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# Control of Gonadotropin Hormone Synthesis and Release in the Goldfish Pituitary.

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Control of Gonadotropin Hormone Synthesis and Release in the Goldfish Pituitary." submitted by Zeinur Khakoo in partial fulfillment of the requirements for the degree of Master of Science.

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**Abstract**

Hypothalamic gonadotropin-releasing hormone (GnRH) and gonadal hormones are important regulators of pituitary gonadotropin (GtH) release and synthesis in vertebrates. The brain of all vertebrates with the exception of placental mammals contain multiple forms of GnRH, and in goldfish there are two forms of GnRH, salmon GnRH ([$Trp^7$, $Leu^8$]-GnRH; sGnRH) and chicken GnRH-II ([$His^5$, $Trp^7$, $Tyr^8$]-GnRH; cGnRH-II). This study investigated the control of release and synthesis of maturational GtH (GtH-II) by native GnRH molecules and gonadal steroids in the goldfish pituitary. The initial set of studies focused on the differences between sGnRH- and cGnRH-II-induced synthesis and release of GtH-II in the goldfish pituitary. The findings provide a strong support for the hypothesis that sGnRH and cGnRH-II function through different receptor-effector mechanisms in the goldfish pituitary. Further experiments were carried out to investigate the effects of steroids on GtH-II subunit gene expression. The findings demonstrate, for the first time, a biphasic regulation of GtH-II subunit mRNA levels by steroids in the goldfish pituitary. The GtH-II subunits were stimulated at lower physiological levels, and inhibited at higher doses of testosterone and estradiol.

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To

My Parents

Zera and Nurally

## Table of Contents

# List of Tables

## List of Figures

## List of Abbreviations

| | |
|---|---|
| AA | Arachidonic Acid |
| CaM | Calmodulin |
| cDNA | Complementary DNA |
| cGtH | carp GtH |
| DA | Dopamine |
| DAG | Diacylglycerol |
| E-2 | 17β-estradiol |
| FSH | Follicle-stimulating hormone |
| GABA | γ-amino-butryic acid |
| GnRH | Gonadotropin-releasing hormone |
| GnRH-R | GnRH receptor |
| cGnRH-II | Chicken GnRH-II |
| sGnRH | Salmon GnRH |
| GtH | Gonadotropin |
| GtH-I | Vitellogenic gonadotropin (FSH-like) |
| GtH-II | Maturational gonadotropin (LH-like) |
| $IP_3$ | Inositol triphosphate |
| LH | Luteinizing hormone |
| mRNA | Messenger RNA |
| pfu | Plaque-forming units |
| $PLA_2$ | Phospholipase $A_2$ |
| PLC | Phospholipase C |

| | |
|---|---|
| PKC | Protein Kinase C |
| T | Testosterone |
| VSCC | Voltage-sensitive calcium channels |
| 11β-HA | 11β-hydroxyandrosterone |

# Chapter 1

# Introduction

## 1.1. *GnRH and Its Receptors*

The secretion of pituitary gonadotropins (GtH) is mediated by the hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH). Eight forms of native GnRH peptides have been characterized (Sower et al., 1993; Table I). The brain of all vertebrate classes contain more than one molecular form of GnRH. These include Agnatha (Sherwood *et al.*,1986a; Ngamvongchon *et al.*, 1992), Chondrichthyes (Powell *et al.*,1986b), Osteichthyes (King and Millar, 1985; Powell *et al.*,1986b; Sherwood *et al.*,1984; Yu *et al.*, 1988), Amphibia (King and Millar, 1986; Sherwood *et al.*,1986b), Reptilia (Powell *et al.*, 1985, 1986a; Sherwood and Whittier, 1988), Aves (Mikami *et al.*,1988; Sherwood *et al.*, 1988), and Mammalia (King *et al.*, 1989; Gautron *et al.*, 1992). However, to date only one form of GnRH has been demonstrated in the brain of placental mammals. The physiological significance for the presence of multiple GnRH forms in the brain of a single species is at present unclear. It is interesting to note the universal presence of cGnRH-II as the second form of GnRH in most of these species suggesting that this form evolved early and is highly conserved across the various vertebrate species (Millar and King, 1994). Goldfish brain and pituitary contains two

**Table I.** Primary structure of native GnRH peptides and their comparison to mammalian GnRH form.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Mammal GnRH(**mGnRH-I**) | pGlu | His | Trp | Ser | Tyr | Gly | Leu | Arg | Pro | Gly-$NH_2$ |
| Mammal GnRH (**mGnRH-II**) | pGlu | His | Trp | Ser | Tyr | Gly | Leu | Arg | **Pro-OH** | Gly-$NH_2$ |
| Salmon GnRH(**sGnRH**) | pGlu | His | Trp | Ser | Tyr | Gly | **Trp** | **Leu** | Pro | Gly-$NH_2$ |
| Chicken GnRH(**cGnRH-I**) | pGlu | His | Trp | Ser | Tyr | Gly | Leu | **Gln** | Pro | Gly-$NH_2$ |
| Chicken GnRH(**cGnRH-II**) | pGlu | His | Trp | Ser | **His** | Gly | **Trp** | **Tyr** | Pro | Gly-NH2 |
| Catfish GnRH(**cGnRH**) | pGlu | His | Trp | Ser | **His** | Gly | Leu | **Asn** | Pro | Gly-$NH_2$ |
| Dogfish GnRH(**dGnRH**) | pGlu | His | Trp | Ser | **His** | Gly | **Trp** | **Leu** | Pro | Gly-$NH_2$ |
| Lamprey GnRH(**lGnRH-I**) | pGlu | His | **Tyr** | Ser | **Leu** | **Glu** | **Trp** | **Lys** | Pro | Gly-$NH_2$ |
| Lamprey GnRH (**lGnRH-II**) | pGlu | His | Trp | Ser | **His** | **Asp** | **Trp** | **Lys** | Pro | Gly-$NH_2$ |

molecular forms of GnRH; [$Trp^7$,$Leu^8$]-GnRH, (sGnRH) and [$His^5$,$Trp^7$,$Tyr^8$]-GnRH (cGnRH-II) (Peter *et al.*, 1987; Yu *et al.*,1988). Unlike other vertebrates, teleosts lack the hypothalamo-hypophyseal portal system and control of GtH release is exerted directly through nerve fibers extending from hypothalamus to the pars distalis (Kaul and Vollrath, 1974; Ball, 1981).

GnRH action is mediated through high affinity membrane receptors. Photoaffinity labelling studies have revealed the presence of one high affinity binding site with molecular weight of 53 and 42 kDa in rat, 42 kDa in bovine, and 39 kDa protein in ovine pituitaries (Catt et al., 1985). Characterization of GnRH receptors in the goldfish pituitary has demonstrated the presence of two classes of binding sites, a high affinity/low capacity site and a low affinity/high capacity site (Habibi *et al.*, 1987;for review Habibi and Peter, 1991). Photoaffinity labeling demonstrated the presence of two major bands of molecular weight 71 kDa and 51 kDa. The displacement characteristics of the 51 kDa band was found to be consistent with that of the high affinity binding sites involved in the control of GtH release in the goldfish pituitary (Habibi *et al.*, 1989a, 1990).

