Pak fractions.

About 70% of the GnRH was successfully digested by pyroglutamyl aminopeptidase. The purified catfish-I $GnRH_{2-10}$ fragment eluted at 14.916 min compared to the undigested peptides at 16.350 (Fig. 3.3). Similarly, the catfish-II fragment eluted at 19.990, whereas the retention time of the undigested molecule was 21.671 (Fig. 3.3). Edman degradation of the catfish-I $GnRH_{2-10}$ fragment yielded the sequence

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

The sequence of the catfish-II $GnRH_{2-10}$ fragments was determined to be

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

Figure. 3.1. Elution of immunoreactive GnRH from the Sep-Pak column. GnRH in 20 μl aliquots from each fraction was assayed using antiserum GF-4. A large peak in fractions 22-27 (Catfish-I = CF I; ca 29,000 ng irGnRH) and a smaller one in fractions 28-32 (Catfish-II = CF II; ca 500 ng irGnRH) were observed. The quantity of material in catfish-I may depend on the use of a nonhomologous assay. Reservoir B consisted of 0.05% TFA in 80% acetonitrile.

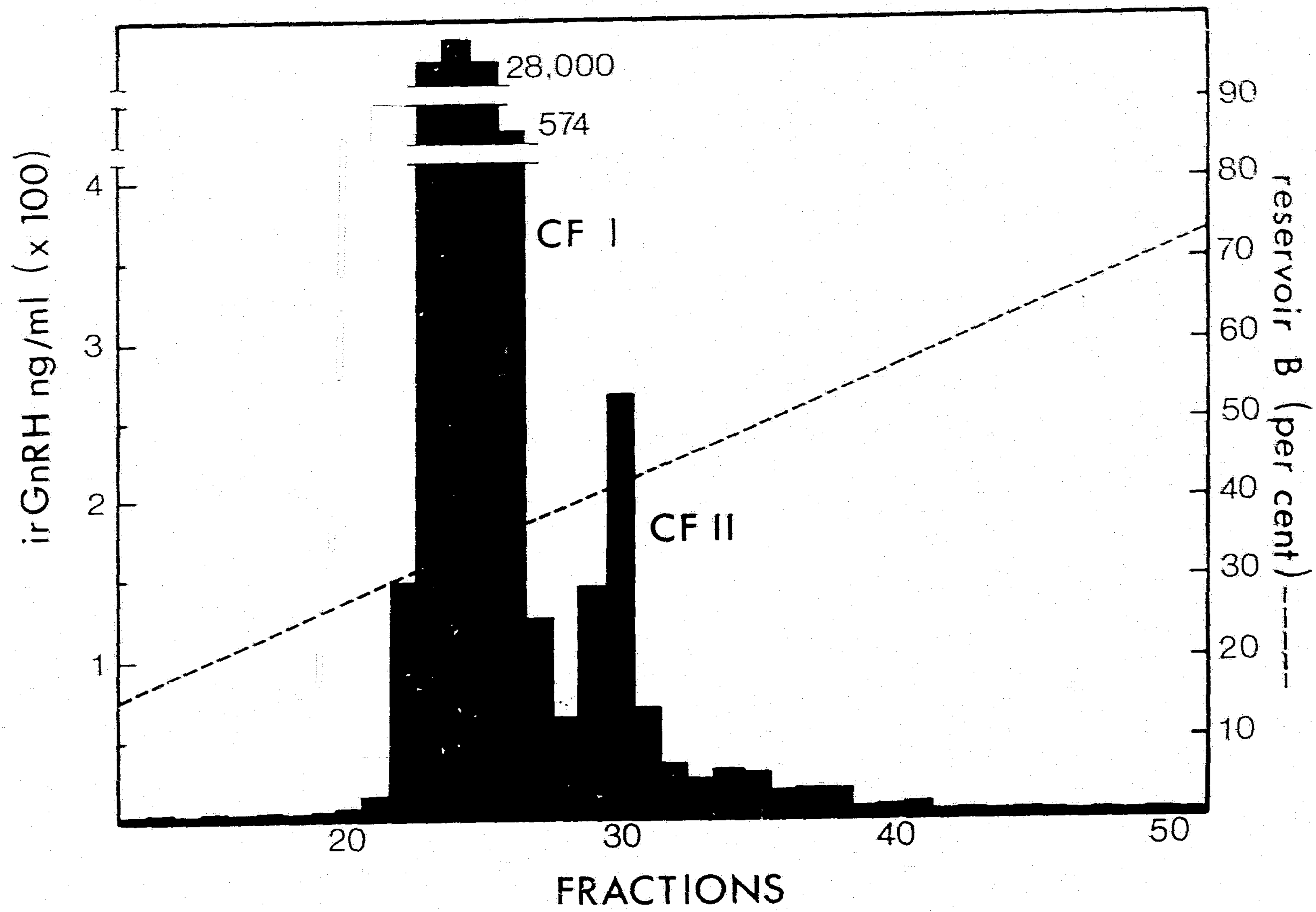

28,000
574
CF I
CF II
irGnRH ng/ml (x 100)
reservoir B (per cent) ----
FRACTIONS
1
2
3
4
10
20
30
40
50
60
70
80
90
20
30
40
50

Figure. 3.2. Final stage of catfish GnRH-I (CF I) and -II (CF II) purification. The GnRH peptides were resolved on a Vydac diphenyl analytical column. Full scale absorbance was 600 mAU. The immunoreactive HPLC fractions are denoted by the histogram surrounding the chromatogram peak. Peak fractions were used for structural characterization. Note the change in the vertical scale for CF I and CF II. Reservoir B consisted of 0.05% TFA in 80% acetonitrile.

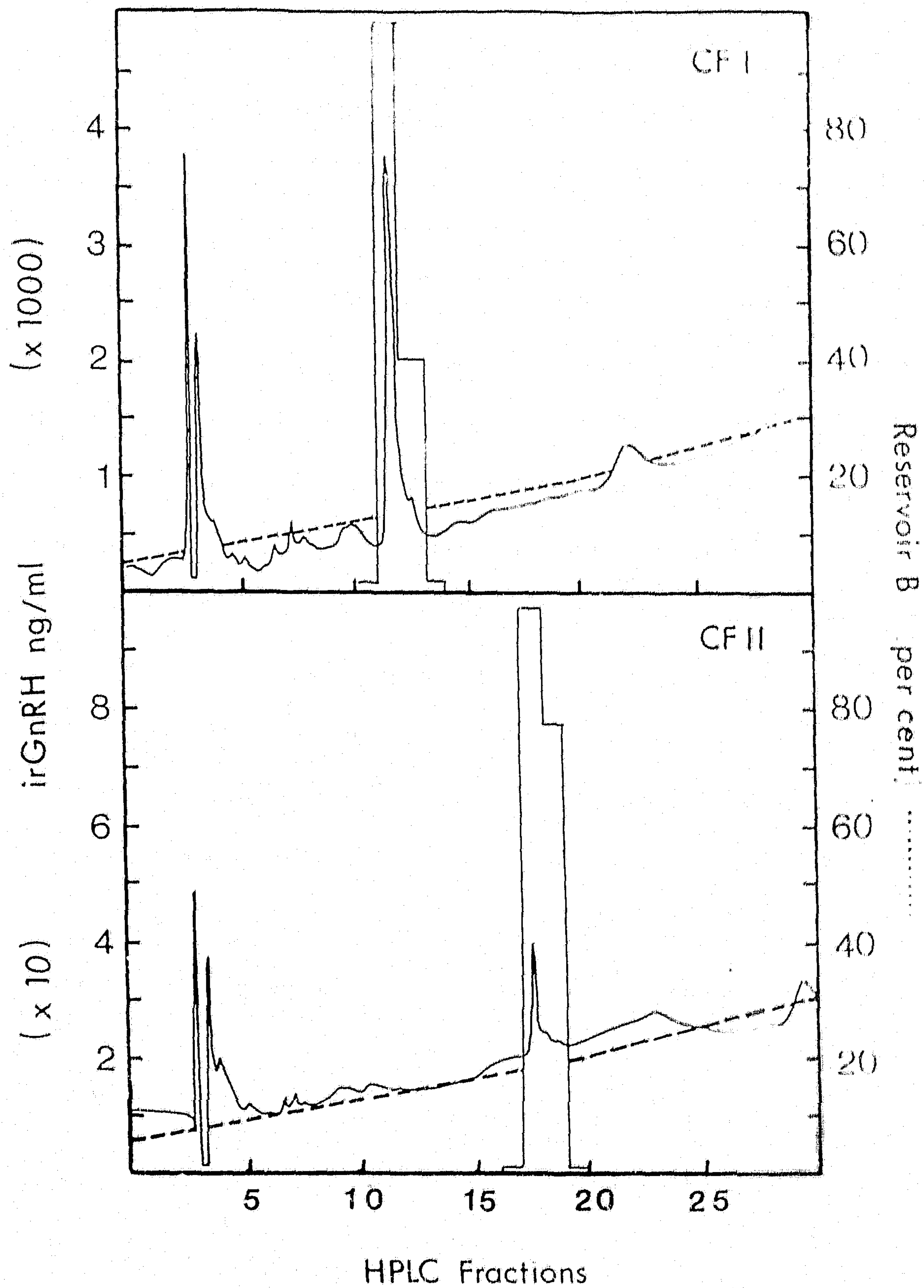
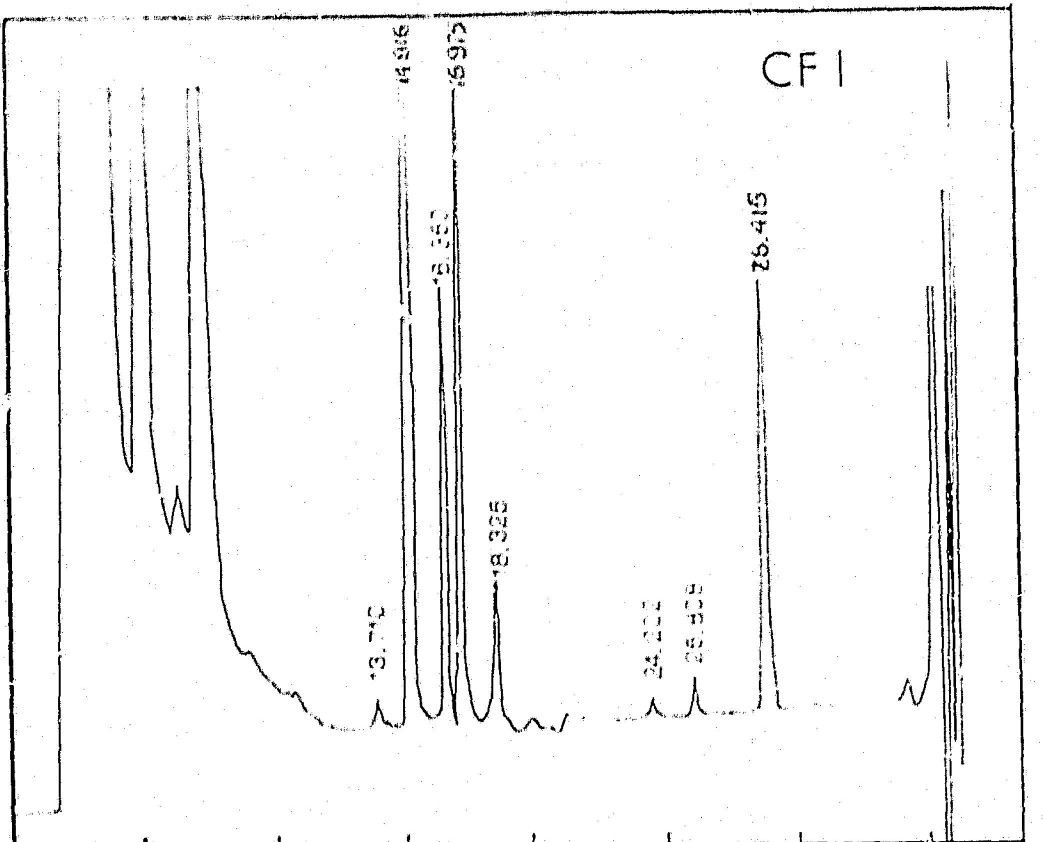
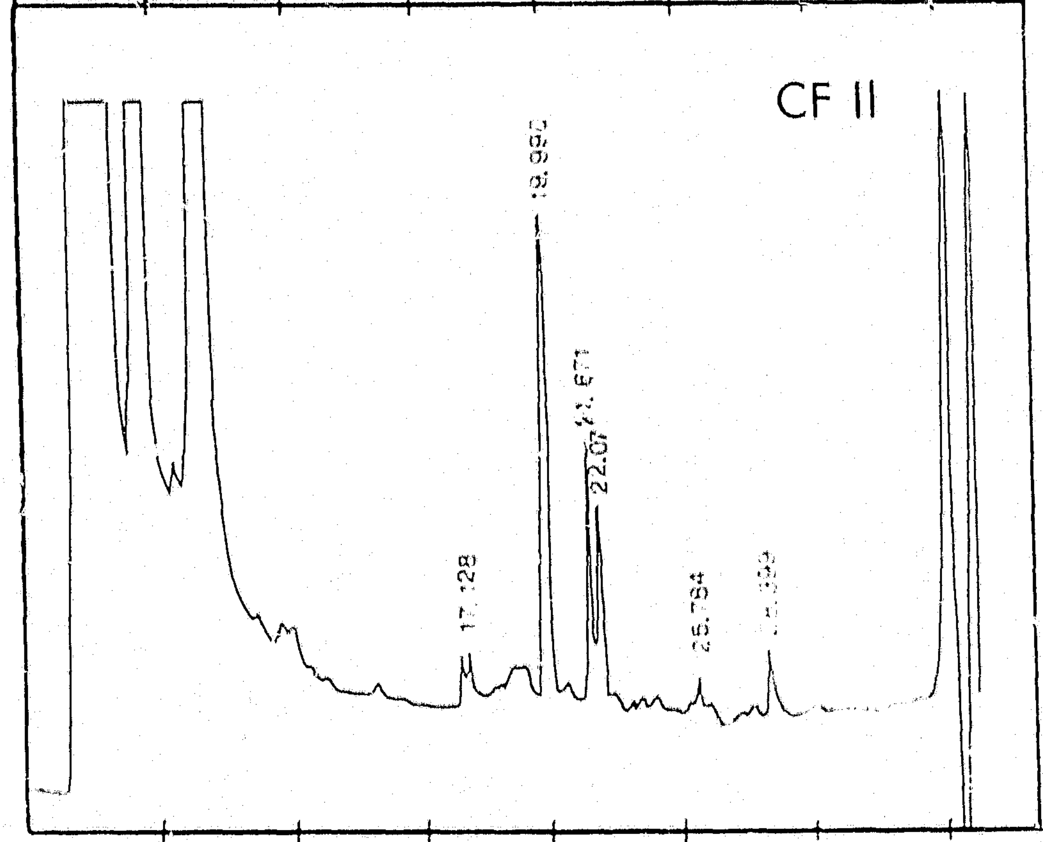

CF I
CF II
irGnRH ng/ml
(x 1000)
(x 10)
Reservoir B per cent
HPLC Fractions
4
3
2
1
8
6
4
2
80
60
40
20
80
60
40
20
5
10
15
20
25

Figure. 3.3 Purification of the catfish $GnRH_{2-10}$ fragment after digestion with pyroglutamyl aminopeptidase. The digested mixture was separated on a Vydac C18 microbore column using 0.05% TFA in 95% acetonitrile. The peaks at 14.916 min (CF I) and 19.990 (CF II) represent the $GnRH_{2-10}$ fragments. The material in these peaks was used for Edman degradation and mass spectral analysis.


CF I
absorbance


CF II
17.128
25.784
minutes after injection

The yields of the individual amino acids ranged from 5.6 to 69 pmol.

An elemental compositon of $C_{50}H_{68}N_{17}O_{13}$ gives a calculated mass of 1114.5 Da, which is consistent with the measured monoisotopic mass of 1114.5 Da obtained for catfish GnRH-I. The mass measurement is consistent with C-terminal amidation of the proposed sequence. There was insufficient material for the mass spectral analysis of catfish GnRH-II. Native and synthetic catfish GnRH-I and -II were found to coelute on HPLC and capillary zone electrophoresis under conditions that gave baseline separation of the two peptides.

DISCUSSION

This study provides the primary structure for a new member of the GnRH family of peptides. The structure is distinguished from the known forms by an asparagine residue at position 8. Also, it varies from mammalian GnRH by the presence of histidine in position 5. It is also clear that the brain and pituitary of the Thai catfish, *C. macrocephalus* contains a second form of GnRH. The primary structure of the second GnRH peptide is identical to that of chicken GnRH-II ([His^5, Trp^7, Tyr^8]GnRH). Peptide sequence data from the Thai catfish

brain has not been previously reported. Moreover, it is the first time that chicken GnRH-II has been confirmed in a species of bony fish and demonstrates the highly conserved nature of chicken GnRH-II. This work supports previous studies that provided indirect evidence that this peptide was found in bony fish (Sherwood, 1986; Yu *et al.*, 1988; Sherwood and Lovejoy, 1989; Okuzawa *et al.*, 1990).

The structural characterization was based on three main lines of evidence. First, the presence of the N-terminal pyroglutamyl residue was ascertained by successful Edman degradation after cleavage of the first amino acid with pyroglutamyl aminopeptidase. Sequence analysis could not be performed on the undigested peptide, because of the blocked N-terminus.

Second, the sequences of the $GnRH_{2-10}$ fragments were identified using normal Edman degradation. Finally, the molecular mass of the novel form was determined by mass spectral analysis. This confirmed the sequence analysis and indicated that the carboxyl terminus was amidated.

The distinct catfish peptide clearly belongs to the family of GnRH peptides. In all seven forms of GnRH, the length of chain, the amino and carboxyl termini, and the amino acids in positions 1, 2, 4, 9, and 10 have remained stable (Chapter 1, Table 1.1). The position 8 residue is

distinctive for each peptide. In mammals, GnRH residues 1-3 are necessary for the release of the gonadotropins from the pituitary (Schally and Coy, 1977; Rivier *et al.*, 1981). In catfish GnRH-I and -II, residues 1-3 are conserved.

The structural relationship of catfish GnRH to that of the other forms is shown in Figure 3.4. It is difficult to deduce whether catfish GnRH evolved due to an amino acid substitution from one of the other GnRH forms. This is because catfish GnRH is identical to all GnRH peptides in jawed vertebrates except in positions 5, 7 and 8. The catfish GnRH molecule differs from salmon GnRH in all 3 positions, but differs from mammalian, chicken I, chicken II and dogfish GnRH in 2 of the 3 positions. The problem with using amino acids to determine the order in which the peptides appeared in evolution can be illustrated by looking at position 5. It is not clear whether Tyr^5 in mammalian GnRH, chicken GnRH-I, and salmon GnRH or His^5 in chicken GnRH-II and catfish GnRH evolved first. Also, it is not known if the Leu^5 in lamprey GnRH is derived or if it is an ancestral condition of the molecule. The frequency of the amino acid replacement at position 5, His to Tyr or vice versa, is very low (Schulz and Schirmer, 1979), although only one nucleotide base substitution is required for this change between His^5 and

Figure. 3.4. The variable amino acid positions in GnRH peptides for jawless and jawed vertebrates.

* represents identical amino acids.

	1	2	3	4	5	6	7	8	9	10
Jawless Vertebrates										
Lamprey	*	*	Tyr	*	Leu	Glu	Trp	Lys	*	*
Jawed Vertebrates										
Mammalian	*	*	*	*	Tyr	*	Leu	Arg	*	*
Chicken I	*	*	*	*	Tyr	*	Leu	Gln	*	*
Salmon	*	*	*	*	Tyr	*	Trp	Leu	*	*
Chicken II	*	*	*	*	His	*	Trp	Tyr	*	*
Catfish	*****	*****	*****	*****	**His**	*****	**Leu**	**Asn**	*****	*****
Dogfish	*	*	*	*	His	*	Trp	Leu	*	*

Tyr^5. In conclusion, there is no obvious peptides from which catfish GnRH was derived unless the phylogenetic relationships of the fishes ia also considered as discussed below.

The fishes from which catfishes might have evolved contain either mammalian GnRH and chicken GnRH-II or salmon GnRH and chicken GnRH-II (Sherwood *et al.*, 1991). Because catfishes contain chicken GnRH-II also, but lack mammalian or salmon GnRH, it seems possible that catfish GnRH evolved due to a nucleotide substitution in the mammalian or salmon GnRH gene. This would require changing a minimum of three nucleotide bases (mammalian GnRH to catfish GnRH) or two nucleotide bases (salmon GnRH to catfish GnRH).

Elucidation of the two molecular forms of GnRH from *C. macrocephalus* is consistent with evidence suggesting that most vertebrates, with the exception of placental mammals, possess at least two forms of GnRH (Sherwood and Lovejoy, 1989). Despite the presence of two forms of GnRH in teleosts, distinct functions have not been delineated. Additional forms of GnRH in catfish brain cannot be discounted. However, only two forms of GnRH were detected in catfish brain extract using antisera that together recognize the seven forms of GnRH (Ngamvongchon *et al.*, 1992). Also, these immunological

studies do not discount that additional forms of GnRH might be identified by bioassays of HPLC fractions.

The determination of the primary structure of two catfish GnRH peptides is important for future research. The peptides can be used to understand GnRH evolution, by studying the functions of multiple GnRH forms within a species. Also, the information will enable us to improve synthetic GnRH analogs for specific species. This may increase their effectiveness for aquaculture.

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CHAPTER 4

DISTRIBUTION OF GONADOTROPIN-RELEASING HORMONES IN DISCRETE BRAIN AREAS AND PITUITARY OF FEMALE AND MALE THAI CATFISH

Parts of this chapter have been submitted for publication:

Ngamvongchon, S., Warby, C.M. and Sherwood, N.M. 1992. Localization of two forms of gonadotropin-releasing hormone (GnRH) in different brain areas and pituitary of female and male catfish, *Clarias macrocephalus*.

INTRODUCTION

The structure of gonadotropin-releasing hormone (GnRH) has been determined and seven distinct forms have been identified (Ngamvongchon *et al.*, 1992, Chapters 2, and 3). In teleosts, two forms of immunoreactive GnRH (irGnRH) have been detected in most brain extracts using high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) (King and Millar, 1985; Sherwood, 1986; Sherwood and Lovejoy, 1989). GnRH not only causes the release of gonadotropic hormones from the pituitary,

but also may function as a neurotransmitter or neuromodulator in neurons of the hypothalamus, olfactory lobes, ventral telencephalon, sympathetic ganglia and retina (see Sherwood, 1986).

The major forms of immunoreactive GnRH that have been found in teleosts are salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II) (Yu *et al.*, 1988; Sherwood and Lovejoy, 1989; Okuzawa *et al.*, 1990). In catfish brain, two forms of GnRH have been purified; one form (catfish GnRH) is distinct and the other is identical to cGnRH-II (Ngamvongchon *et al.*, 1992; Chapters 2, and 3). The physiological effects on ovulation of both forms of GnRH and their analogs will be described in Chapter 5.

In addition, it has been suggested that different forms of GnRH in the brain have different functions. However, the nature of these functions in the brain is still not clear. It is hypothesized that a differential localization of these two forms will give clues regarding differences in function. Therefore, this experiment was designed to localize GnRHs in different brain areas, spinal cord and pituitary gland of female and male catfish, *Clarias macrocephalus*, by using the combined methods of HPLC and RIA. In identification of chicken GnRH-II in HPLC fractions, both a homologous and heterologous RIA were used. The latter has an antiserum

with high relative (67%) cross-reactivity for chicken GnRH-II. A specific antiserum was not available for catfish GnRH, and hence two heterologous assays were used, one of which has high (50%) cross-reactivity with catfish GnRH.

MATERIALS AND METHODS

Collection of Tissues

Mature female and male catfish, *Clarias macrocephalus*, were purchased during the spawning season (July, 1991) from a private fish farm near Bangkok, Thailand. The fish were reared in a 1600 m^2 pond for 2 months before their collection in September 1991 at which time their body weights (BW) were 150 to 350 g. The water temperature ranged from 29°C to 32°C.

Fifty male and 45 female fish were collected. Whole brains and pituitaries from fish of both sexes were rapidly dissected into 8 different areas as showed in Figures 4.1, and 4.2. The tissues were rapidly frozen on dry ice and kept at -80°C until used.

Figure 4.1. Dorsal view of catfish brain showing parts dissected for study (adapted from Nieuwenhuys and Pouwels, 1983, p. 28). The parts were (1) olfactory tracts, (2) telencephalon = Tel, (3) hypothalamus; not shown, (4) pituitary gland; not shown, (5) thalamus+cerebellum+optic tectum = OT, (6) cerebellum, (7) medulla and (8) spinal cord.

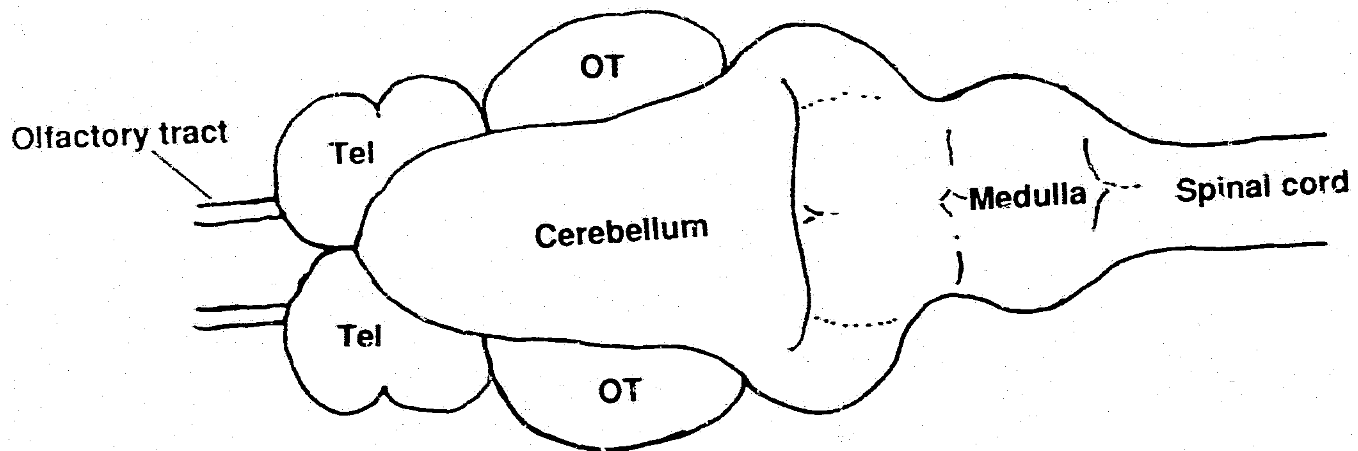
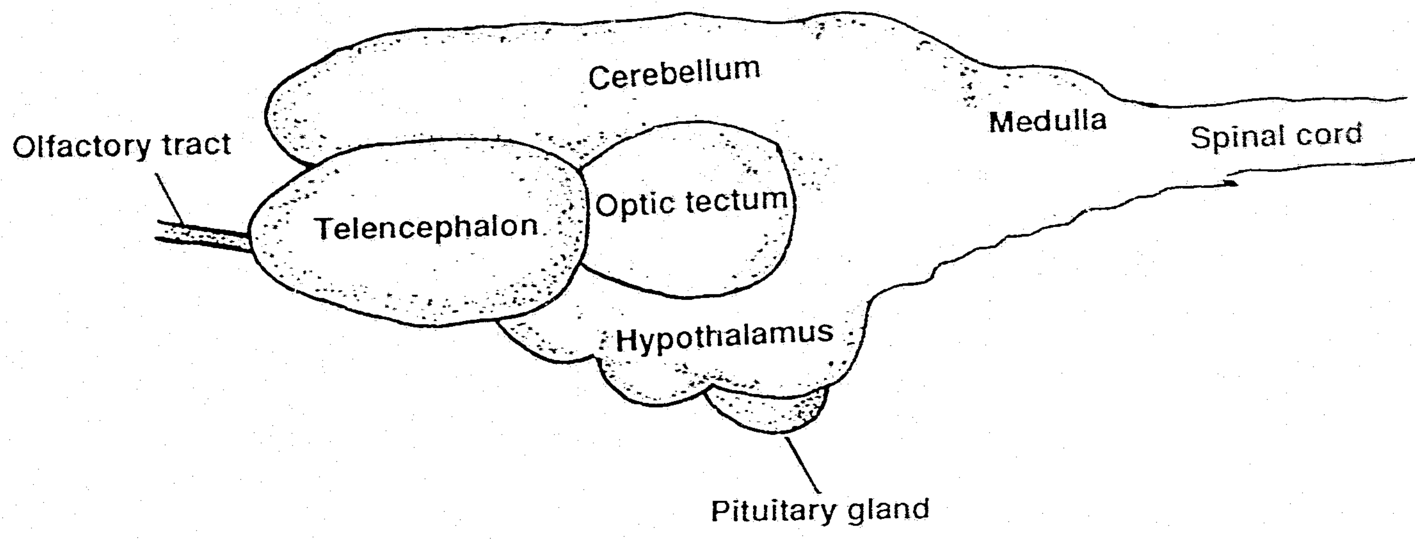

Olfactory tract
Tel
Tel
OT
OT
Cerebellum
Medulla
Spinal cord

Figure 4.2. Lateral view of catfish brain showing parts dissected for study (adapted from Timmers *et al.*, 1988, p. 20).


Cerebellum
Medulla
Spinal cord
Olfactory tract
Telencephalon
Optic tectum
Hypothalamus
Pituitary gland

Extraction of Tissues

Frozen dissected brain areas, spinal cord and pituitary glands were pooled and crushed to a fine powder with a prechilled mortar and pestle. Tissues from female and male fish were treated separately in all experiments. The weight of dissected brain areas in both sexes is shown in Table 4.1.

The tissue was treated as described (Sherwood *et al.*, 1989). In summary, the powdered tissue was added to 1 *N* HCl and acetone (3:100, v/v) in a ratio of 1 g frozen tissue to 5 ml of solution. Frozen tissue weighing less than 1 g was added to 10 ml of solution, since this volume was considered to be a minimum for extraction. The mixture was stirred for 3 hrs with dry ice around the container and filtered through Whatman No. 4 paper. The insoluble material was reextracted in 0.01 *N* HCl and acetone (1:4, v/v), in 40% of the original extraction volume, then stirred for 5 min and refiltered. In order to remove the hydrophobic substances, the combined filtrate was treated with petroleum ether (bp 30-60°C), with a ratio of 4:1 (v/v; filtrate to petroleum ether), for five successive extractions. The final extracted volume was reduced in a refrigerated vacuum concentrator (Savant) to 2.0 ml and filtered through a low-protein-

Table 4.1. Weight of various brain areas, spinal cord and pituitary gland of catfish in this study.

Brain areas	Female (mg/fish)	Male (mg/fish)
Olfactory tract	4.7	3.8
Telencephalon	54.7	35.4
Hypothalamus	40.8	31.4
Pituitary Gland	2.7	1.0
Thalamus+Cerebellum +Optic Tectum	106.7	76.4
Cerebellum	91.1	77.4
Medulla	21.3	20.4
Spinal Cord (0.3cm)	12.4	17.[illegible]

absorbing filter (Millipore Millex-GV, 0.22 μm). An aliquot of 5% of the total volume of each extracted filtrate was kept for RIA and the rest was used for further purification.

High Performance Liquid Chromatography (HPLC)

The concentrated tissue extracts of the male and female fish were injected onto a Supelco C-18 column (0.5μm, 0.46 x 25 cm) with a C-18 guard column (0.5μm, 0.46 x 2cm) of the same material. A Varian 5000 liquid chromatograph was programmed for a flow rate of 1 ml/min. The tissue filtrate was injected at the beginning of a 5 min period of 4% acetonitrile (CH_3CN) in 0.1 *M* heptafluorobutyric acid (HFBA) pH 2.0. Acetonitrile was then increased as a linear gradient from 4% to 48% CH_3CN for 60 min (0.8% increase/min). Sixty fractions of 1 ml were collected from each extraction.

A blank run, in which 800μl Milli-Q water was injected, preceded each injection of the tissue extract. Sixty fractions of 1 ml were collected under the same conditions as for the extracts. All blank fractions were vacuum dried and assayed with antiserum GF-4 to determine whether the previously injected extracts were being carried over to subsequent runs.

Synthetic standards of the seven known forms of GnRH were chromatographed after the sample runs for comparison with the brain tissue extracts. Synthetic mammalian, salmon, chicken-I and chicken-II GnRH peptides were purchased from Peninsula Laboratories (Belmont, CA). Lamprey GnRH was a gift from Dr. D. Marshak; catfish and dogfish GnRHs were a gift from Dr. J. Rivier. The synthetic standards (200 ng each) individually and combined were diluted with Milli-Q water to a final volume of 800 μl and injected onto the HPLC column. The elution positions were determined, individually and combined, by measuring their absorbance at 280 nm

Radioimmunoassay (RIA)

Heterologous assay. GnRH immunoreactivity (irGnRH) in the HPLC fractions (1000μl each) of extracted tissues was measured by RIA using a 200μl sample of each fraction and antiserum GF-4 (made against salmon GnRH) and synthetic mammalian GnRH (mGnRH) as the iodinated tracer and standard (Sherwood *et al.*, 1983; 1986). This assay resulted in 24-30% binding of ^{125}I mGnRH and at 50% B/B$_o$ was equal to 10-31 pg at 1:25,000 final dilution of the antiserum. Fractions with high concentrations of irGnRH were reassayed in 1:2 serial dilutions; the values

closest to 50% binding are reported.

The percentage of relative cross-reactivity between the seven known GnRHs with 3 antisera, B-6, Bla-4, and GF-4, was determined. Antiserum B-6, made against mGnRH, had 57% binding at 1:5000 final dilution, whereas Bla-4, made against lamprey GnRH, had 13% binding at 1:5000 final dilution, and antiserum GF-4, made against salmon GnRH, had 32% binding at 1:25,000 final dilution.

Homologous assay. A 200μl sample from the HPLC fractions of each tissue extract was assayed by RIA using synthetic chicken GnRH-II as iodinated tracer and standard. Three antisera made against chicken GnRH-II were used. Antiserum 34-3 resulted in 18% binding at 1:5000 final dilution; 8NW4 resulted in 14% binding at 1:5000 final dilution; and 8NP5 resulted in 0.01% binding at the same final dilution. Antiserum 34-3 was a gift from Dr. Renato De Leeuw; 8NP5 and 8NW4 were gifts from Dr. R.E. Peter. For antiserum 34-3, which was selected for the assay based on % binding, 80% B/B_o was equal to 125-310 pg. Fractions with high concentrations of irGnRH were reassayed in 1:2 serial dilutions. Values closest to 50% tracer binding are reported. The percent of relative cross-reactivity of the 7 known forms of GnRH with antiserum 34-3 (made against cGnRH-II) using ^{125}I cGnRH-II as tracer is also reported.

Radioiodination

The radiolabelled GnRH peptides were iodinated using the chloramine T method. In brief, 5 μg (1 μg/μl) of synthetic cGnRH-II or mGnRH was added into 25 μl of 0.5 M phosphate buffer, pH 7.5, prior to the addition of 10 μl chloramine T (25 mg in 12.5 ml 0.05 M phosphate buffer pH 7.0). Then 1 mCi (10 μl) of ^{125}I-Na was added to the mixture. The reaction was allowed to continue for 60 seconds and then stopped by adding 50 μl potassium metabisulfate solution (KMBS, 29.2 mg in 12.5 ml 0.05 M phosphate buffer pH 7.0).

For the cGnRH-II iodination, 1 g of fine carboxymethyl cellulose (CMC, Sigma) was swollen overnight in 100 ml of 0.3 M ammonium acetate pH 4.5 and packed into a 12x1 cm column. The packed column was conditioned with 250 ml of 0.3 M ammonium acetate. The radiolabelled mixture and a rinse of 200 μl of 0.02 M ammonium acetate were added onto the column. The radiolabelled GnRH was eluted from the column by adding 15 ml of 0.02 M ammonium acetate followed by 80 ml of 0.3 M ammonium acetate. Forty fractions of 2 ml were collected and a 5 μl aliquot of each fraction was counted.