Recently, pituitary GnRH receptors (GnRH-R) have been cloned from mouse (Tsutsumi et al., 1992; Reinhart at al., 1992), human (Kakar et al., 1992; Chi et al., 1993), and sheep (Illing et al., 1993). GnRH-R cDNA codes for a 327 amino acid polypeptide. The receptor has seven transmembrane domains typical of G-protein-linked receptors with two to three N-linked glycosylation sites. However, unlike a typical G-protein linked receptor, it lacks a C-terminal intracellular domain presumed to be involved in desensitization. It is likely that another region of GnRH-R is involved in the process of desensitization since prolonged administration of GnRH is known to cause refractoriness (Belchetz *et al.*,1978; Valk *et al.*, 1980; Wildt *et al.*, 1981). Northern blot analyses demonstrated the presence of two GnRH-R mRNAs (1.6 and 3.5 kb in length) in the mouse pituitary, while only one mRNA (4.6 kb) was found in the rat pituitary (Kakar et al., 1992). Presence of more than one transcript may imply the presence of multiple GnRH-R forms in some species. In this context, goldfish pituitary contains GnRH-R on both gonadotropes and somatotropes (Cook et al., 1991) with different molecular requirement for post-receptor activation (Habibi et al., 1992). This indicates that multiple GnRH-R mRNA transcripts

may be produced in the goldfish pituitary.

### 1.2. *GnRH Desensitization*

In mammals, GnRH is released in a pulsatile fashion corresponding with episodic release of LH (Carmel *et al.*, 1976; Clark and Cummins, 1982). In ovariectomized sheep, LH pulse in the jugular vein is preceeded by GnRH pulse in the hypothalamic portal system (Clark and Cummins, 1982). Similar findings have also been reported in rats using the push-pull cannulae technique (Levine and Ramirez, 1982). A neuronal construct in the central nervous system is believed to be responsible for the rhythmic activation of GnRH cells and release of the neuropeptide. This is commonly referred to as the GnRH pulse generator, however, its cellular nature remains unknown (Knobil, 1990). The pulsatile release of GnRH is crucial for sustaining secretion of gonadotropins, and is the basis for use of GnRH for induction of ovulation. Studies have shown that pulsatile administration of GnRH to GnRH-deficient human or monkey maintains pituitary sensitivity to this releasing factor for several weeks, whereas continuous administration of GnRH agonists results in desensitization and reduction of LH serum level (Belchetz *et al.*,1978; Valk *et al.*, 1980; Wildt *et al.*, 1981). Other

studies have also shown that LH secretion progressively decreases with continuous or frequent (high frequency) infusion of GnRH (Rivier *et al.*, 1978; Rivier *et al.*, 1979; Schuling *et al.*, 1976; Sandow *et al.*,1978). Studies using rat hemipituitaries (de Koning *et al.*, 1978) and cultured pituitary cells (Rivier *et al.*, 1979) have shown that initial rise in LH and FSH begins to decline even in the continued presence of GnRH. Mechanisms underlying desensitization involve multiple components such as receptor down regulation, uncoupling of receptor-effector systems, depletion of gonadotropin stores and decrease in gonadotropin synthesis (for review see Noar, 1990)

Desensitization of pituitary gonadotropes through continuous administration of GnRH agonists has been shown in mammals (Badger *et al.*, 1983; Jinnah and Conn, 1985; Smith and Conn, 1983; Smith *et al.*,1983; Smith and Vale, 1981), chicken (King *et al.*, 1986), turtles (Licht and Porter, 1985), amphibians (Tsai and Licht, 1993) and goldfish (Habibi, 1991a,b). Desensitization studies in the goldfish pituitary have shown that both sGnRH and cGnRH-II treatments result in biphasic GtH-II release, characterized by initial sharp peak followed by a lower sustained release (Habibi, 1991b). GnRH desensitization in goldfish is an agonist-

induced process, since occupancy of receptors by a GnRH antagonist with higher binding affinity than sGnRH and cGnRH-II does not result in pituitary refractoriness (Habibi, 1991a). Significant differences between sGnRH- and cGnRH-II-induced GtH-II release have been observed with respect to dependence on extracellular calcium, pulse frequency, and peptide concentrations (Habibi 1991 a,b; Habibi *et al.*,1991). These studies show significant differences in the biphasic pattern of GtH-II release; the GtH-II release during the second sustained phase is less pronounced for cGnRH-II-, than for sGnRH-,treated group. Also, cGnRH-II was found to exert a greater degree of desensitization at high frequency of GnRH pulse administration than sGnRH.

### 1.3 *Postreceptor Mechanisms in the GnRH Action*

Post-receptor mechanisms mediating GnRH action have been investigated in detail in mammals. Multiple mechanisms are involved including phosphoinosotide turnover regulating intracellular $Ca^{2+}$ levels, protein kinase C (PKC) activation, and arachidonic acid metabolism (for reviews, see Chang and Jobin, 1994; Naor, 1990; Fig.1.1). Briefly, GnRH stimulates phospholipase C (PLC) through a G-protein

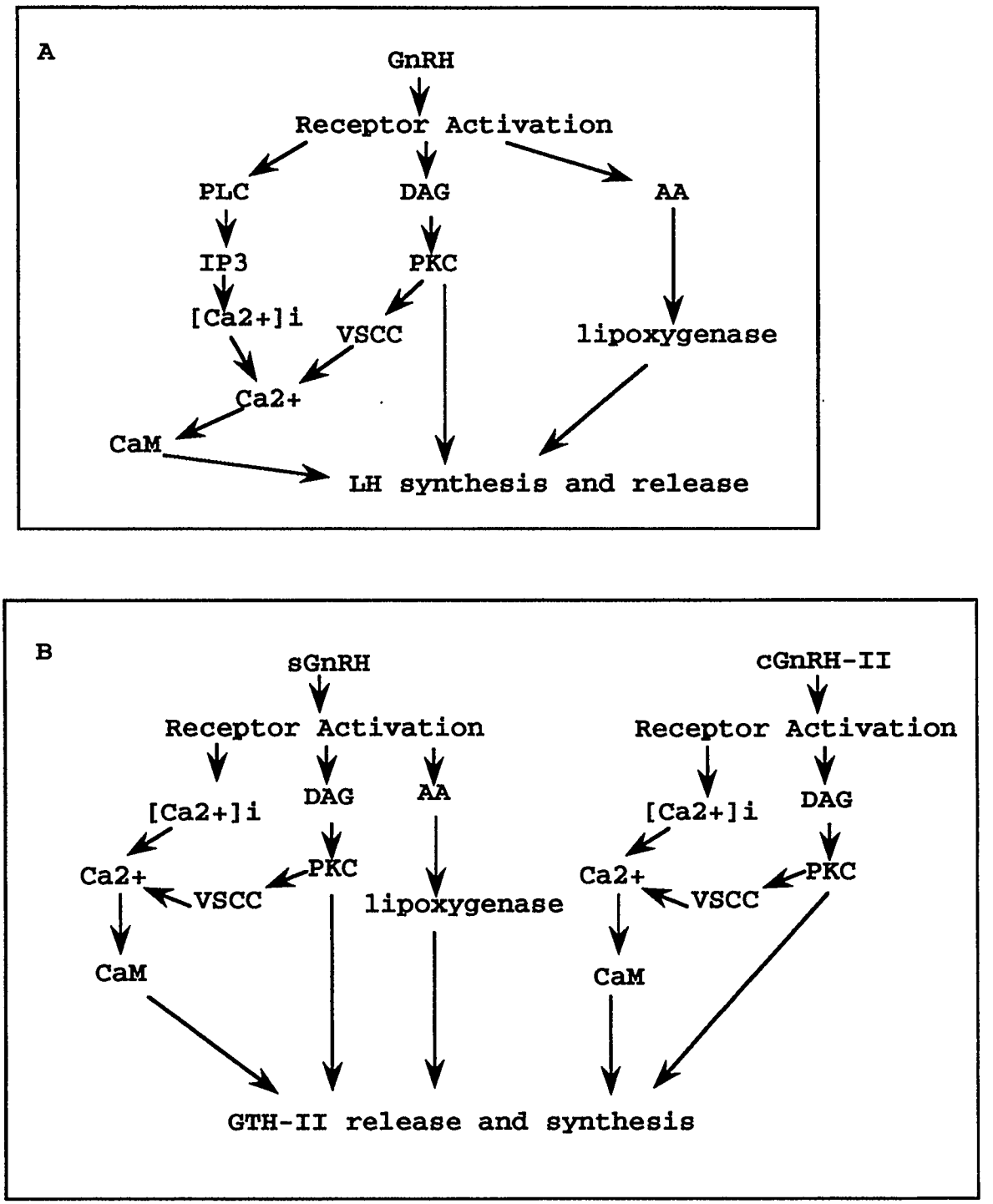



**Figure 1.1** Summary of post-receptor mechanisms involved in GnRH action in mammals (A) and goldfish (B). modified from Chang et al., 1994

linked GnRH-R. This results in increased levels of inositol triphosphate ($IP_3$), which in turn releases calcium from the intracellular stores. Receptor activation also results in increased entry of calcium through receptor-gated channels during the spike phase of the release. During the plateau phase of LH release, calcium entry through voltage-gated channels plays an important role. In addition, activation of PLC generates diayclglycerol (DAG) which then activates protein kinase C (PKC). Arachidonic acid (AA) pathways also participate in GnRH action. Mobilization of AA may be through phopholipase A2 ($PLA_2$) or DAG. The lipoxygenase metabolites of AA seem to be important in mediating GnRH response. In goldfish, the main pathways for GnRH-induced GtH release involve PKC activation, arachidonic acid metabolism, and calcium mobilization (for review see Chang and Jobin, 1994). Recent studies have demonstrated differences in the post-receptor mechanisms activated by the two native GnRH peptides in the goldfish pituitary (Chang et al., 1994; Jobin and Chang 1992 a,b; Chang and Jobin, 1991; Chang *et al.*,1991 a,b; 1990). sGnRH stimulatory action on long term GtH-II release involves both PKC and AA-dependent pathways. cGnRH-II action also involves the PKC pathway, however in contrast to sGnRH, long term cGnRH-II action does

not include mobilization of AA pathway. Furthermore, PKC plays a more prominent role in cGnRH-II-induced GtH-II release compared to sGnRH. Also calcium-deficient media revealed calcium independence in sGnRH-induced response but not in cGnRH-II-induced response.

### 1.4. *Structure of Gonadotropin Subunit Genes*

Pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) along with thyroid stimulating hormone and placental chorionic gonadotropin constitute the glycoprotein hormone family. LH and FSH are critical for normal gonadal function, while chorionic gonadotropin is responsible for maintainence of corpus luteum during pregnancy. Thyroid-stimulating hormone is important for regulation of thyroid cell activity. All members of this family are heterodimers consisting of two distinct subunits, $\alpha$ and $\beta$, non-covalently bound by weak forces such as hydrogen bonding, van der Waals forces and electrostatic bonding. Within a given species, $\alpha$-subunit is identical , while the $\beta$-subunit is unique and confers the biological activity of the hormone. These subunits are encoded on different genes. (Naylor *et al.*,1983; Pierce and Parsons, 1981).

The complementary DNAs (cDNA) and genes for LH, and FSH have been isolated and characterized in several species. The α-subunit gene and cDNA have been isolated and characterized in human (Fiddles and Goodman, 1979; 1981), bovine (Goodwin *et al.*, 1983; Erwin *et al.*, 1983; Nilson *et al.*, 1983), mouse (Chin *et al.*, 1981; Gordon *et al.*, 1988), and rat (Burnside *et al.*, 1988; Godine *et al.*, 1982). In all these species, α-subunit is encoded on one gene and is composed of 4 exons and 3 introns (Boothby *et al.*,1981). The size of α-subunit varies between 8-16.5 kb due to variation in the size of the first intron. The mature α-subunit protein is 96 amino acids in length in all species except human, which encodes 92 amino acids (Chin *et al.*, 1983). LH-β genes in human (Talmadge *et al.*, 1983, 1984; Fiddes and Talmadge, 1984), rat (Jameson *et al.*, 1984; Chin *et al.*, 1983; Tepper and Roberts, 1984), and bovine (Virgin *et al.*, 1985; Maurer, 1985) were found to be approximately 1.5 kb in length, possessing 3 exons and 2 introns. The mature protein is 121 amino acids in length. FSH-β subunit gene in human (Watkins *et al.*, 1987; Jameson *et al.*, 1988), bovine (Kim *et al.*, 1988), and rat (Gharib *et al.*, 1989) varied from 3.5-5 kb in length and comprised of 3 exons and 2 introns. The mature protein contains 110 amino acids.

Until recently, it was believed that teleosts had only one form of gonadotropin hormone (GtH) which was homologous to the mammalian LH/FSH family. However, recent studies provide evidence for duality of the gonadotropins. Distinct gonadotropins, referred to as GtH I and GtH II (structurally FSH-like and LH-like respectively), possessing different β-subunits, have recently been isolated and characterized in chum and coho salmon (Itoh *et al.*, 1988; Suzuki *et al.*, 1988a,b; Sekine *et al.*, 1989; Swanson *et al.*, 1991), common carp (Van Der Kraak *et al.*, 1992), and killifish (Lin *et al.*, 1992). Molecular weights of chum salmon GtH I and GtH II were estimated to be 50 KDa and 36 KDa, respectively, while those of common carp were 45 KDa and 36 KDa. Although in mammals, α-subunit is encoded by a single gene and only one α-subunit is involved in covalent bonding with all the glycoprotein hormones, two distinct α-subunits have been demonstrated in piuitary extracts from salmon (Kitahara et al., 1988; Kawauchi et al., 1989; Itoh *et al.*, 1990) and bonito (Koide et al., 1993). In salmon the two types of α-subunit give rise to two isoforms of GtH-I, GtH-Iα1 and GtH-Iα2 (Itoh *et al.*, 1990), while in bonito the reverse is observed (Koide et al., 1993). In carp, although two different α-mRNAs were isolated through cDNA cloning (cGtH-

α1 and cGtH-α2), only one of the α-subunits (cGtH-α1) was present in the pituitary protein extracts (Chang et al., 1988). The two α cDNAs are structurally different. However, the amino acid sequences are very similar (96 % homology). Bioassay studies demonstrated that only cGtH-α1 is functionally active while the cGtH-α2 is not. The relationship of the two carp α-subunits mRNAs with GtH-Iβ and GtH-IIβ is not clear yet. The first complete characterization of the primary structure of GtH β-subunit was done in chinook salmon (Trinh et al., 1986). cDNAs encoding both GtH-I and GtH-II β-subunits have so far been cloned and sequenced in salmon (Suzuki et al., 1988a,b; Sekine et al., 1989) and killifish (Lin et al., 1992). In carp, only the GtH-II β subunit has been cloned and sequenced (Chang et al., 1988). In the present study, cGtH-IIβ and cGtH-α cDNAs were used as probes in Northern analysis.