For iodination of mGnRH, 8 g of Sephadex G-25 (fine) swollen overnight in 48 ml phosphate buffered saline

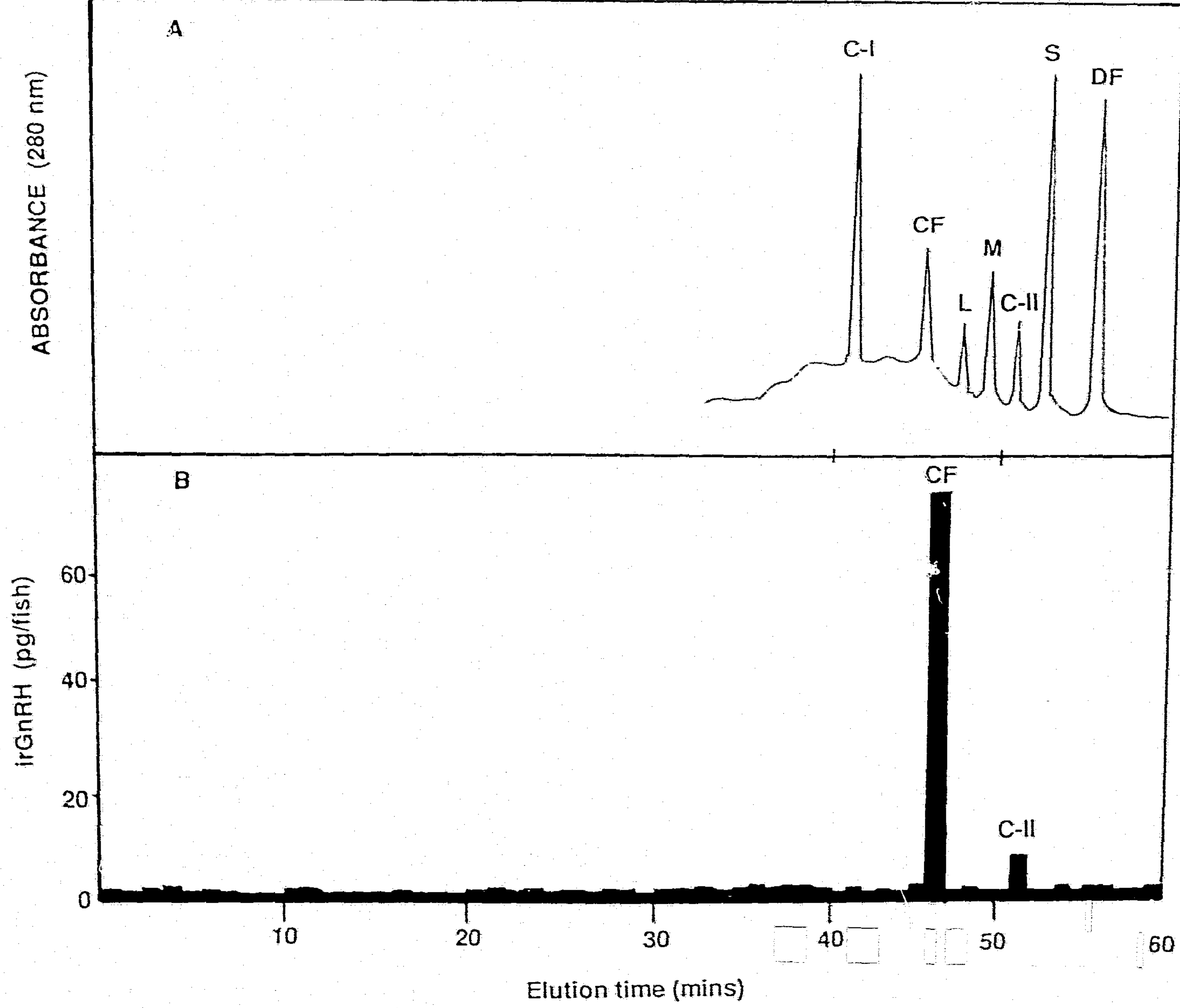
(PBS) was packed into the 60x1 cm column one day prior to the iodination. The column was conditioned with 20 ml of 1% gelatin/PBS followed by 60 ml PBS. The radiolabelled mixture was added onto the column, then eluted with the 1% gelatin/PBS solution. Fifty fractions of 2 ml were collected and a 5 µl aliquot of each fraction was counted.

RESULTS

The results from combined methods of RIA and reverse phase HPLC show that the elution of the early peak (fractions 46, 47) is coincident with the catfish GnRH standard and the elution of the late peak (fractions 52, 53) coincides with chicken GnRH-II (cGnRH-II) standard. The positions of the standard peaks (Fig. 4.3A) are compared with the extract peaks (Fig. 4.3B) run under the same conditions. The synthetic standards of chicken-I, catfish, lamprey, mammalian, chicken-II, salmon and dogfish GnRH eluted at fractions 42, 46, 48, 49, 51, 53 and 56 respectively (Fig. 4.3A).

To ensure that antiserum GF-4, made against salmon GnRH, detects catfish GnRH and cGnRH-II, the percentage of relative cross-reactivity of 7 known GnRH peptides and 3 antisera (B-6, Bla-4 and GF-4) was measured

Figure 4.3. (A) shows the seven known synthetic standards of GnRH eluted from the HPLC system. (B) indicates the immunoreactive GnRH detected from a tissue extract of male hypothalamus using a heterologous assay. C-I = chicken GnRH-I; CF = catfish GnRH; L = lamprey GnRH; M = mammalian GnRH; C-II = chicken GnRH-II; S = salmon GnRH and DF = dogfish GnRH.



A
ABSORBANCE (280 nm)
C-I
CF
L
M
C-II
S
DF
B
irGnRH (pg/fish)
0
20
40
60
CF
C-II
10
20
30
40
50
60
Elution time (mins)

(Table 4.2). Antiserum GF-4 showed 50% cross-reactivity with catfish GnRH and 67% cross-reactivity with cGnRH-II compared with mammalian GnRH. However, antisera B-6 and Bla-4 showed 1% cross-reactivity with catfish GnRH and chicken GnRH-II. Hence, GF-4 was selected as the antiserum for the heterologous RIA in this study.

Immunoreactive GnRH (irGnRH) was detected from tissue extracts of various brain areas, spinal cord and pituitaries from both female and male catfish. The measurement of immunoreactive GnRH was calculated as both content (Tables 4.3, 4.5, Figs. 4.4, 4.6, 4.8) and concentration (Tables 4.4, 4.6, Figs. 4.5, 4.7, 4.9) for different brain areas (Figs. 4.4, 4.5, 4.6). The two irGnRH peaks, catfish GnRH and cGnRH-II, were detected using the heterologous assay.

In this assay, catfish GnRH was detected mainly in female pituitary glands, at a content of 34 pg/fish (Table 4.3) and a concentration of 13 pg/mg tissue (Table 4.4). The concentration of catfish GnRH in other parts of the female brain was: olfactory tract (1.2 pg/mg tissue), hypothalamus (0.1 pg/mg tissue), medulla (0.1 pg/mg tissue), and telencephalon (0.1 pg/mg tissue).

Table 4.2. Percentage cross-reactivity of four antisera with seven GnRHs.

GnRH	Antiserum B-6	Antiserum Bla-4	Antiserum GF-4	Antiserum 34-3
Mammalian GnRH	100	100	100	0.04
Lamprey GnRH	<0.05	200	<0.05	24
Chicken GnRH-I	<0.05	100	100	0.002
Chicken GnRH-II	<0.05	1.0	67	100
Salmon GnRH	<0.05	67	50	2
Catfish GnRH	<0.05	1.3	50	0.02
Dogfish GnRH	<0.05	0.4	14	4

Mammalian GnRH was used as the reference peptide for 3 antisera in the calculation of relative activity. Relative cross-reactivity (%) is picomoles of the reference peptide at 50% B/B_0 divided by picomoles of the other peptides at 50% B/B_0 times 100. Mammalian GnRH was also used as ^{125}I tracer for the assays. The final dilutions of the three antisera were 1:5000 (B-6), 1:5000 (Bla-4), and 1:25000 (GF-4).
Chicken GnRH-II was used as the reference peptide for antiserum 34-3 because the antiserum was made against this peptide. The final dilution was 1:5000. cGnRH-II was used as the ^{125}I tracer.

Table 4.3. Content of immunoreactive catfish GnRH and chicken GnRH-II in various brain areas, spinal cord, and pituitary gland of female and male catfish. A heterologous RIA with mammalian GnRH as the iodinated tracer and standard was used with antiserum GF-4. Tissue from each area was pooled and used for analysis. (* = irGnRH <1.0 pg/fish)

Brain areas	Female		Male	
	Catfish GnRH (pg/fish)	cGnRH-II (pg/fish)	Catfish GnRH (pg/fish)	cGnRH-II (pg/fish)
Olfactory Tract	5.6	*	8.3	*
Telencephalon	5.1	1.1	9.6	2.8
Hypothalamus	5.3	*	73.0	7.5
Pituitary Gland	34.2	3.3	*	*
Thalamus +Cerebellum +Optic Tectum	2.3	2.3	1.2	2.2
Cerebellum	3.2	7.8	1.2	4.8
Medulla	2.4	3.3	*	*
Spinal Cord	*	*	*	*

Table 4.4. Concentration of immunoreactive catfish GnRH and chicken GnRH-II in various brain areas, spinal cord, and pituitary gland of female and male catfish. A heterologous RIA with mammalian GnRH as the iodinated tracer and standard was used with antiserum GF-4. Tissue from each area was pooled and used for analysis. (* = irGnRH < 0.01 pg/mg tissue)

Brain areas	Female		Male	
	Catfish GnRH (pg/mg)	cGnRH-II (pg/mg)	Catfish GnRH (pg/mg)	cGnRH-II (pg/mg)
Olfactory Tract	1.19	*	2.18	*
Telencephalon	0.09	0.02	0.27	0.08
Hypothalamus	0.13	*	2.32	0.24
Pituitary Gland	12.67	1.22	*	*
Thalamus +Cerebellum +Optic Tectum	0.02	0.02	0.01	0.03
Cerebellum	0.03	0.08	0.01	0.06
Medulla	0.11	0.15	*	*
Spinal Cord	*	*	*	*

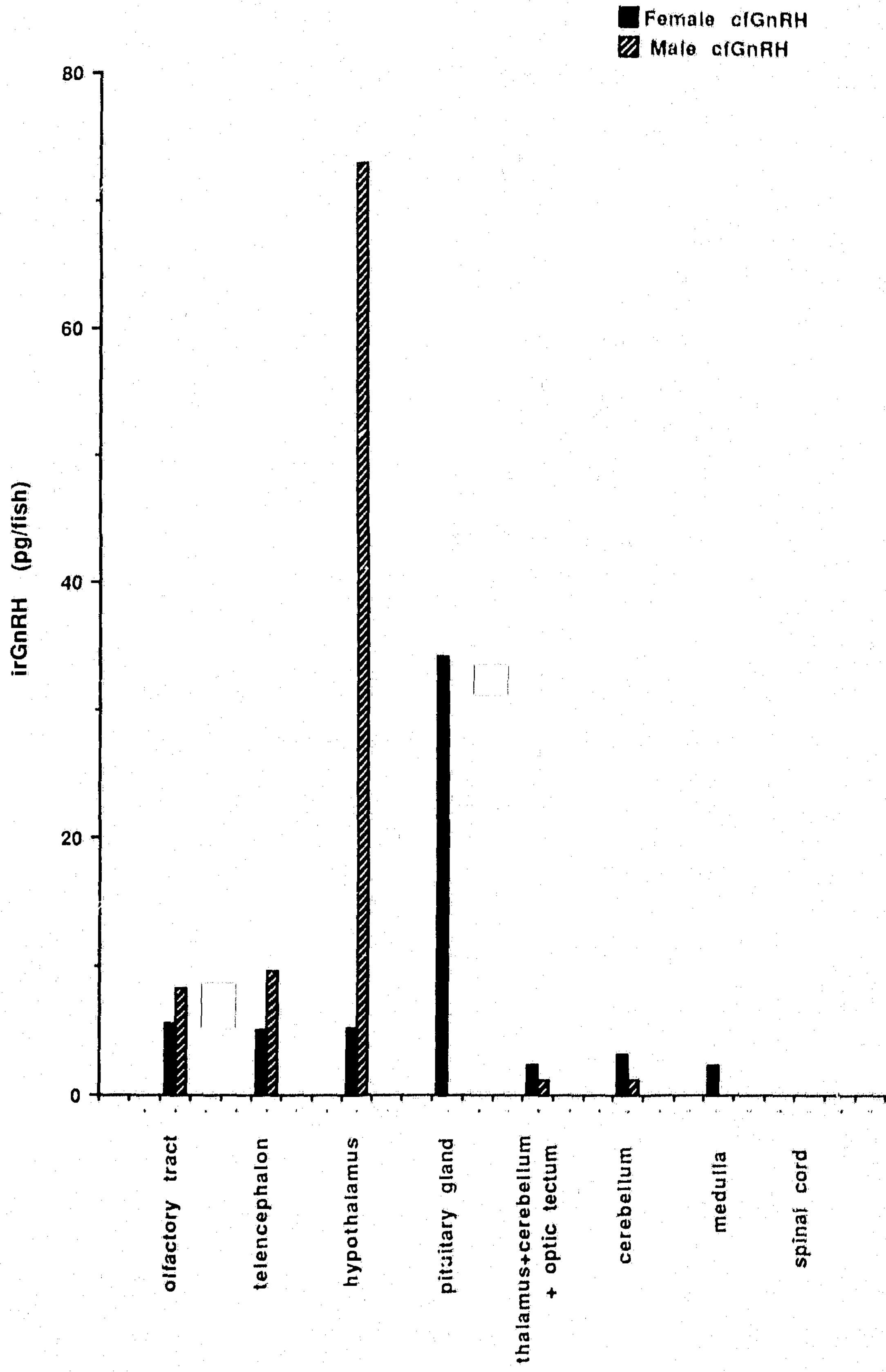
Table 4.5. Content of immunoreactive catfish GnRH and chicken GnRH-II in various brain areas, spinal cord, and pituitary of female and male catfish. A homologous RIA with chicken GnRH-II as iodinated tracer and standard was used with antiserum 34-3. Tissue from each area was pooled and used for analysis.
(* = irGnRH <1.0 pg/fish)

Brain areas	Female		Male	
	Catfish GnRH (pg/fish)	cGnRH-II (pg/fish)	Catfish GnRH (pg/fish)	cGnRH-II (pg/fish)
Olfactory Tract	*	*	*	*
Telencephalon	*	*	*	23.5
Hypothalamus	*	*	*	13.0
Pituitary Gland	*	*	*	*
Thalamus +Cerebellum +Optic Tectum	*	40.4	*	38.0
Cerebellum	*	184.3	*	320.0
Medulla	*	14.5	*	74.0
Spinal Cord	*	*	*	*

Table 4.6. Concentration of immunoreactive catfish GnRH and chicken GnRH-II in various brain areas, spinal cord, and pituitary of female and male catfish. A homologous RIA with chicken GnRH-II as iodinated tracer and standard was used with antiserum 34-3. Tissue from each area was pooled and used for analysis. (* = irGnRH <0.01 pg/mg tissue)

Brain areas	Female		Male	
	Catfish GnRH (pg/mg)	cGnRH-II (pg/mg)	Catfish GnRH (pg/mg)	cGnRH-II (pg/mg)
Olfactory Tract	*	*	*	*
Telencephalon	*	*	*	0.66
Hypothalamus	*	*	*	0.41
Pituitary Gland	*	*	*	*
Thalamus +Cerebellum +Optic Tectum	*	0.38	*	0.50
Cerebellum	*	2.02	*	4.13
Medulla	*	0.68	*	3.63
Spinal Cord	*	*	*	*

Figure. 4.4. Content of catfish GnRH in female and male measured with a heterologous RIA.



Female cfGnRH
Male cfGnRH
irGnRH (pg/fish)
80
60
40
20
0
olfactory tract
telencephalon
hypothalamus
pituitary gland
thalamus+cerebellum
+ optic tectum
cerebellum
medulla
spinal cord

Figure. 4.5. Concentration of catfish GnRH in female and male brains measured with a heterologous RIA.

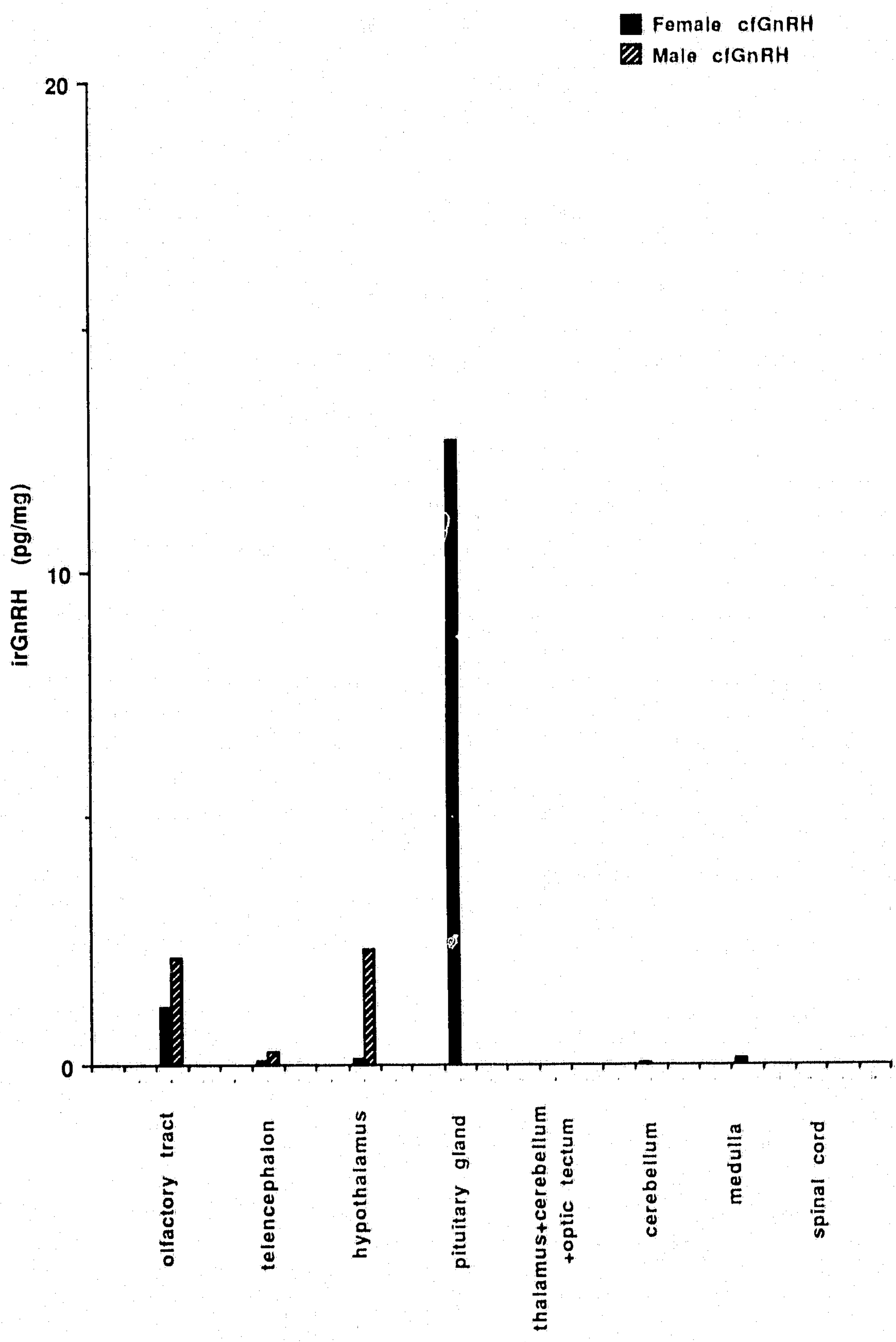

Female cfGnRH
Male cfGnRH
irGnRH (pg/mg)
20
10
0
olfactory tract
telencephalon
hypothalamus
pituitary gland
thalamus+cerebellum
+optic tectum
cerebellum
medulla
spinal cord

Figure 4.6. Content of chicken GnRH-II in female brains measured with heterologous and homologous RIAs.