Complete amino acid sequences of GtH have been determined for chum salmon (Suzuki *et al.*,1988 a,b; Itoh *et al.*, 1988, 1990; Sekine *et al.*, 1989), carp (Chang *et al.*, 1988, 1990; Van Der Kraak *et al.*, 1992), and killifish (Lin *et al.*, 1992). In all cases, the mature α-subunit was found to be 92 and/or 95 amino acids in length depending on

if the species contained two forms of α-subunits. The GtH-I β subunit ranged from 95-113 amino acids and GtH-II β subunit ranged form 115-119 amino acids in length. Information on the gene structure of the glycoprotein hormones in non-mammalian vertebrates is limited. Two different genes encoding two types of α-subunit have been cloned in carp (Huang et al., 1992) and demonstrate similar structure as the mammalian form (Pierce, 1988). With respect to the β-subunits, only GtH-II β genes have been cloned in salmon (Xiong and Hew, 1991) and carp (Chang et al., 1992).

Evolutionarily, it has been observed that the α-subunit shows high conservation between species. Carp α-subunit has 70% homology with mammalian subunit (Chang *et al.*, 1990). However, the β-subunit of carp GtH-II has 75% homology with that of salmon (Chang *et al.*, 1988) and only 40% homology to mammalian LH (Chang *et al.*, 1990). Although the amino acid sequences in the β-subunit are diversified between fish and mammals, all of the 12 half-cystine residues present are aligned at the same positions. It is believed that these similar regions represent the binding site of the α-subunit, while the variable regions are hormone specific and may be involved in binding to the receptor. Hence, the α-subunit of GtH has been highly conserved while the β-subunit is

diversified through evolution.

### 1.5. *Regulation of Gonadotropin Release and Subunit Synthesis*

Regulation of GtH release and synthesis involves a complex network of interplay between neuronal and endocrine factors. To study the role of GnRH and gonadal steroids in this regulation, investigators have utilized various methods, *in vivo* and *in vitro* studies in intact animals, castrated animals, and animals with hypothalamic-pituitary disconnection. These studies have yielded a complex set of results as summarized below.

The pattern of GnRH administration (continuous or pulsatile) is an important regulator of GtH release and synthesis. As outlined earlier in section 1.2, continuous GnRH agonist treatment results in desensitization of GtH release. In rats, continuous GnRH treatment *in vivo* resulted in inhibition of LH-β gene expression and synthesis without affecting α-subunit gene (Lalloz *et al.*, 1988b). Rodin et al. (1989) demonstrated that endogenous GnRH is essential for maintainance of FSH-β mRNA levels in intact and orchidectimized rats. Furthermore, studies in rats and sheep have demonstrated that the mode of GnRH pulstaile

treatment can differentially regulate the gonadotropin subunit mRNA levels. Many *in vivo* studies in rats (Haisenleder et al., 1987; Dalkin et al., 1991) and sheep (Leung et al., 1987) have demonstrated that pulsatile GnRH treatment stimulates $\alpha$ subunit as well as LH-$\beta$ and FSH-$\beta$ subunit mRNA concentrations. In addition, higher frequencies of GnRH pulse administration was found to increase $\alpha$ and LH-$\beta$ mRNA levels, while slower frequencies increased FSH-$\beta$ mRNA levels (Haisenleder et al., 1991).

In a number of studies, hypothalamic-pituitary link was lesioned to study regulation of GtH independent of hypothalamus. Hypothalamo-hypophysial lesioning in ewes resulted in significant and rapid reduction of serum LH levels with lower level of change in serum FSH concentration (Hamernik et al., 1986). Pulsatile replacement of GnRH in hypothalamic-pituitary disconnection ovariectomized ewes or hypothalamus-lesioned monkeys resulted in differential effect whereby low frequency of GnRH adminstration favored FSH release over LH release (Clarke et al., 1984; Wildt et al., 1981).

Castration resulted in increased LH and FSH mRNA levels as well as increased pituitary and serum LH and FSH levels (Gharib *et al.*, 1986, 1987; Wierman *et al.*, 1988). In other

studies, postcastration increase in cytosolic LH subunit mRNA levels were shown to depend on endogenous GnRH since adminstration of a GnRH antagonist abolished this effect (Lalloz *et al.*,1988a; Wierman *et al.*, 1989; Rodin *et al.*, 1989).

Studies *in vitro* have also demonstrated that the mode of GnRH administration differentially regulates the expression of the different gonadotropin subunits. In the rat dispersed pituitary cells, the α-subunit mRNA responded to both pulsatile and continuous administration of GnRH, while LH-β mRNA was not responsive to either (Weiss *et al.*, 1990). In the same study, FSH-β mRNA level was shown to increase following pulsatile treatments and decrease after continuous treatments with GnRH. Furthermore, the amplitude of GnRH pulse was found to be an important regulator of GtH subunit gene expression. Recent *in vitro* studies have demonstrated that LH-β and FSH-β mRNA levels are only stimulated in response to a specific range of pulse amplitudes (Haisenleder et al., 1993).

Extensive studies have been carried out in mammals to study the gonadal steroid regulation of gonadotropin synthesis and release. Both inhibitory and stimulatory effects have been observed, and there is evidence that

steroids influence gonadotropin release and synthesis both through hypothalamic and pituitary pathways. To differentiate between a direct effect at the level of pituitary and indirect pathways, studies have been performed in *in vivo* and *in vitro,* using intact and castrated animals.

In anestrous ewes, *in vivo* studies demonstrated differential regulation of GtH subunits by 17β-estradiol (E-2). It was observed that the α mRNA levels increased while, FSH-β levels decreased, and LH-β levels only slightly increased (Landefeld *et al.*, 1989). Testosterone (T) has been demonstrated to decrease LH secretion *in vivo* (Papavasiliou *et al.*, 1986b; Haisenleder *et al.*, 1987; Steiner *et al.*, 1982).

Many studies have reported a postcastration rise in gonadotropin gene expression (Godine *et al.*, 1980; Counis *et al.*, 1983; Gharib *et al.*, 1986,1987; Wierman *et al.*, 1988). In another study, estradiol-treated pituitaries from ovariectomized rats specifically stimulated synthesis of LH-β gene with no effect on FSH-β and α mRNA levels.(Shupnik *et al.*, 1989b). In castrate rats, T inhibits LH secretion with relatively lower effect on FSH release (Decker *et al.*, 1981).

Cultured pituitary cells from orchidectomized rats treated with T were found to have increased pituitary content of FSH and decreased content of LH (Kitahara et al., 1991). In the same study, the LH-β and α-subunit mRNAs of the cultured cells were decreased and the FSH-β mRNA levels remained unchanged (Wierman et al., 1988). In addition, T has been shown to stimulate FSH-β mRNA levels in rat pituitary cells in the absence of GnRH (Gharib et al., 1990; Winters et al., 1992) and inhibit FSH-β mRNA levels in presence of GnRH (Winters et al., 1992).

In teleosts, several studies have shown a negative feedback effects of gonadal steroids on GtH release. Removal of gonads results in increased serum GtH levels in rainbow trout (Bommelaer et al., 1981), African catfish (Habibi et al., 1989b), and goldfish (Kobayashi and Stacey, 1990). Estrogens and androgens inhibited the activity of pituitary gonadotrophs in the Indian catfish (Sundaraj and Gowswami, 1968). Several studies in goldfish have demonstrated that the underlying mechanisms for the negative feedback effect by the steroids may be regulated by neuronal factors such as GABA and dopamine (DA), known to effect GtH synthesis and release (discussed in detail in chapter 3). Information on the regulation of GtH-II subunit synthesis in the fish

species is limited and will be discussed in detail in chapter 3. Briefly, in adult fish, as in mammals, gonadal steroids have generally exerted an inhibitory effect on gonadotropins as outlined above. However in juvenile fish, it has been observed that steroids induce an increase in pituitary GtH content (Crim and Evans, 1979; Dufour et al., 1983) as well as on GtH subunit mRNA levels (Trinh et al.,1986; Querat et al,. 1991; Xiong et al., 1994).