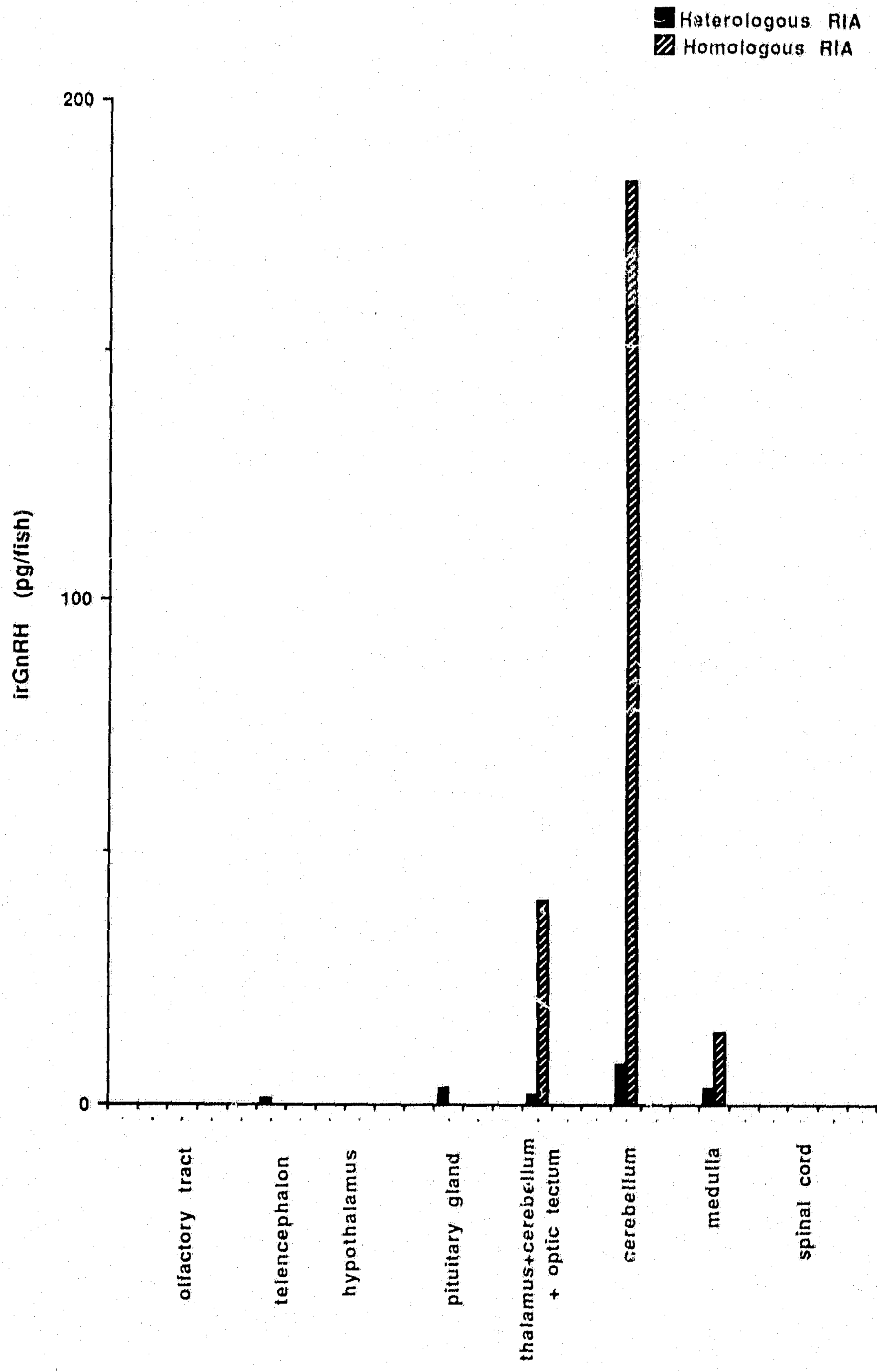

Heterologous RIA
Homologous RIA
200
100
0
irGnRH (pg/fish)
olfactory tract
telencephalon
hypothalamus
pituitary gland
thalamus+cerebellum
+ optic tectum
cerebellum
medulla
spinal cord

Figure 4.7. Concentration of chicken GnRH-II in female brains measured with heterologous and homologous RIAs.

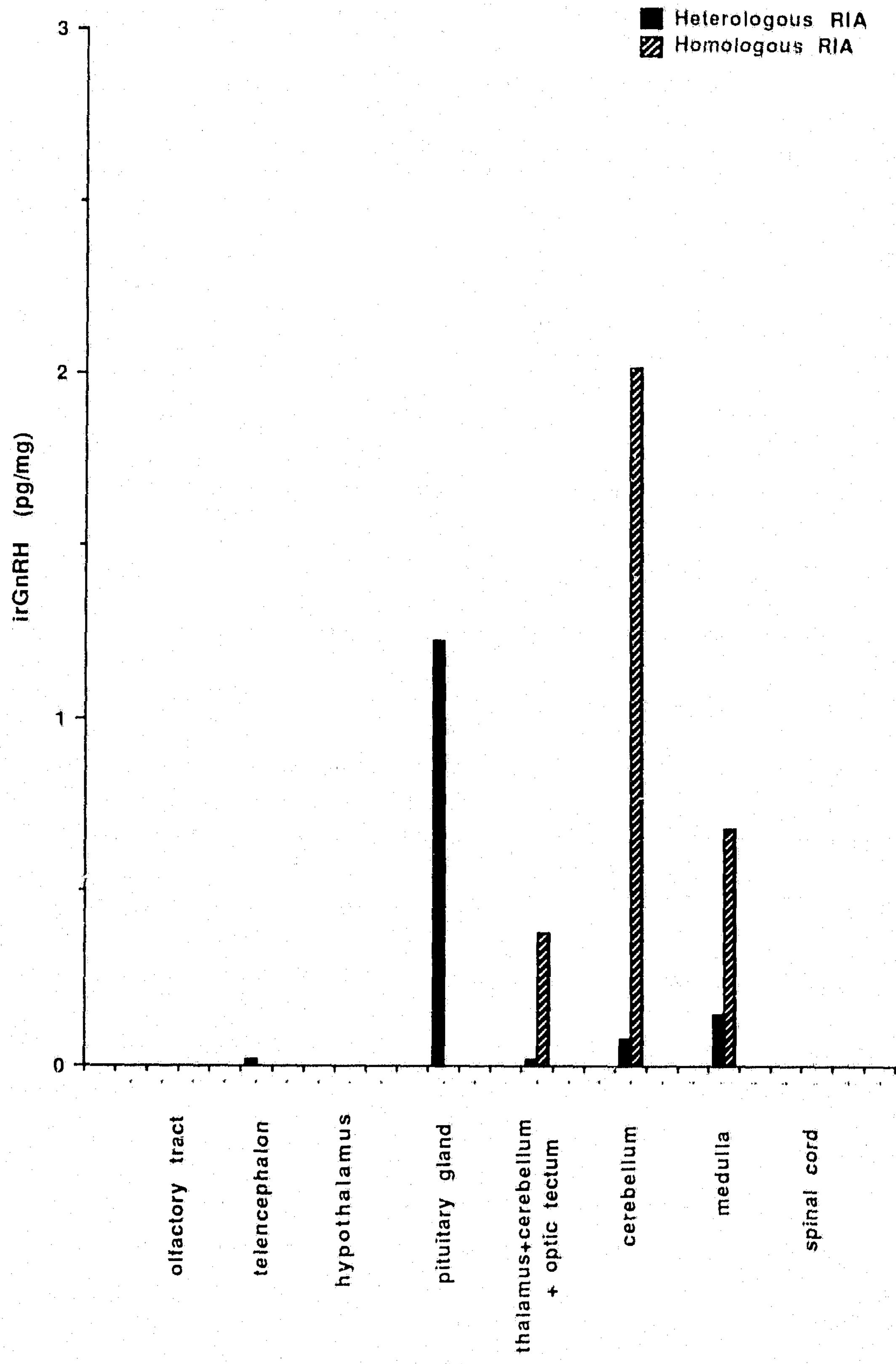

Heterologous RIA
Homologous RIA
irGnRH (pg/mg)
3
2
1
0
olfactory tract
telencephalon
hypothalamus
pituitary gland
thalamus+cerebellum
+ optic tectum
cerebellum
medulla
spinal cord

Figure 4.8. Content of chicken GnRH-II in male brains measured with heterologous and homologous RIAs,

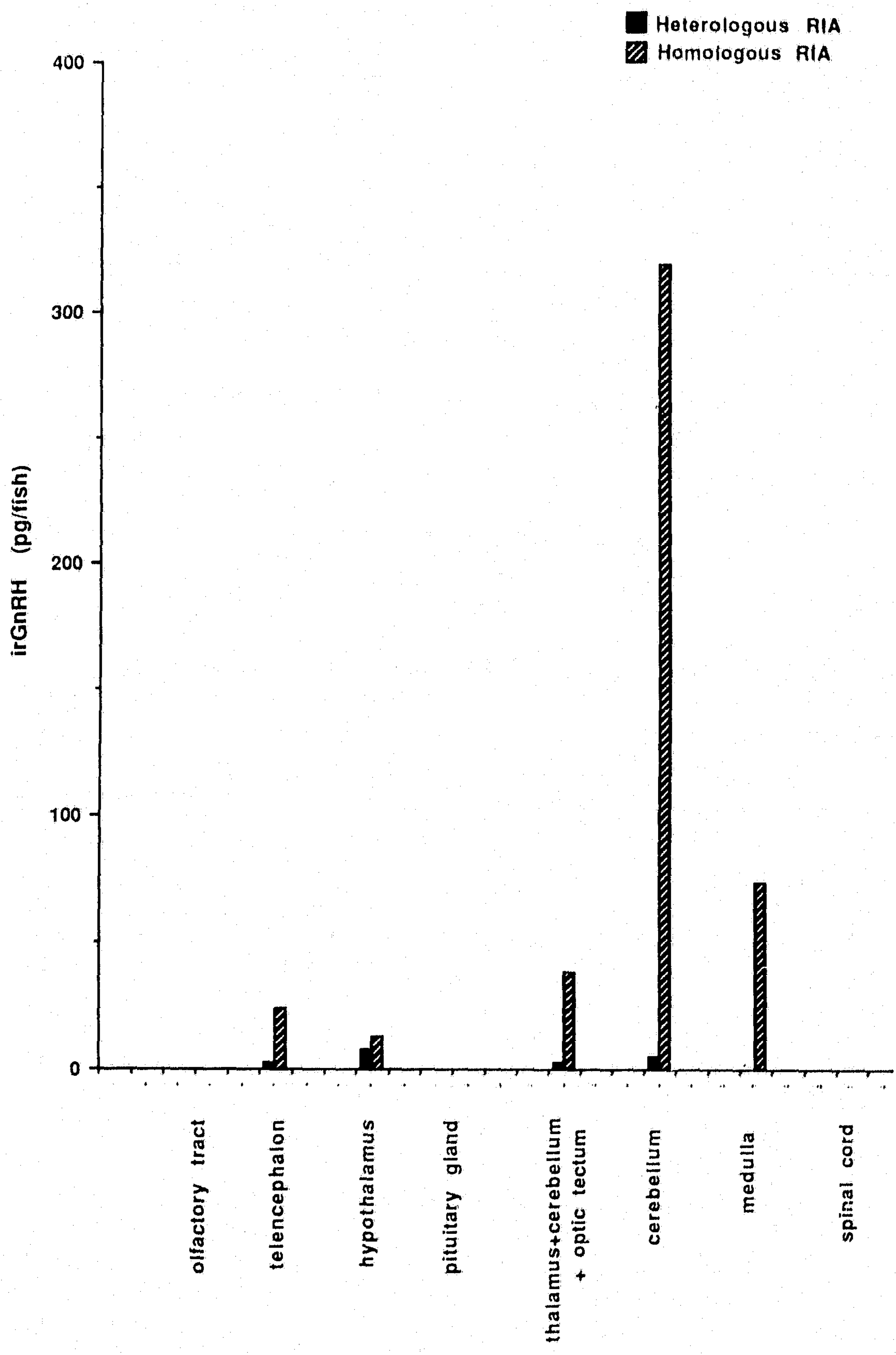
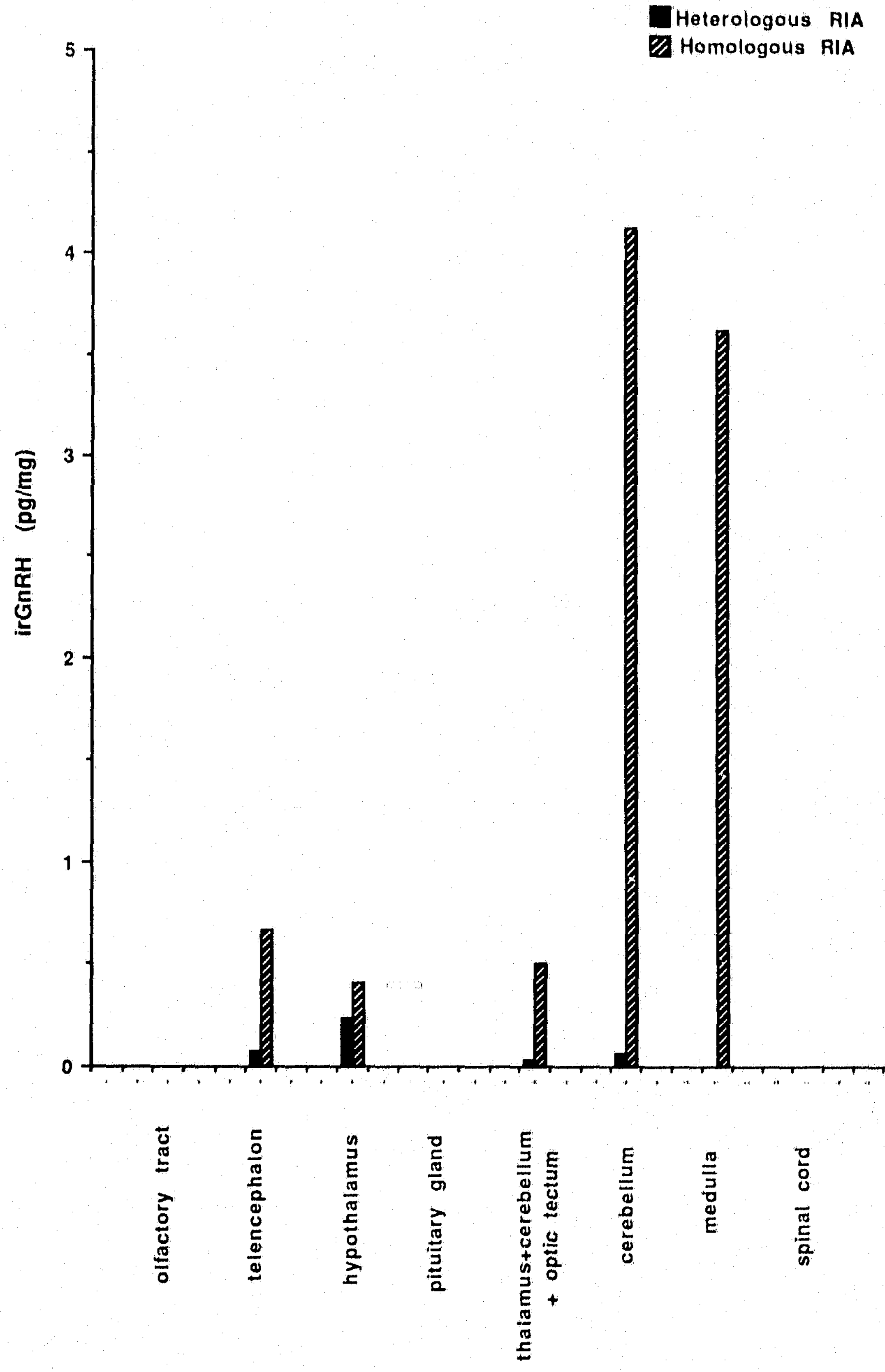

Heterologous RIA
Homologous RIA
400
300
200
100
0
irGnRH (pg/fish)
olfactory tract
telencephalon
hypothalamus
pituitary gland
thalamus+cerebellum
+ optic tectum
cerebellum
medulla
spinal cord

Figure 4.9. Concentration of chicken GnRH-II in male brains measured with heterologous and homologous RIAs.