## 1.6 *Objectives of the Present Study*

At present, there is little known about the physiological significance for the presence of more than one form of GnRH in the brain of vertebrates. In addition, our information on the regulation of gonadotropin synthesis in lower vertebrates and teleosts is rather limited.

This thesis attempts to investigate the regulation of GtH-II release and synthesis by the native GnRH peptides, sGnRH and cGnRH-II, in the goldfish pituitary. Desensitization and gene expression studies were carried out to elucidate differences between sGnRH- and cGnRH-II-induced GtH-II release and synthesis. The present study provides information on the existence of specific receptor-effector systems with preferential specificity for sGnRH and cGnRH-II coupled to release and synthesis of GtH-II in the goldfish pituitary. Furthermore, the findings also provide information on the effects of gonadal steroids on GtH-II gene expression in the goldfish pituitary.

# Chapter 2

## Functional specificity for salmon GnRH and chicken GnRH-II coupled to the gonadotropin release and subunit mRNA level in the goldfish pituitary

## 2.1 *Introduction*

The secretion of gonadotropins (GtH) from the pituitary is mediated by the hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH). To date, the primary structure of eight native GnRH forms have been elucidated: one in mammal (Burgus et al., 1972), one in salmon (Sherwood et al., 1983), two in chicken (King and Millar, 1982), one in dogfish (Lovejoy et al., 1992), two in lamprey (Sherwood et al., 1986a; Sower et al., 1993), and one in catfish (Ngamvongchon et al., 1992). Multiple GnRH forms have been demonstrated in the brain of Agnatha (Sherwood et al., 1986a), Chondrichthyes (Powell et al., 1986b), Osteichthyes (Powell et al., 1986b; King and Millar, 1985; Sherwood et al., 1984; Yu et al., 1988) , Amphibia (King and Millar, 1986; Sherwood et al., 1986b), Reptilia (Powell et al., 1985; Powell et al., 1986a; Sherwood and Whittier, 1988), Aves (Mikami et al.,1988; Sherwood et al., 1988), and Marsupial (King et al., 1988). However, the physiological significance for the presence of multiple forms of GnRH in the brain of any one species is at present unknown. Goldfish brain contains two forms of GnRH, [Trp$^7$, Leu$^8$]-GnRH (sGnRH) and [His$^5$, Trp$^7$, Tyr$^8$]-GnRH (cGnRH-II) (Yu et al., 1988; Peter et al., 1987). As for GtH, there is

immunological and biochemical evidence for the presence of two gonadotropic hormones, GtH-I and GtH-II in cyprinid pituitary (Van Der Kraak et al., 1992). However, specific antisera and genomic probes are only available for the maturational (steroidogenic) GtH which is analogous to and has activities similar to salmon GtH-II (Itoh et al., 1988; Suzuki et al., 1988; Lin et al., 1992) and tetrapod luteinizing hormone (LH), hereinafter described as GtH-II. Both sGnRH and cGnRH-II stimulate the release of GtH-II, although cGnRH-II has a greater GtH-II-releasing activity ($ED_{50}$=3.5±1.5 nM) and binds with a greater affinity ($K_a$=9.2/nM) to the high affinity GnRH receptors than sGnRH ($ED_{50}$=14.6±4.3 nM, $K_a$=3.3/nM) in the sexually mature goldfish pituitary (Habibi, 1991a; Habibi et al., 1992; Chang et al., 1990).

In mammals, GnRH is secreted in a pulsatile manner, leading to episodic secretion of LH and FSH (Carmel et al., 1976; Clark and Cummins, 1982; Drouva and Gallo, 1976). It has been demonstrated that continuous administration of GnRH results in desensitization of pituitary gonadotropes while pulsatile treatment leads to little or no refractoriness (Badger et al., 1983; Jinnah and Conn, 1985; Smith and Conn, 1983; Smith et al., 1983; Smith and Vale, 1981). GnRH

desensitization has also been demonstrated in chicken (King et al., 1986), turtle (Licht and Porter, 1985; Tsai and Licht, 1993), and goldfish (Habibi, 1991a,b). In addition, in mammals it has been demonstrated that maintainance of steady production of LH subunits are dependent on pulsatile GnRH stimulation (Dalkin et al., 1989; Weiss et al., 1990; Haisenleder et al., 1991).

Previous studies in goldfish have demonstrated significant differences between sGnRH- and cGnRH-II-induced desensitization of GtH-II release in terms of dependence on concentration, pulse frequency, and extracellular calcium (Habibi, 1991a,b; Habibi et al., 1991). Furthermore, recent studies also demonstrated differences in the post-receptor mechanisms involved in sGnRH- and cGnRH-II-induced GtH-II release in the goldfish pituitary (Chang et al., 1994; Jobin and Chang 1994, 1992a,b; Chang et al., 1991). These findings indicate that sGnRH action greatly depends on intracellular calcium concentration and is less affected by extracellular calcium concentration. On the other hand, cGnRH-II is largely dependent on extracellular calcium for stimulation of GtH-II release in the goldfish pituitary (Jobin and Chang, 1992a,b). As well, arachidonic acid pathway was shown to be a major component of sGnRH action, while cGnRH-II

action lacked this component (Chang et al., 1991). All together, the above differences are not fully compatible with the view that sGnRH and cGnRH-II interact with the same receptor population coupled to the same post-receptor mechanisms coupled to the release of GtH-II in the same pituitary cell populations. An important question is whether sGnRH and cGnRH-II, physiologically, work through different receptor-effector mechanisms in regulating GtH-II synthesis and release.

The purpose of the present study was to examine the possible existence of GnRH receptor-effector system that respond differentially to sGnRH and cGnRH-II in the goldfish pituitary. The experimental approach was to compare homologous and heterologous GtH-II release desensitization to sGnRH and cGnRH-II in the goldfish pituitary, as well as studying the effect of these native peptides on GtH-II subunit mRNA levels.

## 2.2 *Materials and Methods*

### 2.2.1 *Animals*

Goldfish, *Carassius auratus*, (mixed sex, ranging 8-10 cm in length) were purchased from Ozark Fisheries in Southland, MO. They were maintained in a 1500 L semi-recirculating aquarium at 17 C on a 16 hr light/8 hr dark photoperiod, and were fed a commercial fish diet.

### 2.2.2 *Hormones and other chemicals*

[$Trp^7$,$Leu^8$]-GnRH (sGnRH) and [$His^5$, $Trp^7$, $Tyr^8$]-GnRH (cGnRH-II) were purchased from Peninsula, Belmont, CA. Peptides were solubilzed in 0.1 M acetic acid at the concentration of 10µg/20µl, stored at -20 C. Appropriate concentrations of peptides were prepared through dilution immediately prior to use for the experiment. Carp gonadotropin (GtH-II), purified as described by Peter *et al.* (1983), was a gift from Dr. B. Breton, Laboratoire de physiologie des Poissons, Institute Nationale de la Recherche Agronomique, Rennes, France. This particular GtH-II corresponds to maturational GtH or LH in its physiological function (Van Der Kraak et al., 1992). Antibody specific to cGtH-II was a gift from Dr. R.E. Peter,

from Dept. of Zoology, University of Alberta, Edmonton, Alberta, Canada. Calcium ionophore (A23187) obtained from Calbiochem, San Diego, CA, was solubilzed in dimethlysulfoxide at 25mM stock solution and stored at -20 C.

The carp GtH-II-β cDNA fragment, 0.7-0.8 kB in length and carp GtH-II-α cDNA fragment, 0.8-0.9 kB in length, were provided by Dr. FL Huang from Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan. The trout α-tubulin cDNA fragment (1.5 kB) was provided by Dr. G. Dixon from Department of Medical Biochemistry, University of Calgary, Calgary, Alberta.

### 2.2.3 *Superfusion of pituitary fragments*

The *in vitro* pituitary GtH-II release in response to sGnRH and cGnRH-II were determined using a superfusion system as described previously by Habibi *et al.* (1989a). Pituitary fragments (three pituitary equivalent per column) were treated with sGnRH and cGnRH-II in pulsatile or continuous fashion at various concentrations. All experiments involved running eight columns simultaneously with automatic fraction collection at 2, 5, or 10 min intervals. Samples were frozen at -20 C until determination

of GtH-II concentration by a radioimmunoassay as described by Peter *et al.* (1984). The GtH-II release was quantified by determination of area under the curve as described in Habibi *et al.* (1989, 1991b, 1992). Briefly, basal GtH-II level prior to each treatment was subtracted from hormone levels following treatment. The GtH-II concentrations are expressed as the mean ± SEM, and were analyzed statistically by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test with $P< 0.05$ degree of significance. For the area under the curve, we also estimated the slope of each line (GtH % initial response vs. pulse number) ± 95% confidence intervals using regression analysis. Please note that the GtH-II release experiments were not designed to provide a comparison between sGnRH and cGnRH-II potency; $ED_{50}$ values and receptor binding affinities for these peptides were reported in previous studies (Habibi, 1991a).

### 2.2.4 *Determination of GtH-II subunit mRNA*

Goldfish of mixed sex at two different stages of gonadal development were treated with either sGnRH or cGnRH-II in order to investigate regulation of GtH-II subunit gene expression. Female goldfish with follicle diameter 0.18-0.25 mm were classified as sexually regressed, while fish with

follicle diameter size 1.08-1.34 mm were classified as sexually mature. Male goldfish containing transparent, non-spermiating testis were classified as immature (regressed), while fish with sperm containing or spermiating testis were classified as sexually mature. In regressed goldfish basal circulating GtH-II level is 3.5 ± 0.7 ng/ml, whereas in sexually mature goldfish the basal circulating GtH-II level is 10.6 ± 1.5 ng/ml).

Following IP injections of sGnRH, cGnRH-II, or saline (control) (1 or 4 μg/fish), groups of fish were anesthetized and sacrificed at various time intervals in accordance with the Animal Care Regulations of the University of Calgary (7-10 animals per treatment group). Total RNA was extracted from the pituitaries using guanidine thiocyanate-phenol-chloroform extraction method (Chomczyski and Sacchi, 1987). Five μg of total RNA was loaded in separate wells, resolved on a 1.2% agarose/formaldehyde gel and transferred onto Hybond-N nylon membrane (Amersham) using 20X SSPE. Membranes were prehybridized at 60 C overnight in 5 ml of 6X SSC, 10X Denhardt, 10 mM EDTA (pH 8), 0.5% SDS, 0.05% sodium pyrophosphate, and 100 μg/ml of sheared and denatured *E. coli* DNA. The membranes were hybridized initially with complementary DNA for cGtH-II-β, stripped and subsequently

hybridized with cGtH-II-α and α-tubulin from trout. Please note that α-tubulin expression is not affected by sGnRH and cGnRH-II and was therefore used as a internal marker for all experimental groups. The cDNAs were labeled by random primer method ($T^7$ Quick Prime, Pharmacia). Hybridization was carried out for 24-28 hours at 60 C. The membranes were subsequently washed with a series of stringent washes up to 0.1X SSPE in presence of 0.1% SDS and 0.1% sodium pyrophosphate. The autoradiograms were scanned using a computerized densitometer scanner, gelscan, and quantified using the Image program provided by NIH (Bethesda, MD). The program computes the area and average density of given selection. The values were transferred to a spreadsheet and statistical analysis program for further analysis of the data. The quantified mRNA levels for GtH-IIβ and GtH-IIα were each divided by α-tubulin mRNA value in the corresponding lane. The relative mRNA levels shown in Fig. 8 and 9 represent difference from the control group within each experiment. The values were statistically analyzed by student's t-test. Blood samples were also obtained from the same groups of regressed goldfish used for determination of mRNA levels. Serum samples were frozen at -20 C until determination of GtH-II concentration by a radioimmunoassay

as described by Peter *et al.* (1984). Circulating GtH-II concentration was expressed in terms of ng/ml (mean ± S.E.), and were analyzed statistically by one-way ANOVA followed by Duncan's multiple range test with $P<0.05$ degree of significance.

## *2.3 Results*

### 2.3.1 *Pulsatile treatments with sGnRH and cGnRH-II:*

Sexually mature goldfish pituitary fragments were exposed to pulsatile treatments (3 min pulses) of sGnRH and cGnRH-II at different doses and frequencies. In these studies, peptides were administered either in a homologous (sGnRH/sGnRH or cGnRH-II/cGnRH-II) or heterologous fashion. In the heterologous paradigm, sGnRH and cGnRH-II were either administered together (sGnRH+cGnRH-II) or in an alternate fashion, so that a sGnRH treatment was followed by cGnRH-II treatment or vice versa. With respect to the sequence of alternate pulse application, experiments were carried out using both paradigms (sGnRH/cGnRH-II/sGnRH/.., or cGnRH-II/sGnRH/cGnRH-II/..,) and no significant differences were observed between the two modes of treatment. In the case of combined treatments (sGnRH+cGnRH-II), the concentration of each peptide was half the indicated total concentration, giving combined final concentrations indicated. Each experiment involved running eight superfusion columns (grouped into 4 pairs), each pair receiving one of the following pulsatile treatments: sGnRH/sGnRH, cGnRH-II/cGnRH-II, sGnRH/cGnRH-II, or sGnRH+cGnRH-II. First set of

experiments involved pulsatile homologous and heterologous treatments every 60 min at $10^{-7}$ M, $10^{-8}$ M, and 5 X $10^{-9}$ M. A total of six pulses (3-minute) were administered every 60 minutes in each experiment. Fractions were collected every five minutes for the first 20 minutes following a treatment and thereafter every 10 minutes until the next pulse. Pulsatile treatments at $10^{-7}$ M, resulted in significant desensitization of the pituitary GtH-II release with no consistent differences between the desensitization pattern induced by homologous and heterologous treatments (Figs.2.1, 2.2). Determination of the slope values indicated significant ($P<0.05$) regression from horizontal line (slope=0) for all treatment groups (Fig. 2.3). Similar pulsatile treatments at lower concentrations of $10^{-8}$ M and $5x10^{-9}$ M did not result in sufficient GtH-II release desensitization and therefore were unsuitable to reveal differences between homologous and heterologous desensitization (results not shown). However, treatments administered at a higher frequency (3 minute pulses every 30 minutes) at $10^{-8}$ M resulted in desensitization of the pituitary GtH-II release with significant differences between homologous and heterologous desensitization; alternate treatments with sGnRH and cGnRH-II resulted in a