Heterologous RIA
Homologous RIA
irGnRH (pg/mg)
5
4
3
2
1
0
olfactory tract
telencephalon
hypothalamus
pituitary gland
thalamus+cerebellum
+ optic tectum
cerebellum
medulla
spinal cord

Less than 0.1 pg/mg tissue was detected in the cerebellum and thalamus+cerebellum+optic tectum (Table 4.4). In male brain areas, catfish GnRH was mainly detected in the hypothalamus with a content of 73 pg/fish and a concentration of 2.3 pg/mg tissue (Table 4.4). The concentrations were 2.2 pg/mg tissue (olfactory tract), 0.3 pg/mg tissue (telencephalon) and only 0.01 pg/mg tissue or less in the remaining areas (Table 4.4). The measurement of both content and concentration of catfish GnRH in the anterior areas of the brain was higher in the male than female (Tables 4.3 and 4.4).

Chicken GnRH-II was also present in the female pituitary glands (1.2 pg/mg tissue), and medulla (0.2 mg/mq tissue), and cerebellum (0.1 pg/mg tissue) but not in the olfactory tract, hypothalamus and spinal cord. Only 0.02 pg/mg tissue was in the telencephalon and remaining brain areas (Table 4.4). Chicken GnRH-II was detectable in the male hypothalamus with a concentration of 0.2 pg/mg tissue and less than 0.1 pg/mg tissue in the remaining areas (Table 4.4).

In the homologous assay, antiserum 34-3 was selected in this experiment because it had a higher percentage of binding compared to the other 2 antisera. This assay was useful for chicken GnRH-II but not for catfish GnRH because antiserum 34-3 has a cross-reactivity of only

0.02% compared with chicken GnRH-II (Table 4.2).

The amount of immunoreactive chicken GnRH-II was high in the homologous assay (Tables 4.5, 4.6) compared to the heterologous assay. The measurements of both the content and the concentration of chicken GnRH-II were highest in the cerebellum of both sexes. The concentration of chicken GnRH-II was 4.1 pg/mg tissue in the male cerebellum (Table 4.6). The other concentrations were: medulla, 3.6 pg/mg tissue; telencephalon, 0.7 pg/mg tissue; thalamus+cerebellum+optic tectum, 0.5 pg/mg tissue; and hypothalamus, 0.4 pg/mg tissue. Chicken GnRH-II was not detectable in the spinal cord, pituitary and olfactory tract (Table 4.6). In female catfish, the concentrations of chicken GnRH-II were: cerebellum (2.0 pg/mg tissue), medulla (0.7 pg/mg tissue) and thalamus+cerebellum+optic tectum (0.4 pg/mg tissue). Female brain areas had lower quantities of cGnRH-II than the corresponding male parts. However, statistical analysis could not be done because the brain tissues had to be pooled before extraction to detect GnRH in the HPLC fractions.

DISCUSSION

The results obtained from this experiment show that

immunoreactive catfish GnRH and cGnRH-II are differentially distributed in the brain. Catfish GnRH is of considerable interest because it has been identified to date in only 3 species of catfish (*C. macrocephalus*, *C. batrachus* and *C. gariepinus*). The present results from the heterologous RIA show that this form of GnRH is present predominately in the pituitary gland of females at a level that is 12 fold higher in concentration than any other region (Table 4.4). This suggests that catfish GnRH is the physiological releaser of gonadotropins from the pituitary in catfish. GnRH axons grow directly into the pituitary in teleosts so that stored GnRH in the nerve terminals would be measured in the pituitary sample. Catfish GnRH is also present in the olfactory tract and telencephalon, areas associated with the terminal nerve (Demski *et al.*, 1983).

In males, the distribution of catfish GnRH is similar to females in the sense that the detectable immunoreactive GnRH is in the anterior regions of the brain. The concentration in the male hypothalamus rather than the pituitary may reflect the presence of GnRH in cell bodies in the hypothalamus in contrast to a storage site in the axon terminals in the pituitary. This location, however, could also be due to GnRH axons that are transversing the hypothalamus to terminate in a

region other than the pituitary. Again both males and females have a higher content of immunreactive catfish GnRH in the olfactory tract and telencephalon and a higher concentration in the olfactory tract compared with the posterior brain regions.

The distribution of catfish GnRH forms a pattern much like that found for salmon GnRH in goldfish (Yu *et al.*, 1988) and rainbow trout (Okuzawa *et al.*, 1990), and for chicken GnRH-I in chickens (Mikami *et al.*, 1988). In all cases these forms of GnRH, unlike chicken GnRH-II, are mainly in the hypothalamic-pituitary regions and in the anterior parts of the brain. However, if given *in vitro* or *in vivo*, chicken GnRH-II is more potent than or equipotent to the catfish, salmon or chicken-I forms in releasing gonadotropins.

In fish, identification of the GnRH form that releases gonadotropins in natural conditions is based on localization studies. However, in chickens, it has been shown that chicken GnRH-I is the natural gonadotropin releaser because antiserum against chicken GnRH-I, but not against chicken GnRH-II, inhibits reproduction and suppresses plasma LH (Sharp *et al.*, 1990). Thus, even though chicken GnRH-I is less potent than chicken GnRH-II, chicken GnRH-I appears to be the physiological releasing hormone. Catfish GnRH is in the expected

location to be the natural releasing hormone for catfish, but immunological studies like those above are needed to support this idea.

Chicken GnRH-II has a distinct pattern of distribution compared with catfish GnRH. The concentration of the two forms of GnRH can be compared in the heterologous assay in which the antiserum detects 50% of catfish GnRH and 67% of chicken GnRH-II compared with mammalian GnRH. In this assay, chicken GnRH-II is present in both sexes in lower content and concentration in the anterior parts of the brain but in higher content and concentration in the posterior regions compared with catfish GnRH. This distribution is similar to that reported for goldfish, trout, and chicken and is the evidence used to support a neurotransmitter role for chicken GnRH-II.

The homologous RIA for chicken GnRH-II, compared with the heterologous RIA, detects higher content and concentration of chicken GnRH-II in most brain areas. An unexpected result in the homologous assay, however, was the high content and concentration of chicken GnRH-II in the cerebellum in the male, and to a lesser extent in the female. GnRH fibers have been reported in the cerebellum for goldfish (Kah *et al.*, 1986) and chickens (Mikami *et al.*, 1988), but not in quantities to match the male catfish. One possibility is that the high level of

chicken GnRH-II in both male and female catfish cerebellum is related to its role as an electrodetector (Bennett, 1971; Hopkins, 1983) or sensory organ (Finger, 1983). The catfish family of Clariidae has receptors for detection of low frequency electrical signals. This function distinguishes catfish from goldfish and trout, although it does not explain the 2-fold difference in chicken GnRH-II levels between the male and female cerebellum unless it is related to the role of the male during the reproductive period. In this study statistical comparisons are not included because each sample contains pooled brains (n = 50) to ensure accurate measurement of the GnRH peptides in the HPLC fractions. However, the measurement of GnRH content and concentration in pooled brain samples provides preliminary data suggesting that the distribution of the two forms of GnRH varies and should be investigated by immunocytochemistry with specific antibodies.

The HPLC method used for determining the form of GnRH in different brain regions is specific in that the primary structures and HPLC elution positions for the two endogenous forms of GnRH in this species of catfish have been reported (Ngamvongchon *et al.*, 1992). The two forms elute 5 fractions apart and in positions distinct from the other known GnRHs (Fig. 4.3B). The quantity of each

form of GnRH is more difficult to determine. A homologous assay for catfish GnRH does not exist because this molecule was just identified. However, the RIA which uses antiserum GF-4 had 50% cross-reactivity with catfish GnRH. This may be due to the nature of the antiserum in that it detects 6 of 7 known GnRHs, including chicken GnRH-II. Only lamprey GnRH is not detected (Table 4.2). In contrast, antisera B-6, Bla-4, and 34-3 have little cross-reactivity with catfish GnRH. Therefore, the analysis is based on the GF-4 assays. Chicken GnRH-II is detected by both antiserum GF-4 (67%) and 34-3 (100%). Antiserum 34-3 is more specific than GF-4 as it detects mainly chicken GnRH-II (100%) and lamprey GnRH (24%). Its cross-reactivity is minimal with dogfish (4%), salmon (2%), mammalian (0.04%), catfish (0.02%) and chicken-I GnRH (0.002%). This may reflect the presence of Trp^{7} in the first 4 GnRHs but not in the latter 3 undetectable forms. The hydrophobic Leu^{8} in dogfish and salmon GnRHs also appears to decrease cross-reactivity compared with lamprey GnRH (Lys^{8}) and cGnRH-II (Tyr^{8}).

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CHAPTER 5

FUNCTIONAL STUDIES OF FIVE NATIVE AND EIGHT ANALOGS OF GONADOTROPIN-RELEASING HORMONES ON THE INDUCTION OF OVULATION IN THE THAI CATFISH (*Clarias macrocephalus*)

Parts of this chapter have been prepared for publication as three papers. One paper is published, the second is accepted for publication and the third has been submitted:

Ngamvongchon, S., Sherwood, N.M., Warby, C.M. and Rivier, J.E. 1992. Gonadotropin-releasing hormone from Thai catfish: Chromatographic and physiological studies. General and Comparative Endocrinology 87 (in press).

Ngamvongchon, S., Lovejoy, D.A., Fischer, W.H., Craig, A.G., Narhorniak, C.S., Peter, R.E., Rivier, J.E. and Sherwood, N.M. 1992. Primary structure of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. Molecular and Cellular Neurosciences 3:17-22.

Ngamvongchon, S., Rivier, J.E. and Sherwood, N.M. Structure-function studies of novel catfish and four other forms of gonadotropin-releasing hormone on

reproduction in Thai catfish (*Clarias macrocephalus*). Submitted.

INTRODUCTION

In aquaculture, the main purpose of controlled reproduction is to achieve sexual maturation and spawning at the time of the year that is normal to that species. In captivity some species of catfish do not breed because of culture conditions or environmental factors.

One substance that mediates environmental influences is gonadotropin-releasing hormone (GnRH), synthesized in the preoptic nucleus of the brain. In recent years there has been an increased effort to utilize GnRH in place of pituitary extracts for induction of ovulation in teleosts (Crim *et al.*, 1986). However, more effective than the native forms of mammalian GnRH (mGnRH) is an analog of mGnRH (mGnRH-a), which stimulates a more prolonged increase in circulating gonadotropin level (Peter *et al.*, 1986, 1988; Van Der Kraak *et al.*, 1983).

More recently 6 forms of GnRH in addition to mGnRH have been shown to exist throughout the vertebrates. The structure of GnRH has been determined for mammals including the human (Seeburg and Adelman, 1984; Adelman *et al.*, 1986), pig (Matsuo *et al.*, 1971), sheep, rat

(Adelman *et al.*, 1986), and mouse (Seeburg *et al.*, 1987). The structure is also known for the chicken (King and Millar, 1982; Miyamoto *et al.*, 1982, 1983, 1984), salmon (Sherwood *et al.*, 1983), lamprey (Sherwood *et al.*, 1986), catfish (Ngamvongchon *et al.*, 1992), and dogfish (Lovejoy *et al.*, 1992).

Catfish of the genus *Clarias* is an important aquaculture species in Thailand. This species does not spawn in the confined waters of fish ponds unless hormonal injections are used. In earlier studies, I found two forms of GnRH in the brain of the Thai catfish, *C. macrocephalus* (Ngamvongchon, *et al.*, 1992). One was novel and the other was identical to chicken GnRH-II (cGnRH-II).

The purpose of this study is to investigate *in vivo* reproductive effects in Thai catfish of several GnRHs and their analogs, particularly the novel catfish GnRH and dogfish GnRH for which the primary structures have been identified recently. Moreover, the *in vitro* release of gonadotropin and growth hormone from pituitary gland fragments is also studied in response to catfish GnRH.

MATERIALS AND METHODS

Mature spawners, female and male Thai catfish,

C. macrocephalus were purchased from a private fish farm near Bangkok, Thailand and transferred immediately to a hatchery. Hormonal injections began in August 1991, which was about the mid-spawning season and about 30 days after transfer. The spawners weighed between 120 to 250 g. The fish were maintained at a water temperature of 29-32°C throughout the experiment.

The peptides used in this experiment are listed in Table 5.1. The salmon GnRH analog and the native catfish, chicken-II, and dogfish GnRHs and their analogs were provided by J. Rivier. The native forms of mammalian and salmon GnRH, and the mammalian GnRH analog were purchased from Peninsula Laboratories (Belmont, CA).

The peptides were dissolved in 0.9% saline. The native peptides were tested at doses of 100, 150, and 300 μg/kgBW, whereas the analogs were tested at 20 and 30 μg/kgBW. Intramuscular injections were used. Each peptide at a single dose was tested in 5-24 fish, divided into 1-3 testing dates. For comparison of one or two injections, each peptide was given at the same total dose, but in the latter case the peptide was divided into equal amounts for injections 6 h apart. Four fish were used for each group. Control female spawners were injecte. with 0.5 ml of 0.9% saline.

Table 5.1. Structure and identification code for GnRH peptides used for this study.

GnRHs	1	2	3	4	5	6	7	8	9	10
Mammalian-n	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	$Gly.NH_2$
Mammalian-a	pGlu	His	Trp	Ser	Tyr	**D-Ala**	Leu	Arg	Pro	**NEt**
Salmon-n	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	$Gly.NH_2$
Salmon-a	pGlu	His	Trp	Ser	Tyr	**D-Arg**	Trp	Leu	Pro	**NEt**
Chicken II-n	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	$Gly.NH_2$
Chicken II-a_1	pGlu	His	Trp	Ser	His	**D-Arg**	Trp	Tyr	Pro	**NEt**
Chicken II-a_2	pGlu	His	Trp	Ser	His	**D-Nal**	Trp	Tyr	Pro	**NEt**
Catfish-n	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	$Gly.NH_2$
Catfish-a_1	pGlu	His	Trp	Ser	His	**D-Arg**	Leu	Asn	Pro	**NEt**
Catfish-a_2	pGlu	His	Trp	Ser	His	**D-Nal**	Leu	Asn	Pro	**NEt**
Dogfish-n	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	$Gly.NH_2$
Dogfish-a_1	pGlu	His	Trp	Ser	His	**D-Arg**	Trp	Leu	Pro	**NEt**
Dogfish-a_2	pGlu	His	Trp	Ser	His	**D-Nal**	Trp	Leu	Pro	**NEt**

n = native form of GnRH
a = analog form of GnRH
a_1 = analog form with $D\text{-}Arg^6$
a_2 = analog form with $D\text{-}Nal^6$
NEt = ethylamide

Pituitary homogenate was also prepared in 0.9% saline and injected two times for the female spawners as in previous studies (Ngamvongchon *et al.*, 1988; Ngamvongchon *et al.*, 1992). One "dose" was the equivalent of one pituitary gland from a one-kilogram donor fish given to a one kilogram recipient fish. Each female spawner was given one dose of pituitary homogenate as a first injection and two doses as a second injection, six hours afterward. Males were injected with a half dose only once at the time of the female's second injection.

Ovulation was determined by gentle hand stripping at 16-18 hours after hormonal injection. The release of uniform reddish-brown eggs at this time was the criterion for ovulation. If the fish had not ovulated, they were checked again two hours later. Fish were not checked for ovulation more than two times because they appeared to be weak, possibly due to the stress of captivity, handling, and injections.

Fertilization was done by mixing the collected eggs with a solution containing sperm from an 8-10 month old mature male. The sperm were obtained by grinding fresh testis in 0.9% saline. The supernatant was then mixed with the eggs. A random sample of fertilized eggs was taken for investigation of the fertilization rate, hatching rate and 7-day survival rate. The fertilization

rate was determined by examining eggs 8 hours after they were mixed with sperm. The hatching rate was examined 72 hours after fertilization and fry survival was observed 7 days later. The water temperature was 29-32°C throughout the experiment.

In Vitro Biological Activity

Goldfish pituitary fragments were perifused with increasing concentrations of either synthetic catfish GnRH or salmon GnRH for 2 min every 60 min. This method was previously described by Marchant *et al.*(1989). Five-minute fractions were collected. Gonadotropin-II and growth hormone were measured in each fraction using a radioimmunoassay for carp gonadotropin-II (Peter *et al.*, 1984) and growth hormone (Marchant *et al.*, 1989).

Statistical Analysis

The statistical significance of the differences observed was determined by using one way ANOVA followed by Tukey's multiple comparison test (Zar, 1984).

RESULTS

Experiment I

The results are shown in Tables 5.2 and 5.3. An injection of pituitary homogenate was the most effective treatments resulting in 100% (100±0%) ovulation. Chicken GnRH-II and dogfish GnRH at a dose of 300 μg/kg showed an effect on ovulation (75.0±23.4% and 54.0±2.2%) that was not significantly different from pituitary homogenate but was different from fish injected with 0.9% saline solution, catfish GnRH at 3 doses (100, 150, 300 μg/kg) and chicken GnRH-II at 100 μg/kg (Table 5.3). The native mammalian, salmon and catfish GnRHs at the same 3 doses were without effect on the induction of ovulation or other reproductive parameters (Tables 5.2, 5.3). This study also shows the percentage of fertilization, hatch and 7-day survival (Table 5.2). However, this information was not used for statistical analysis because of insufficient data.

The results of GnRH analogs on ovulation are shown in Tables 5.4 and 5.5. The analogs of chicken GnRH-II at a dose of 20 μg/kg BW showed no significant difference compared with pituitary homogenate for induction of ovulation (92.5±15.1%, 92.5±15.1% and 96.5±10.2 %

ovulation, respectively) (Table 5.5). The next most effective analog was salmon GnRHa (76.7±42.7 % ovulation) followed by the dogfish GnRH-a_1 (55.0±14.0 % ovulation) and mammalian GnRH analog (27.6±49.7 % ovulation) (Table 5.5). Fish injected with catfish GnRH-a_1, a_2 and dogfish GnRH-a_2 had a percentage of ovulation that was not significantly different from the saline injected control group. GnRH analogs at a dose of 30 μg/kg are not reported because the number of fish tested are insufficient. Figure 5.1 shows the summary of the average percentage of ovulation in this study.

Experiment II

In this experiment, female spawners that received only one hormonal injection did not show major differences in reproductive effects from the fish receiving two hormonal injections (Table 5.6). This was true in the experiments using either native or analog forms of catfish, dogfish and chicken-II GnRHs.

The 0.9% saline injected fish (control group) did not ovulate. Meanwhile, the fish injected with pituitary homogenate showed 100% ovulation in all injected fish.

Table 5.2. Effects of injecting native forms of GnRH into *Clarias macrocephalus*.