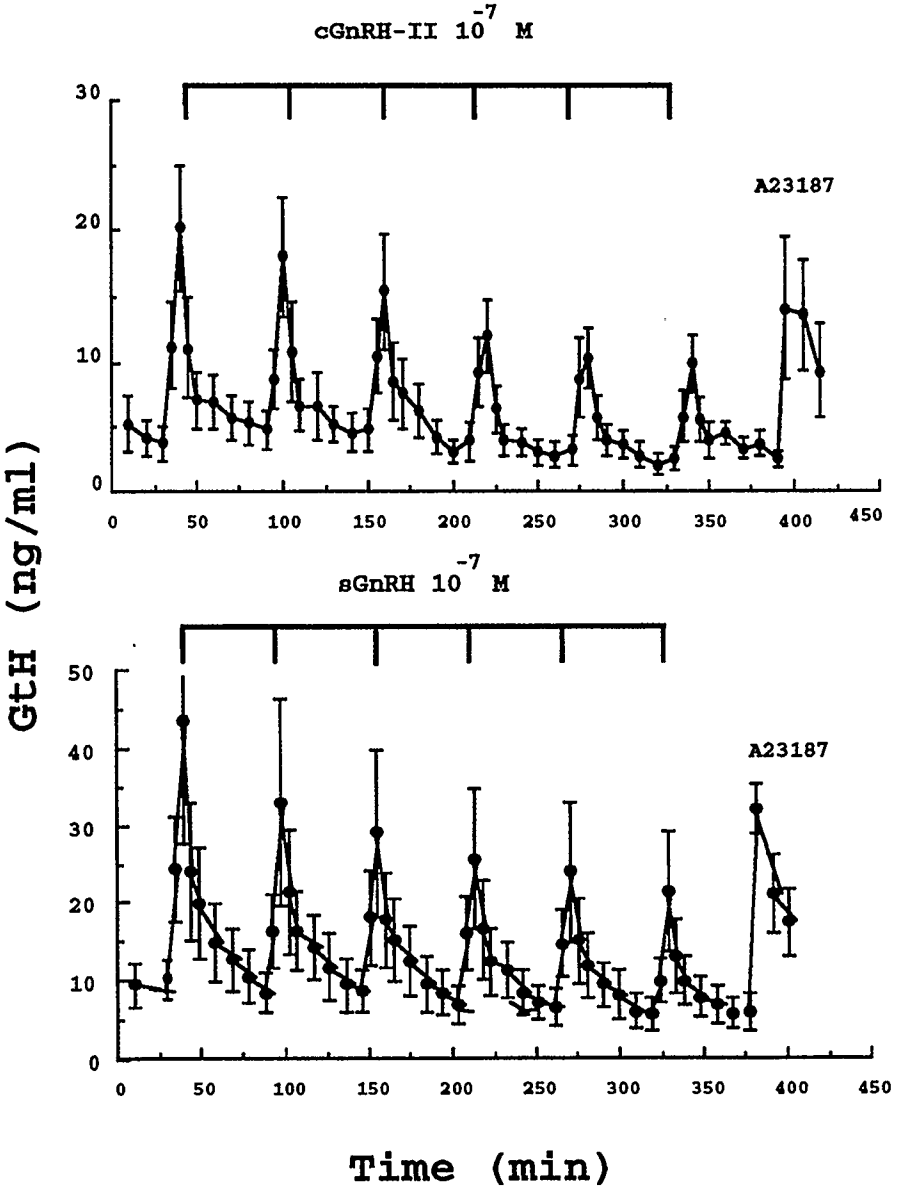



**Figure 2.1** Gonadatropin (GtH-II) release from superfused goldfish pituitary fragments following homologous pulsatile treatment at 10-7 M (3 min pulses every 60 minutes). At the end of the experiment, pituitary fragments were exposed to 3 min pulse of 0.1 mM calcium ionophore (A23187). Fractions were collected every five minutes for the first 20 minutes of each treatment and thereafter every 10 minutes. GtH-II concentration was measured by radioimmunoassay. Each value represents mean ± S.E. of 4 observations (4 different columns).

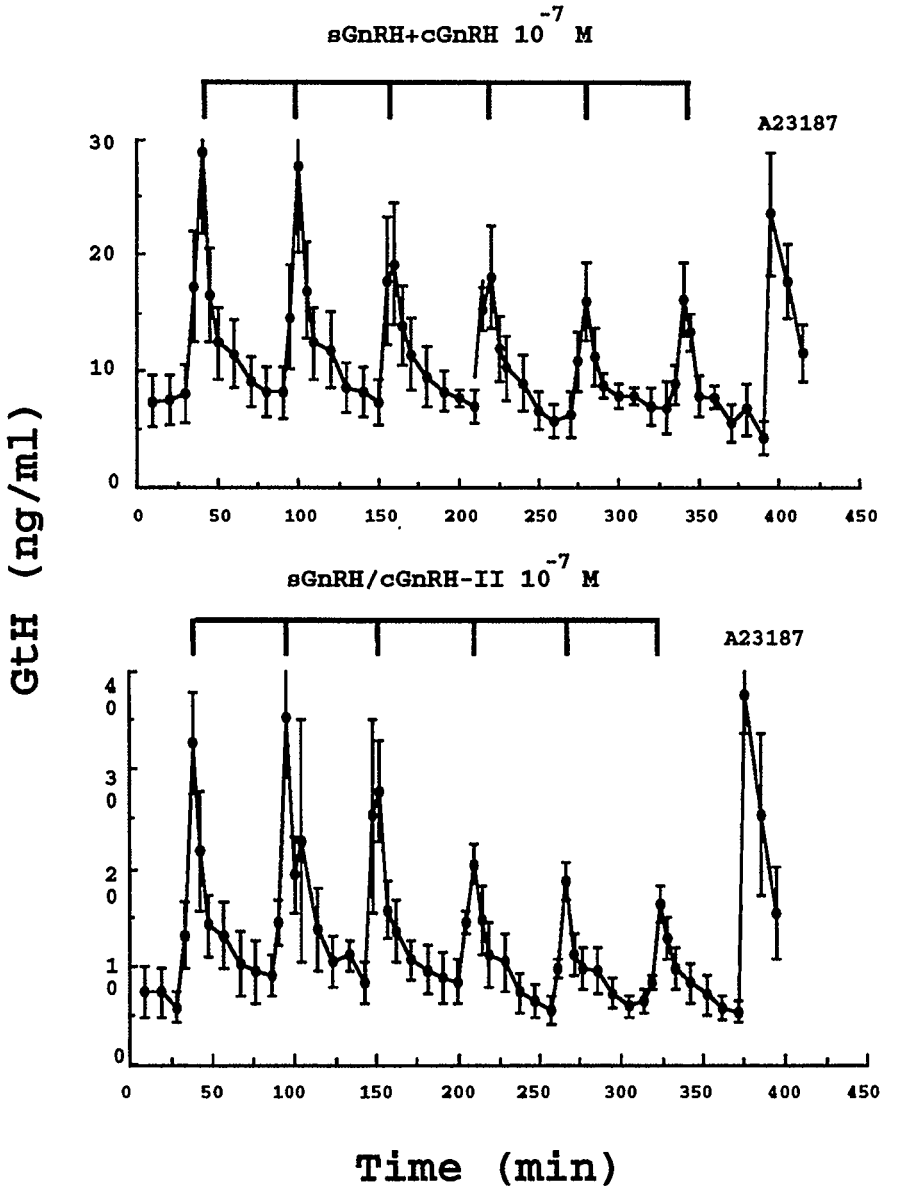



**Figure 2.2** Gonadatropin (GtH-II) release from superfused goldfish pituitary fragments following heterologous pulsatile treatment at 10-7 M (3 min pulses every 60 minutes). Heterologous treatments included a combination of peptides (both peptides present in the treatment; s+cGnRH) or an alternation of peptides (s/cGnRH) during the pulsatile treatment. At the end of the experiment, pituitary fragments were exposed to 3 min pulse of 0.1 mM calcium ionophore (A23187). Fractions were collected every five minutes for the first 20 minutes of each treatment and thereafter every 10 minutes. GtH-II concentration was measured by radioimmunoassay. Each value represents mean ± S.E. of 4 observations (4 different columns).