Injection		No. Ovulated			%Fertilization			%Hatch			%Survival		
		I	II	III	I	II	III	I	II	III	I	II	III
Control: 0.9% saline		0/10	0/7	0/7	*	*	*	*	*	*	*	*	*
Pituitary Homogenate		10/10	7/7	7/7	87	86	80	59	72	80	57	16	57
cfGnRH:	100 µg/kg	0/10	0/7	0/7	*	*	*	*	*	*	*	*	*
	150 µg/kg	0/10	0/7	0/7	*	*	*	*	*	*	*	*	*
	300 µg/kg	0/10	1/7	0/7	*	97	*	*	60	*	*	17	*
cGnRH-II:	100 µg/kg	4/10	2/7	0/7	93	90	*	53	87	*	67	15	*
	150 µg/kg	8/10	0/7	4/7	86	*	93	28	*	96	61	*	73
	300 µg/kg	10/10	2/7	5/7	90	94	97	53	67	94	83	20	61
dfGnRH:	100 µg/kg	0/10	2/7	1/7	*	85	97	*	43	99	*	26	75
	150 µg/kg	1/10	0/7	3/7	84	*	90	20	*	85	24	*	48
	300 µg/kg	4/10	5/7	3/6	79	81	99	39	49	85	64	22	55
sGnRH:	100 µg/kg	0/10	*	*	*	*	*	*	*	*	*	*	*
	150 µg/kg	0/7	*	*	*	*	*	*	*	*	*	*	*
	300 µg/kg	0/7	*	*	*	*	*	*	*	*	*	*	*
mGnRH:	100 µg/kg	0/7	*	*	*	*	*	*	*	*	*	*	*
	150 µg/kg	0/7	*	*	*	*	*	*	*	*	*	*	*
	300 µg/kg	0/7	*	*	*	*	*	*	*	*	*	*	*

* = no fish tested; I = experiment on 2/08/91, II = experiment on 19/08/91, III = experiment on 27/08/91, different experimental dates and different fish were used.

Table 5.3. Effect of native forms of GnRH on ovulation in *Clarias macrocephalus*.

Treatment		Ovulation Success			Mean ± SD # (%)
		I	II	III	
		2/08/91	19/08/91	27/08/91	
Control: 0.9% saline		0/10	0/7	0/7	0.0 ± 0.0 (1)
Pituitary Homogenate		10/10	7/7	7/7	100.0 ± 0.0 (3)
cfGnRH	100 µg/kg	0/10	0/7	0/7	0.0 ± 0.0 (1)
	150 µg/kg	0/10	0/7	0/7	0.0 ± 0.0 (1)
	300 µg/kg	0/10	0/7	0/7	0.0 ± 0.0 (1)
cGnRH-II	100 µg/kg	4/10	2/7	0/7	16.3 ± 12.8 (1)
	150 µg/kg	8/10	0/7	4/7	37.1 ± 30.1 (1,2,3)
	300 µg/kg	10/10	2/7	5/7	75.0 ± 23.4 (2,3)
dfGnRH	100 µg/kg	0/10	2/7	1/7	9.7 ± 8.1 (1,2)
	150 µg/kg	1/10	0/7	3/7	11.5 ± 12.2 (1,2)
	300 µg/kg	4/10	5/7	3/6	54.0 ± 2.2 (2,3)
sGnRH	100 µg/kg	0/7	*	*	
	150 µg/kg	0/7	*	*	
	300 µg/kg	0/7	*	*	
mGnRH	100 µg/kg	0/7	*	*	
	150 µg/kg	0/7	*	*	
	300 µg/kg	0/7	*	*	

* = no fish tested
I = experiment on 2/08/91, II = experiment on 19/08/91, III = experiment on 27/08/91
# = mean and standard deviation calculated with arcsine transformed data. Different superscripts indicate significant differences at $\alpha = 0.05$ as determined by Tukey's multiple comparison test.

Table 5.4. Effects of injecting analog forms of GnRH into *Clarias macrocephalus*. (* = no fish tested. I, II, III = different experiments and fish used)

Injection	No. Ovulated			%Fertilization			%Hatch			%Survival		
	I	II	III	I	II	III	I	II	III	I	II	III
Control: 0.9% saline	0/5	0/7	0/7	*	*	*	*	*	*	*	*	*
Pituitary Homogenate	5/7	7/7	7/7	92	86	80	57	72	80	23	16	57
cfGnRH-a1: 20 μg/kg	0/5	0/7	0/7	*	*	*	*	*	*	*	*	*
30 μg/kg	*	*	0/7	*	*	*	*	*	*	*	*	*
cfGnRH-a2: 20 μg/kg	3/5	0/7	0/7	79	*	*	13	*	*	0	*	*
30 μg/kg	1/5	*	0/7	87	*	*	77	*	*	23	*	*
cGnRH II-a1: 20 μg/kg	5/5	5/7	*	73	80	*	15	53	*	8	25	*
30 μg/kg	4/5	*	*	97	*	*	84	*	*	58	*	*
cGnRH II-a2: 20 μg/kg	5/5	5/7	*	77	88	*	8	64	*	2	20	*
30 μg/kg	4/5	*	*	40	*	*	9	*	*	11	*	*
dfGnRH-a1: 20 μg/kg	4/5	2/7	*	70	76	*	8	23	*	10	18	*
30 μg/kg	4/5	*	*	94	*	*	58	*	*	15	*	*
dfGnRH-a2: 20 μg/kg	*	*	1/7	*	*	95	*	*	82	*	*	70
30 μg/kg	*	*	0/7	*	*	*	*	*	*	*	*	*
sGnRH-a: 20 μg/kg	5/5	2/7	*	80	88	*	23	93	*	24	18	*
30 μg/kg	2/5	*	*	73	*	*	27	*	*	14	*	*
mGnRH-a1: 20 μg/kg	4/5	0/7	*	79	*	*	12	*	*	26	*	*
30 μg/kg	*	*	*	*	*	*	*	*	*	*	*	*

Table 5.5. Effect of GnRH analogs on ovulation in *Clarias macrocephalus*.

Treatment	Ovulation Success			Mean ± SD # (%)
	I	II	III	
	13/08/91	19/08/91	27/08/91	
Control: 0.9% saline	0/5	0/7	0/7	0.0 ± 0.0 (1)
Pituitary Homogenate	5/7	7/7	7/7	96.5 ± 10.2 (5)
cfGnRH-a1 20 µg/kg	0/5	0/7	0/7	0.0 ± 0.0 (1)
cfGnRH-a2 20 µg/kg	3/5	0/7	0/7	8.5 ± 24.0 (1,2)
cGnRH-II-a1 20 µg/kg	5/5	5/7	*	92.3 ± 15.1 (5)
cGnRH-II-a2 20 µg/kg	5/5	5/7	*	92.3 ± 15.1 (5)
dfGnRH-a1 20 µg/kg	4/5	2/7	*	55.0 ± 14.0 (3,4)
dfGnRH-a2 20 µg/kg	*	*	1/7	14.3 ± 0.0 (1,2)
sGnRH-a 20 µg/kg	5/5	2/7	*	76.7 ± 42.7 (4,5)
mGnRH-a 20 µg/kg	4/5	0/7	*	27.6 ± 49.7 (2)

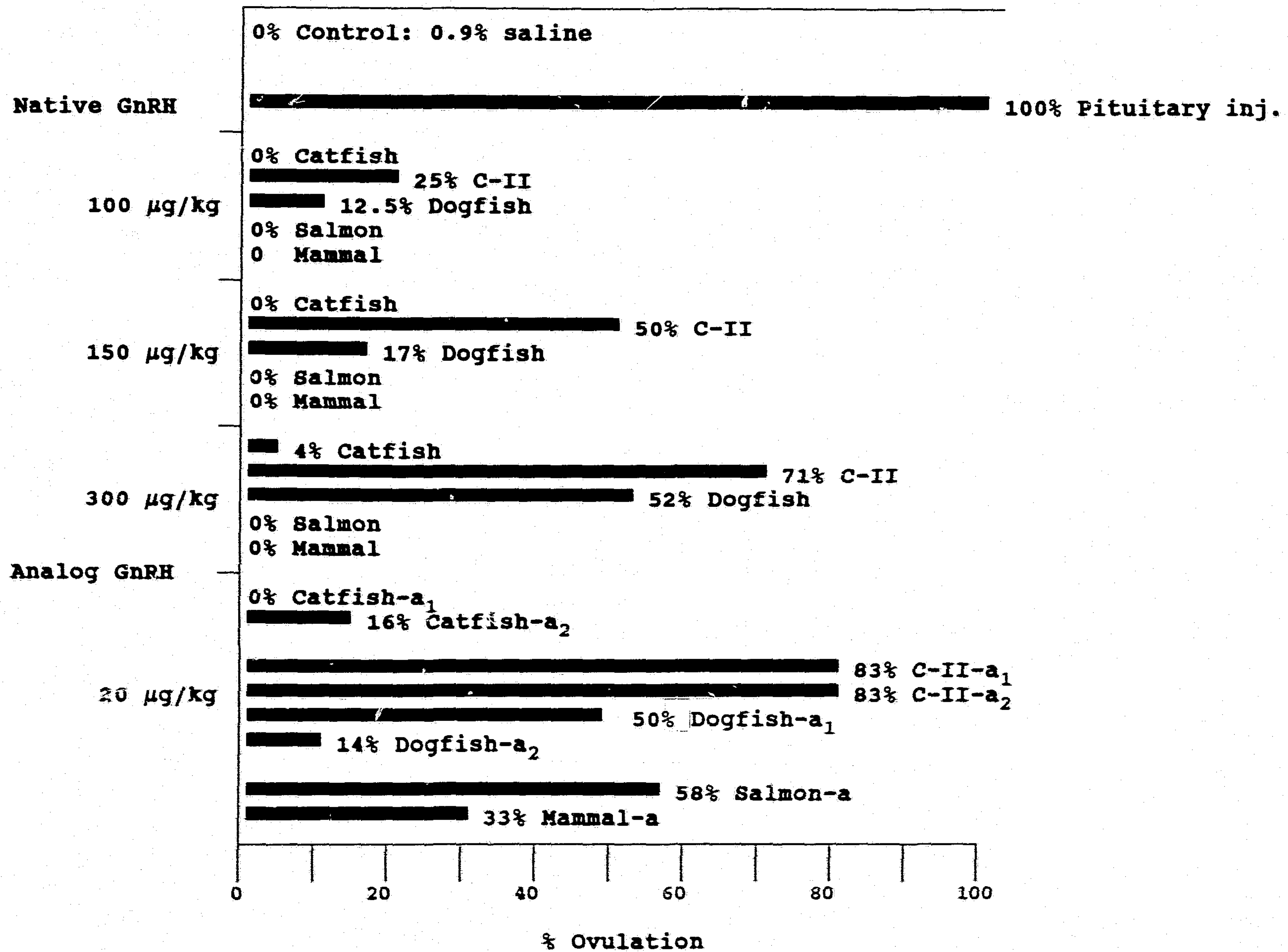
* = no fish tested, a = analog form, a1 = D-Arg^6, a2 = D-Nal^6
I = experiment on 13/08/91, II = experiment on 19/08/91, III = experiment on 27/08/91
\# = mean and standard deviation calculated with arcsine transformed data. Different superscripts indicate significant differences at $\alpha = 0.05$ as determined by Tukey 's multiple comparison test.

Table 5.6. Effects of one or two GnRH injection(s) on ovulation in *Clarias macrocephalus*. (experiment on 30/08/91)

Injections	No. Ovulated		%Fertilization		%Hatch		%Survival	
	one inject	two inject	one inject	two inject	one inject	two inject	one inject	two inject
Control: 0.9% saline	0/4	0/4	*	*	*	*	*	*
Pituitary Homogenate	*	4/4	*	82	*	83	*	0
Native Forms:								
cfGnRH: 150 μg/kg	0/4	0/4	*	*	*	*	*	*
cGnRH-II: 150 μg/kg	4/4	4/4	91	66	55	92	21	15
dfGnRH: 150 μg/kg	3/4	2/4	85	76	38	45	0	0
Analog Forms:								
cfGnRH-a1: 20 μg/kg	0/4	0/4	*	*	*	*	*	*
cfGnRH-a2: 20 μg/kg	0/4	0/4	*	*	*	*	*	*
cGnRH II-a1: 20 μg/kg	4/4	4/4	69	59	45	38	15	12
cGnRH II-a2: 20 μg/kg	3/4	3/4	78	21	35	0	10	*
dfGnRH-a1: 20 μg/kg	4/4	4/4	73	90	21	43	17	17
dfGnRH-a2: 20 μg/kg	4/4	4/4	91	90	55	20	10	25

* = no fish tested
one inject = total dose given in one injection
two inject = total dose divided in half and injected six hours apart
cfGnRH = catfish GnRH, cGnRH-II = chicken GnRH-II, dfGnRH = dogfish GnRH

Figure 5.1. Summary of the effect of GnRHs on ovulation of *Clarias macrocephalus*. (a_1 = analog with D-Arg^6 and a_2 = analog with D-Nal^6).



0% Control: 0.9% saline
Native GnRH
100% Pituitary inj.
0% Catfish
25% C-II
100 μg/kg
12.5% Dogfish
0% Salmon
0 Mammal
0% Catfish
50% C-II
150 μg/kg
17% Dogfish
0% Salmon
0% Mammal
4% Catfish
71% C-II
300 μg/kg
52% Dogfish
0% Salmon
0% Mammal
Analog GnRH
0% Catfish-a1
16% Catfish-a2
83% C-II-a1
20 μg/kg
83% C-II-a2
50% Dogfish-a1
14% Dogfish-a2
58% Salmon-a
33% Mammal-a
0
20
40
60
80
100
% Ovulation

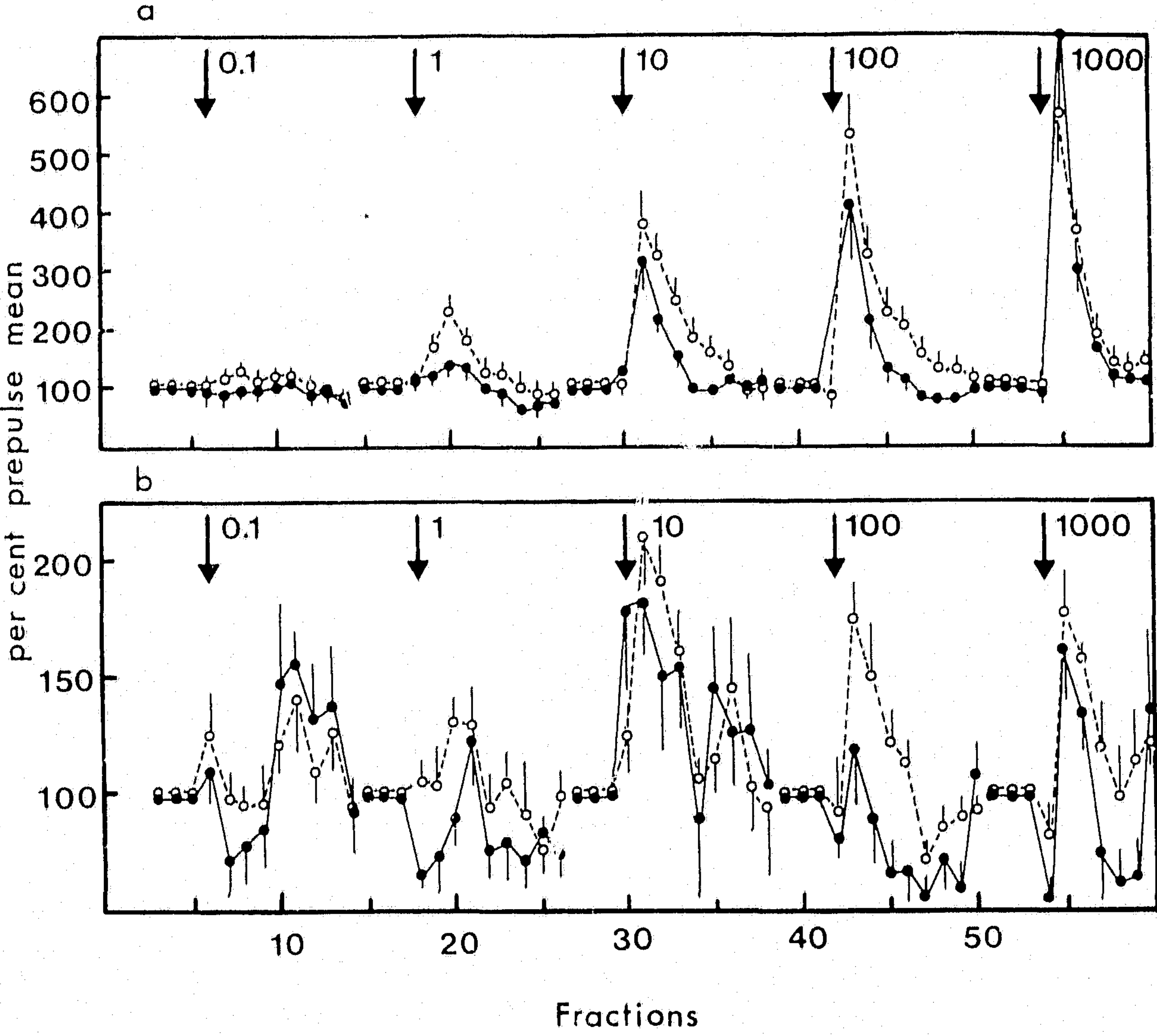
In Vitro Biological Activity

Gonadotropin-II was released from goldfish pituitary fragments perifused with various concentrations of synthetic catfish GnRH (Fig. 5.2). Pulses of 0.1 and 1.0 n*M* of catfish GnRH did not result in gonadotropin release, but pulses of 10, 100, or 1000 n*M* produced release of gonadotropin in the range of three to seven times that of the prepulse release. Catfish GnRH also released growth hormone from goldfish pituitaries. A maximal increase in growth hormone of approximately twofold compared with the prepulse release was produced by catfish GnRH and salmon GnRH (Fig. 5.2). The growth hormone response was more variable than the gonadotropin response.

DISCUSSION

This study provides the first data on the *in vivo* effects of two novel forms of GnRH, catfish and dogfish GnRH, on the induced ovulation of catfish, *C. macrocephalus*. Chicken GnRH-II and dogfish GnRH induced ovulation in a dose dependent manner. There are no significant differences between them at 300 μg/kg but all are significant difference from the controls that

Figure 5.2. Release of gonadotropin-II (a) and growth hormone (b) from goldfish pituitary fragments by synthetic catfish GnRH (●) and salmon GnRH (o). The release of the hormones is expressed as a percentage of the mean (±SE) hormone concentration in the three fractions immediately preceding the GnRH pulse. This mean was defined as 100%. The marks at the top of the figure denote the time of each pulse.


a
0.1
1
10
100
1000
600
500
400
300
200
100
per cent prepulse mean
b
0.1
1
10
100
1000
200
150
100
10
20
30
40
50
Fractions

received 0.9% saline (Table 5.3). Also, the native forms of mammalian, salmon and catfish GnRHs did not induce ovulation at any of 3 doses in any fish. This was the same as the control group, 0.9% saline injection (Table 5.3).

The relationship between structure and function in the 5 native peptides tested must depend on changes in amino acids 5, 7 or 8 because these are the only positions that differ. Otherwise the 5 peptides have the same length, the same terminal amino acids, and 7 identical residues. A single amino acid does not appear to account for the effectiveness of chicken GnRH-II and dogfish GnRH. Both peptides have histidine in position 5, but so does the ineffectual catfish GnRH. Both chicken GnRH-II and dogfish GnRH have tryptophan in position 7, but so does salmon GnRH, which did not induce ovulation at the doses noted. Finally, position 8 has a different residue in 4 of the 5 peptides, but both the effective dogfish GnRH and ineffective salmon GnRH have leucine. The induction of ovulation must depend on the combination of residues in 5, 7 and 8.

The analog forms of chicken GnRH-II (D-Arg^6 Pro^9 NEt and D-Nal^6 Pro^9 NEt) were significantly more effective for induction of ovulation than any other analog tested (Table 5.5). The average percentage of ovulation

increased up to 83% at a dose of 20 μg/kg BW with both chicken GnRH-II analogs. Next in effectiveness for induction of ovulation were ths salmon GnRH analog (D-Arg^{6} Pro^{9} NEt) and one of the dogfish analogs (D-Arg^{6} Pro^{9} NEt) (Table 5.5). The average value for percentage of ovulation was 58% and 50% for the salmon and dogfish GnRH respectively (Fig. 5.1). In contrast, (D-Nal^{6} Pro^{9} NEt) dogfish GnRH and (D-Nal^{6} Pro^{9} NEt) catfish GnRH at a dose of 20 μg/kg BW induced only 14% and 16% ovulation, respectively (Fig. 5.1). (D-Arg^{6} Pro^{9} NEt) catfish GnRH did not induce any ovulation. Surprisingly, one of the analogs most commonly used in aquaculture, (D-Ala^{6} Pro^{9} NEt) mammalian GnRH at a dose of 20 μg/kg BW produced only 33% ovulated fish (Fig. 5.1). These four analogs (mammalian GnRH-a, catfish GnRH-a1, a2 and dogfish GnRH-a2) produced significant fewer ovulated fish than other four analogs (Table 5.5). The striking effectiveness of both chicken GnRH-II analogs for induction of ovulation must depend on the native structure of chicken GnRH-II as the dogfish and catfish analogs had identical substitutions in the 6th and 10th positions of the peptide. The D-Arg^{6} substitution appears to be particularly effective as this change in chicken GnRH-II, dogfish and salmon GnRH produced three of the most effective analogs (Table 5.5).

One injection of the native or analog forms of GnRH produced almost identical results compared to two injections in inducing ovulation (Table 5.6). This result was not expected because the pituitary homogenate is ineffective unless given as 2 doses 6 hours apart (Ngamvongchon, personal observation). Mature fish may not require the additional priming of an early injection of GnRH. The single injection of GnRH may be sufficient to stimulate both gonadotropin release and synthesis so that a long-term release of gonadotropin results. If a single dose of pituitary homogenate is given, the gonadotropin in the preparation may not be available for a sufficient period of time to induce ovulation. There was, however, no statistical difference between the percentage of ovulation for fish given two injections of pituitary homogenate compared with one injection of chicken GnRH-II analogs.

The fertilization, hatching and survival rates were in parallel to those for ovulation in each group. Of particular interest was the high rate of fertilization, 40-97% in the group of fish injected with cGnRH-II or 70-99% in the group injected with dogfish GnRH (Tables 5.2, 5.4). The most important criterion in aquaculture is probably survival of the hatchlings. However, factors other than GnRH treatment such as water quality and feed,

could also affect survival. Native cGnRH-II and dogfish GnRH produced high survival rates in some groups. However, of the analogs, only chicken GnRH-II-a_2 produced survival rates equivalent to those for the pituitary homogenate-injected group. The high survival rate noted in the analog dogfish GnRH-a_2 was based on the offspring of only 1 fish (Table 5.4). The results indicate that induced ovulation in *C. macrocephalus* occurs in a dose- and form-dependent manner.

In contrast to the action of GnRH on the spontaneous release of gonadotropin, dopamine appears to serve as an inhibitory factor (Peter *et al.*, 1986). A combined treatment using GnRH-a and a dopamine receptor antagonist such as pimozide or domperidone is essential to induce ovulation in cyprinids, but not in salmonids. It has been proposed that there is a dichotomy (Crim and Peter, 1988) between freshwater species, and marine species. In the former a combined treatment of GnRH-a and a dopamine antagonist is thought to be the best method for induction of ovulation, whereas in the marine species a slow-release preparation of GnRH-a alone is effective. In our experiments, it was not known whether catfish require this antagonist to block dopamine inhibition in addition to GnRH. Clearly cGnRH-II and dogfish GnRH alone induced ovulation of *C. macrocephalus*, although the catfish GnRH

did not produce ovulation at any dose. One possibility is that novel catfish GnRH is effective for induction of ovulation only if combined with a dopamine antagonist. This remains to be tested.

Both forms of GnRH in catfish brain, however, are biological active peptides *in vitro*. Like several other family members they release gonadotropin-II as well as growth hormone (Fig. 5.2). The releasing activity of catfish GnRH was assessed *in vitro* by adding pulses of GnRH to the media perifused around goldfish pituitary fragments via column. Catfish GnRH stimulates the release of gonadotropin-II in a dose-dependent manner at concentrations ranging from 10 to 1000 n*M*. This response was similar when compared to that for salmon GnRH. The second form of GnRH in catfish, chicken GnRH-II, was reported previously to release gonadotropin from dispersed goldfish pituitary cells in a static system. Chicken GnRH-II compared with salmon GnRH had a higher efficacy in releasing gonadotropin, but was equipotent in inducing growth hormone secretion (Chang *et al.*, 1990). These data suggest a similar response profile for the two catfish GnRHs in the goldfish pituitary system.

The presence of two distinct forms of GnRH in the brain of the catfish implies that more than one function is associated with these peptides. There are at least 3

possible explanations for the function of the novel catfish form. Firstly, catfish GnRH may release gonadotropin only if a dopamine antagonist is also present. Secondly, catfish GnRH may have another distinct function such as growth hormone release. In this study, I found that catfish GnRH like dogfish, salmon, mammalian and chicken-II GnRH releases growth hormone in addition to gonadotropin, but the relative importance is not known. Thirdly, a possibility that cannot be discounted is that catfish GnRH does not have a clear function. The novel structure may be due to a random change in the DNA coding region of one of the GnRH genes in an ancestral catfish. This ancestral gene may have been salmon GnRH (see Sherwood and Coe, 1991). The continued presence of cGnRH-II in the ancestral and modern catfish would have allowed normal reproduction.

The results of the present paper suggest that several of the tested analogs can be used in aquaculture. Previously, sexually mature broodfish were induced to spawn by using freshly homogenized or acetone-dried common carp (*Cyprinus carpio*) pituitary glands. However, often the glands were collected from dead fish preserved on ice or collected prior to the spawning season. These practices lowered the pituitary gonadotropic potency and led to reproductive failure. Another approach was to

inject human chorionic gonadotropin or Synahorin, a mixture of chorionic gonadotropin and mammalian pituitary extract (Weil *et al.*, 1986). In this study, however, it is shown that the use of a synthetic hypothalamic decapeptide analog (GnRH-a) is a good alternative for induction of ovulation and spawning in catfish species. Thus the form and dose of the GnRH hormone is important in addition to the stage of fish maturity, and the temperature of the water. These experimental results provide a promising indication that cGnRH-II or its analogs will induce ovulation of catfish. However, it is still necessary to determine if a dopamine antagonist or a change in formulation will increase the effectiveness of the peptides.

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CHAPTER 6

GENERAL CONCLUSION AND DISCUSSION

The brain is an important organ for the control of reproduction in all vertebrates including teleost fish. In teleosts, it is generally accepted that gonadotropin-releasing hormone (GnRH) in the brain is a major mediator for neural control of reproduction (see Ball, 1981; Peter *et al.*, 1987; Sherwood, 1987). However, our understanding of the brain GnRH system has come primarily from studies in mammals (Blackwell and Guillemin, 1973). The structure and function of GnRH in teleost fish and other lower vertebrates is still poorly understood. This research provides the first determination of the primary structure for two GnRHs from the brain of a freshwater fish, catfish (*Clarias macrocephalus*). Additionally, it provides a more complete story of GnRH in terms of studies on structural characterization and *in vivo* and *in vitro* physiological testing. This chapter, therefore, will briefly discuss the major findings of the research. It will also consider new synthetic neurohormones for the improvement of aquaculture.

The Structures of Catfish GnRH: One Unique, One Conserved

The study of GnRH in two species of Thai catfish, *C. batrachus* and *C. macrocephalus* (Chapter 2) provided preliminary information that GnRH existed in these catfish and that further research might be fruitful. Two distinct peaks were eluted from the HPLC system and the immunoreactive GnRHs were detected by a heterologous RIA using antisera against salmon GnRH (GF-4), mammalian GnRH (B-6, R-42) and lamprey GnRH (Bla-4). In Chapter 2, the results showed cross-reactivity of antisera GF-4 and R-42 only with the GnRHs in the catfish brain, and this led to the research in Chapter 3.

The characterization of the structure of GnRH in the catfish brain in Chapter 3 was made by using a pool of 1400 brains. In view of the small quantities of GnRH in brain tissue, isolation and sequence analysis was at the edge of protein chemistry techniques. There was sufficient material to do mass spectral analysis of catfish GnRH-I, but not catfish GnRH-II. The structural analysis revealed that both catfish GnRHs have a linear sequence of ten amino acids, which is a common feature of GnRHs distributed throughout the vertebrates. One catfish GnRH is distinct from the six known forms shown in Table 6.1 and the other GnRH is identical to chicken

Table 6.1. Primary structures of seven known forms of GnRH. The two forms isolated from Thai catfish brain are in bold. The boxes indicate the variable positions.

	1	2	3	4	5	6	7	8	9	10
Chicken GnRH-I	pGlu-	His-	Trp-	Ser-	Tyr	-Gly-	Leu-	Gln	-Pro-	Gly-NH_2
Mammalian GnRH	pGlu-	His-	Trp-	Ser-	Tyr	-Gly-	Leu-	Arg	-Pro-	Gly-NH_2
Catfish GnRH	**pGlu-**	**His-**	**Trp-**	**Ser-**	**His**	**-Gly-**	**Leu-**	**Asn**	**-Pro-**	**Gly-NH_2**
Chicken GnRH-II	**pGlu-**	**His-**	**Trp-**	**Ser-**	**His**	**-Gly-**	**Trp-**	**Tyr**	**-Pro-**	**Gly-NH_2**
Dogfish GnRH	pGlu-	His-	Trp-	Ser-	His	-Gly-	Trp-	Leu	-Pro-	Gly-NH_2
Salmon GnRH	pGlu-	His-	Trp-	Ser-	Tyr	-Gly-	Trp-	Leu	-Pro-	Gly-NH_2
Lamprey GnRH	pGlu-	His	Tyr	Ser-	Leu	-Glu	Trp-	Lys	-Pro-	Gly-NH_2

GnRH-II. This is the first direct evidence for the structure of chicken GnRH-II (catfish GnRH-II) in teleost fish.

Catfish GnRH, therefore, is a decapeptide with 80% sequence identity to mammal, chicken I, chicken II and dogfish GnRH, 70% to salmon GnRH and 50% to lamprey GnRH (Table 6.1). This new member of the GnRH family, catfish GnRH, is the seventh member of the family. This study shows the portion of GnRH that has been retained for millions of years. The conserved positions are still at residues 1 and 2 at the N-terminus, at residue 4, and residues 9 and 10 at the C-terminus in all vertebrates. In jawed vertebrates positions 1-4, 6, 9, and 10 are conserved.

The Distribution and Quantitation of GnRH: Beginnings

The distribution of types of GnRH has been studied in about 26 species of teleosts (see Chapter 1; Sherwood and Coe, 1991). Teleosts do not appear to have lamprey GnRH. Chicken I-like material has been reported in only three species of teleosts and has not been confirmed by determination of primary structure (see Sherwood, 1988). The mammalian form of GnRH has been detected in primitive bony fish (Crim *et al.*, 1985; Sherwood *et al.*, 1991) but

in only one teleost, the eel (Shih *et al.*, 1988; King *et al.*, 1990). Flounder GnRH elutes near mammalian GnRH from HPLC (Idler and Everard, 1987), but this may be a novel form. Otherwise, mGnRH does not appear in other teleosts studied to date. In many teleost fish the salmon form is present, except in 3 species of catfish: *Clarias gariepinus*, (Sherwood *et al.*, 1989), *C. macrocephalus* (Chapters 2, 3), and *C. batrachus* (Chapter 2). A chicken II-like form of GnRH is present in cartilaginous fish (Sherwood and Sower, 1985; Millar and King, 1987; Lovejoy and Sherwood, 1989) and in many bony fish (Sherwood, 1988).

The ratio of the quantity of GnRH for the two catfish forms may provide a clue about function. The second form of GnRH, chicken GnRH-II, appears to be present in very small amounts in fish compared to the first form. However, the quantity of GnRH may be misleading in many studies. Quantitation of GnRH is not accurate unless GnRH is purified and measured by amino acid composition. Usually, sufficient brain material is not available for purification. Quantitation by the use of an antiserum is acceptable if the structure of the GnRH is known so that the synthetic form can be used as a standard and tracer and an antiserum specific for that form of GnRH is available. However, isolation of a novel form requires

use of an antiserum that cross-reacts with a variety of GnRH peptides including the unknown form. In this case quantitation is not accurate until the above conditions are met. Hence levels of chicken GnRH-II reported in heterologous assays can be used to show chicken GnRH-II is present, but the amounts may not be correct. This is illustrated in the present experiments in which higher levels of chicken GnRH-II were measured in the homologous compared to the heterologous assay. If accurate quantitation is available, then it will be possible to determine the ratio of the GnRH forms in catfish at different seasons or between genders. In the present study the fish were collected in the same season and were reproductively mature. Also, brains from both genders were used. Sherwood *et al.* (1989) reported that the two forms of GnRH were the same in both genders of the African catfish, *C. gariepinus*, although the amounts varied between genders. It remains to be determined if season or gender affect GnRH ratios in individual brains.

This study confirms that two or more forms of GnRH are present in most species (see King and Millar, 1987; Millar and King, 1987; 1988; Sherwood, 1987), but it is not yet certain if multiple forms of GnRH are in individual brains. The small quantity of GnRH in the brain means that the purification and even the HPLC

analysis must be done on pooled brains. An important next step will be to quantitate the two forms of GnRH in individual catfish brains. A more sensitive RIA with specific antiserum is one possible method. A more likely method to prove that two forms are present in one brain is by *in situ* hybridization. This will require isolation of the cDNA or gene for the two catfish GnRHs.

GnRH Locations: Traditional and Not So Traditional

The findings in Chapter 4 show that different amounts of immunoreactive GnRHs are detected in discrete brain areas. A high content and concentration of catfish GnRH (heterologous RIA) was in the hypothalamus or pituitary as expected, but the most striking observation was the high content and concentration of chicken GnRH-II (catfish GnRH-II) in the cerebellum (homologous RIA). The possible function of the latter is discussed below. Another observation was the anterior brain areas had higher content and concentration of catfish GnRH in the males than females. In the homologous RIA, which detects mainly chicken GnRH-II, the concentration of immunoreactive material in males is higher than in females. This study suggests: (1) the same forms of GnRH are present, but not in equal content and concentration

in the two genders, (2) the season or stage of reproduction may be responsible for the difference in the amount of detectable immunoreactive GnRH in the two genders, and (3) the brain location for different forms of GnRH suggests differentiation of function. The higher content and concentration of immunoreactive GnRH in the anterior brain of the male may be related to sexual behavior during the spawning season in that the male maintains milt production for a long period of time. The lower content and concentration of immunoreactive GnRH in the anterior brain of female may be related to the collection of the fish after ovulation. Thus, much of the GnRH may have already been released from the brain. Another gender difference is that immunoreactive GnRH was detected mainly in the female pituitary, but not in the male pituitary. A detailed study on the location of individual GnRH neurons and fibers in both sexes is required to explain this observation. This can be done when specific antisera have been prepared against both catfish GnRH and chicken GnRH-II.

GnRH Functions: Releaser and Neurotransmitter

The function of GnRH has been evaluated primarily by the release of gonadotropins, but it is clear that GnRH

has other functions. In fish, GnRH nerve fibers are widely distributed in the brain, in addition to terminating in the pituitary. In mammals, GnRH receptors have been detected in nonhypothalamic regions of the brain in rats, but have not been reported in fish (see Sherwood, 1988). The distribution of GnRH axons and receptors in the mammalian brain supports the idea that GnRH may be a neurotransmitter in the brain. Additionally, GnRH neurons near the olfactory bulb in most fish have terminal axons in the forebrain, and in some species, in the retina. This evidence suggests GnRH functions in the brain may include: (1) release of pituitary gonadotropins and growth hormone, (2) transduction of olfactory stimuli to reproductive behavior, and (3) modulation of vision.

The results obtained in Chapter 4 show that the novel form of catfish GnRH is distributed widely in the brain from the olfactory tract through the medulla. The male pituitary is an exception. Chicken GnRH-II, the second form of GnRH in the catfish brain, unlike catfish GnRH, is concentrated in the posterior part of the brain, particularly in the cerebellum and medulla. Based on these findings, it is possible that GnRH in the catfish brain is involved in the sensory system. It may play a role in electrodetection or in visual responses.

Physiologically, catfish (family Clariidae: *Clarias* spp.) are bottom feeders with small eyes. During the spawning season, the fish may require more protection and defense in regard to mating behavior. It is not known if catfishes use electrodetection in regard mating behavior but some fish are reported to use weak electrical discharges as part of mating behavior (Kramer, 1990). GnRH may act as a neurotransmitter in addition to its role as a neuromodulator in sexual behavior (King and Millar, 1990; Oka and Ichikawa, 1991). Similarly, in other vertebrates, an increase of sexual receptivity due to GnRH has also been reported, but not yet studied in fish. Finally, GnRH neurons in the midbrain have axons that travel in the spinal cord to terminate on the caudal neurosecretory neurons (Miller and Kriebel, 1986). Therefore, it is suspected that the GnRH fibers may modulate the release of a secretion controlling contraction of oviducts and sperm ducts. Currently, there is no evidence of irGnRH in the spinal cord of the Thai catfish using heterologous and homologous RIAs (Chapter 4).

Surprisingly, the functional significance of multiple forms of GnRH is still not clear. In birds, both chicken GnRH-I and chicken GnRH-II stimulate secretion of LH and FSH at concentrations appropriate for physiological

regulation. However, chicken GnRH-I is present in the median eminence, whereas chicken GnRH-II predominates in extrahypothalamic areas of the brain. This evidence suggests that only chicken GnRH-I can exert hypophysiotropic control, while the role of chicken GnRH-II is that of a neuromediator. The situation is different in several species of teleost fish, where chicken GnRH-II occurs together with salmon, mammalian, dogfish or catfish GnRHs in the pituitary and/or brain areas. Whether one or several forms of GnRH are involved in the physiological control of pituitary gonadotropic function is not known.

The use of GnRH for induced ovulation has now been tested in a variety of fish. One advantage of GnRH is that its molecules are simple. They can be synthesized, and different amino acids can be inserted at any position to produce artificial analogs that are characteristically 50 to 100 times more potent than the natural form (see Crim *et al.*, 1987; Horvath *et al.*, 1986; Van Der Kraak *et al.*, 1987; Zohar *et al.*, 1989). Many analogs of mammalian and fish GnRH have been made, often by substituting ethylamide for amino acid ten and a D-amino acid for amino in position six. More than 2000 analogs have been synthesized.

Of these analogs, one based on mammalian GnRH and

called LHRH-a, and an analog based on salmon GnRH called sGnRH-a are two superactive forms in fish breeding. In addition there are other active GnRH analogs (Table 6.2). In the analogs, the GnRH peptide is altered at position 6 by substituting a D-residue for the natural L-form of the residue. The D-form is the mirror image of the L-form and makes the molecule more resistant to degradation and therefore longer-lasting.

Once the primary structure was determined for GnRH, the synthetic form and analogs were prepared and used for structure-function studies. These studies are also the basis for designing useful analogs for aquacultural purposes. The physiological studies show that the two forms of GnRH in the catfish differ in their potency for induction of ovulation in catfish. Native and analog forms of catfish GnRH induce ovulation in only a few fish if given by intramuscular injection. In contrast, native chicken GnRH-II (catfish GnRH-II) at dose 300 μg/kg or the analog forms at 20 μg/kg causes a significant increase in the percentage of fish ovulating compared with control fish. This effect of chicken GnRH-II for induction of ovulation is dose-dependent. The relationship of the native and analog forms of catfish GnRH is shown in that the injection of 300 μg/kg of native chicken GnRH-II gives a similar result as

Table 6.2. GnRH analogs which have been tested in fish. (* = analogs used in this study)

GnRH analog		
Mammalian analogs	pGlu-His-Trp-Ser-Tyr-L-Trp	-Leu-Arg-Pro-Gly-NH_2
	pGlu-His-trp-Ser-Tyr-D-hArg(Et_2)	-Leu-Arg-Pro-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-D-Leu	-Leu-Arg-Pro-Aza-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-D-Nal(2)	-Leu-Arg-Pro-Aza-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-D-Trp	-Leu-Arg-Pro-NH-CH_2-CH_3
	*** pGlu-His-Trp-Ser-Tyr-D-Ala**	**-Leu-Arg-Pro-NH-CH_2-CH_3**
	pGlu-His-Trp-Ser-Tyr-D-TLe	-Leu-Arg-Pro-NH-CH_2-CH_3
	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Leu-Arg-Pro-NH-CH_2-CH_3
	pGlu-His-Trp-Ser-Tyr-D-Ser(Bu^t)	-Leu-Arg-Pro-NH-CH_2-CH_3
	pGlu-His-Trp-Ser-Tyr-D-His-(imBzl)	-Leu-Arg-Pro-NH-CH_2-CH_3
	pGlu-His-Trp-Ser-Tyr-D-Nal(2)	-Leu-Arg-Pro-NH-CH_2-CH_3
Salmon analogs	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Trp-Leu-Pro-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-D-Phe	-Trp-Leu-Pro-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-D-Ala	-Trp-Leu-Pro-NH-CH_2-CH_3
	*** pGlu-His-Trp-Ser-Tyr-D-Arg**	**-Trp-Leu-Pro-NH-CH_2-CH_3**
	pGlu-His-Trp-Ser-Tyr-D-Trp	-Trp-Leu-Pro-NH-CH_2-CH_3
	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Trp-Leu-Pro-NH-CH_2-CH_3
Chicken I analogs	pGlu-His-Trp-Ser-Tyr-D-Trp	-Leu-Gln-Pro-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Leu-Gln-Pro-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-L-Phe	-Leu-Gln-Pro-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-D-Phe	-Leu-Gln-Pro-NH-CH_2-CH_3
	pGlu-His-Trp-Ser-Tyr-Gly	-Phe-Gln-Pro-NH-CH_2-CH_3
Chicken II analogs	pGlu-His-Trp-Ser-Tyr-D-Arg	-Trp-Tyr-Pro-Gly-NH_2
	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Trp-Tyr-Pro-Gly-NH_2
	*** pGlu-His-Trp-Ser-His-D-Arg**	**-Trp-Tyr-Pro-NH-CH_2-CH_3**
	*** pGlu-His-Trp-Ser-His-D-Nal**	**-Trp-Tyr-Pro-NH-CH_2-CH_3**
Catfish analogs	*** pGlu-His-Trp-Ser-His-D-Arg**	**-Leu-Asn-Pro-NH-CH_2-CH_3**
	*** pGlu-His-Trp-Ser-His-D-Nal**	**-Leu-Asn-Pro-NH-CH_2-CH_3**
Dogfish Analogs	*** pGlu-His-Trp-Ser-His-D-Arg**	**-Trp-Leu-Pro-NH-CH_2-CH_3**
	*** pGlu-His-Trp-Ser-His-D-Nal**	**-Trp-Leu-Pro-NH-CH_2-CH_3**

injection of 20μg/kg of either of the two chicken GnRH-II analogs; ovulation was induced in 71-83% of the fish (Chapter 5, Fig. 5.1). The present results compare eight analog forms of GnRH which includes not only the 2 analogs of chicken GnRH-II, but also 2 analogs of dogfish GnRH, 2 analogs of catfish GnRH, 1 analog of salmon GnRH and 1 analog of mammalian GnRH. The D-Arg^6 and D-Nal^6 forms of chicken GnRH-II were the most effective peptides for the induction of ovulation under the same condition as the other 6 analogs and 5 native forms. A striking result from the *in vitro* research was the evidence that catfish and dogfish GnRH release growth hormone in addition to gonadotropin release in a dose-dependent manner.

Evolution of Catfish GnRH: Two Divergent Paths

The final question is the origin of catfish GnRH. Ohta (1991) stated that the simplest way for an organism to produce proteins with new functions is by the duplication of a whole gene with subsequent differentiation of one gene by nucleotide substitution. Examples of gene duplications are multigene families such as immunoglobins, cytochrome P450, growth hormone, tubulin and others. The phenomenon of gene duplication might

provide new functions that are useful to the organism without loss of the original gene and its functional product. Duplication might also produce a new gene with a gene product that is not harmful, but not functional.

Gonadotropin-releasing hormone is an example of a peptide whose gene may have duplicated to give rise to an additional protein coding region, which is the source of the second form of the peptide. It is difficult to determine exact when, where and how this duplication occurred.

The evolution of catfish may have begun early in the history of teleosts (Fig. 6.1). Most catfish live in fresh water and are grouped with the Ostariophysi. Other fish in this group, such as milkfish, goldfish and pacu, have salmon GnRH and chicken GnRH-II. Only catfishes to date have this new form of GnRH. Its origin could lie in a duplication of chicken GnRH-II gene with subsequent substituitions in the duplicate gene. A more likely explanation is that catfish GnRH arose simply by substitutions in either the mammalian or salmon GnRH gene. Both peptides are absent in catfish suggesting substituitons changed the gene. Figure 6.1 shows that catfish or an ancestor might have inherited the mammalian GnRH gene from primitive bony fish or inherited the salmon GnRH gene from an early-evolving teleost. Figure

6.2 shows that the replacement of two amino acids in chicken GnRH-II or mammalian GnRH or 3 amino acids in salmon GnRH would result in the novel catfish GnRH. The catfish GnRH peptide was not effective *in vivo*, but released both growth hormone and gonadotropin *in vitro*.

The intention of this research was to study in sequence several aspects of GnRH. It introduces a new neurohormone to the family of GnRH peptides. Catfish GnRH becomes the seventh member of the GnRH family. Thus, it extends the number of GnRH peptides involved in teleost species. It confirms many studies showing that more than one form of GnRH can be found in the teleost brain but is the first one to provide primary structures for both forms. It suggests that there are several factors, such as season and gender that affect the release of GnRH in brain areas.

The high content and concentration of GnRH in the cerebellum are unusual and may give another clue regarding the function of GnRH. The structure-function studies show that chicken GnRH-II is the most effective for induction of ovulation and is also the most conserved form distributed throughout the teleost species. Finally, it provides evidence that GnRH is conserved in vertebrate evolution from lower vertebrates to higher vertebrates. The origin of the catfish peptide is most

Figure 6.1. The evolution of GnRH in teleosts shows that catfish or its ancestor might have inherited the mammalian GnRH gene from primitive bony fish or inherited the salmon GnRH gene.

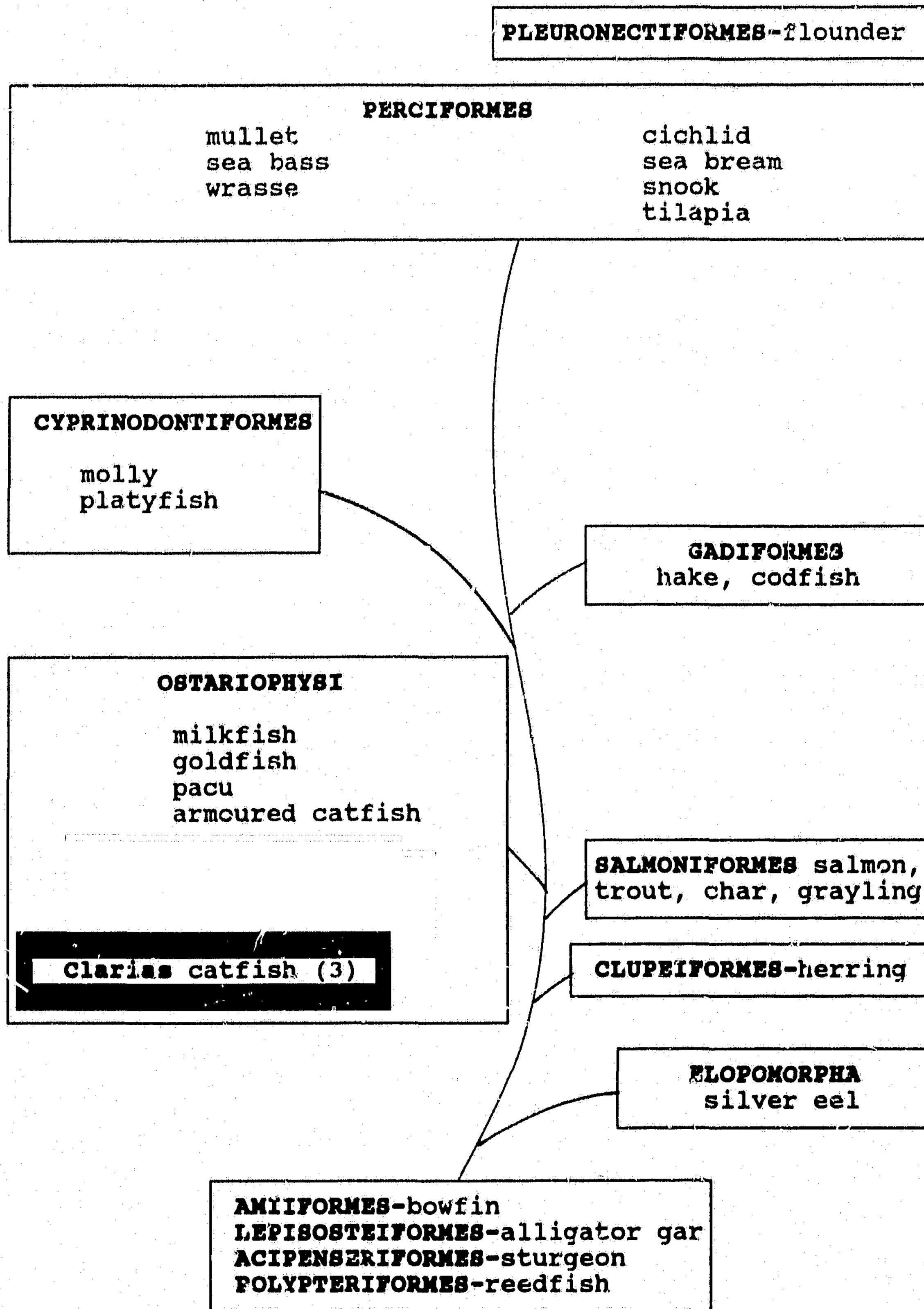
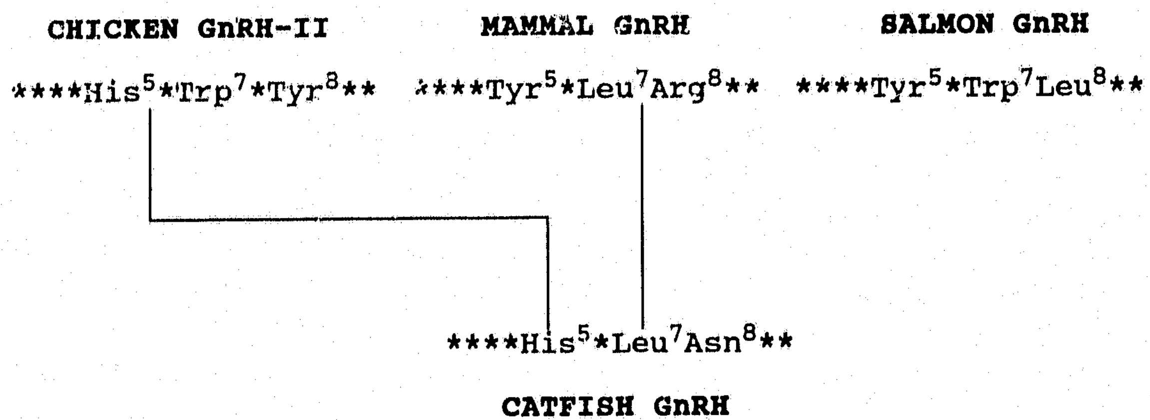

PLEURONECTIFORMES-flounder
PERCIFORMES
mullet
sea bass
wrasse
cichlid
sea bream
snook
tilapia
CYPRINODONTIFORMES
molly
platyfish
GADIFORMES
hake, codfish
OSTARIOPHYSI
milkfish
goldfish
pacu
armoured catfish
Clarias catfish (3)
SALMONIFORMES salmon, trout, char, grayling
CLUPEIFORMES-herring
ELOPOMORPHA
silver eel
AMIIFORMES-bowfin
LEPISOSTEIFORMES-alligator gar
ACIPENSERIFORMES-sturgeon
POLYPTERIFORMES-reedfish

Figure 6.2. The replacement of the two amino acids in chicken GnRH-II or mammalian GnRH or three amino acids result in the novel catfish GnRH.


CHICKEN GnRH-II
MAMMAL GnRH
SALMON GnRH
****His5*Trp7*Tyr8**
****Tyr5*Leu7Arg8**
****Tyr5*Trp7Leu8**
****His5*Leu7Asn8**
CATFISH GnRH

likely due to substitutions in an existing gene for mammalian or salmon GnRH.

FUTURE DIRECTIONS

First, the structure of the GnRH genes needs to be established. Once the DNA sequence is known, it will help to answer how this neuroendocrine peptide is synthesized and regulated at molecular and cellular levels in fish. This will enable us to know whether different forms come from different precursors and have distinct regulatory regions and transcription factors.

Second, the distribution of GnRH cell bodies and axons in the catfish brain will help to localize the origin and termination of individual GnRH neurons. An antiserum made against catfish GnRH is needed to prepare a precise homologous assay. However, *in situ* hybridization with a probe made against regions of the precusor without sequence identity to other GnRHs, such as the cryptic region, may be required for precise localization of the different forms of GnRH in a single brain.

Third, the physiological action of catfish GnRH for induction of ovulation in the Thai catfish needs to be extended. Improved ovulation may require the combination of catfish GnRH with either a dopamine antagonist or with

chicken GnRH-II. This research would indicate whether dopaminergic inhibitory effects occur in the catfish brain.

Fourth, the release of gonadotropins *in vivo* and *in vitro* in Thai catfish in response to GnRH and its analogs need to be studied. Goldfish were used in the present study for bioassay because for Thai catfish neither the pituitary hormones have been identified nor antisera prepared against them. RIAs are required that are homologous for Thai catfish pituitary hormones.

Fifth, intracellular effects of the two catfish GnRH peptides need to be elucidated. Finally, the most difficult research may be to find other functions for catfish GnRH. Structural changes that occur in peptides during evolution are easier to document than changes in the relationship between structure and function. New functions or uses of a peptide may arise, but are often difficult to detect, especially if the function is not clearly related to the original function.

Success of reproduction in cultured fish depends on our understanding of hormonal regulation. Basic research, including molecular biology and physiological studies will lead to the development of technologies designed to improve the management and yield of fish in the aquaculture industry. **The understanding of how to**

transfer new discoveries to the fish farm industry is as important and almost as difficult as the original research. Once the technique is set up at the laboratory level, pilot experiments must be carried out in the field to evaluate the results from a technical view point.

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