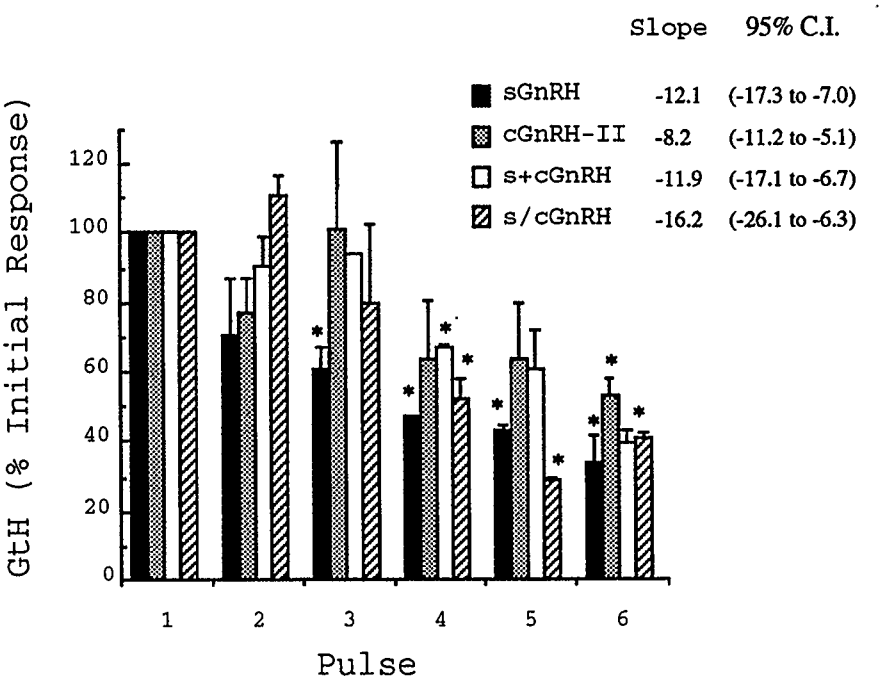



**Figure 2.3** GtH-II response to sGnRH and cGnRH-II pulses at 10-7 every 60 minutes was quantified for each treatment by determination of area under the curve. Values were expressed in terms of percentage control, taking the initial treatment as 100%. Values displaying (*) are significantly different from the initial 100% value. The slope values and 95% confidence intervalls were estimated by regression analysis.

significantly lower degree of desensitization of GtH-II release compared with homologous treatments (sGnRH or cGnRH-II alone), or combination of these peptides (sGnRH + cGnRH-II) (Figs.2.4, 2.5). In terms of time-related overall release, the slope of regression line for alternate sGnRH and cGnRH-II treatment was not significantly different from the horizontal line (slope=0). The slope of other groups, however, were significantly ($P<0.05$) different from horizontal line indicating a gradual decline in GtH-II release (Fig.2.6). In these experiments, total of ten pulses were administered and fractions were collected every five minutes for the full course of the experiment.

### 2.3.2 *Continuous treatments with sGnRH and cGnRH-II:*

Experiments were also carried out to compare homologous and heterologous desensitization induced by continuous treatments with sGnRH and cGnRH-II. For homologous treatment, goldfish pituitary fragments were exposed continuously for three hours to increasing concentrations of sGnRH or cGnRH-II (i.e. 60 min of $10^{-8}$ M, followed by 60 min of $10^{-7}$ M, and finally 60 min of $10^{-6}$ M), while in heterologous treatments the peptides were alternated (i.e. sGnRH/cGnRH-II/sGnRH or cGnRH-II/sGnRH/cGnRH-II) each

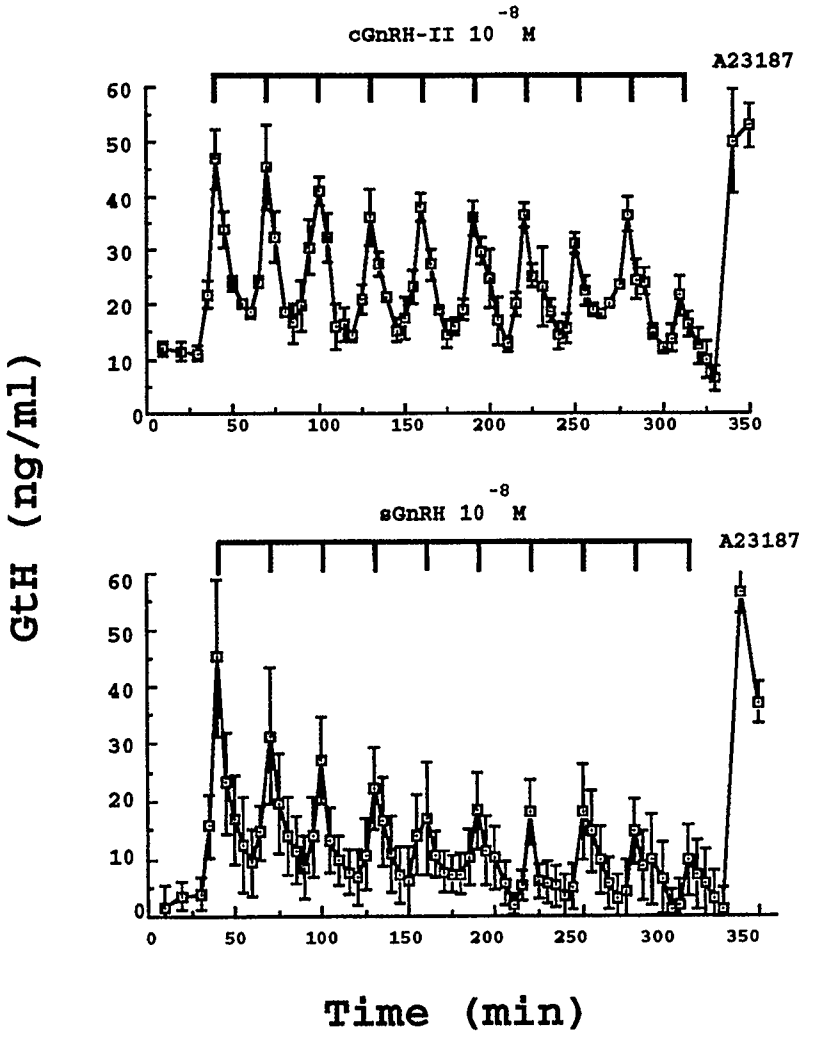



**Figure 2.4** Gonadatropin (GtH-II) release from superfused goldfish pituitary fragments following homologous pulsatile treatment at 10-8 M (3 min pulses every 30 minutes). At the end of the experiment, pituitary fragments were exposed to 3 min pulse of 0.1 mM calcium ionophore (A23187). Fractions were collected every five minutes for the first 20 minutes of each treatment and thereafter every 10 minutes. GtH-II concentration was measured by radioimmunoassay. Each value represents mean ± S.E. of 4 observations (4 different columns).

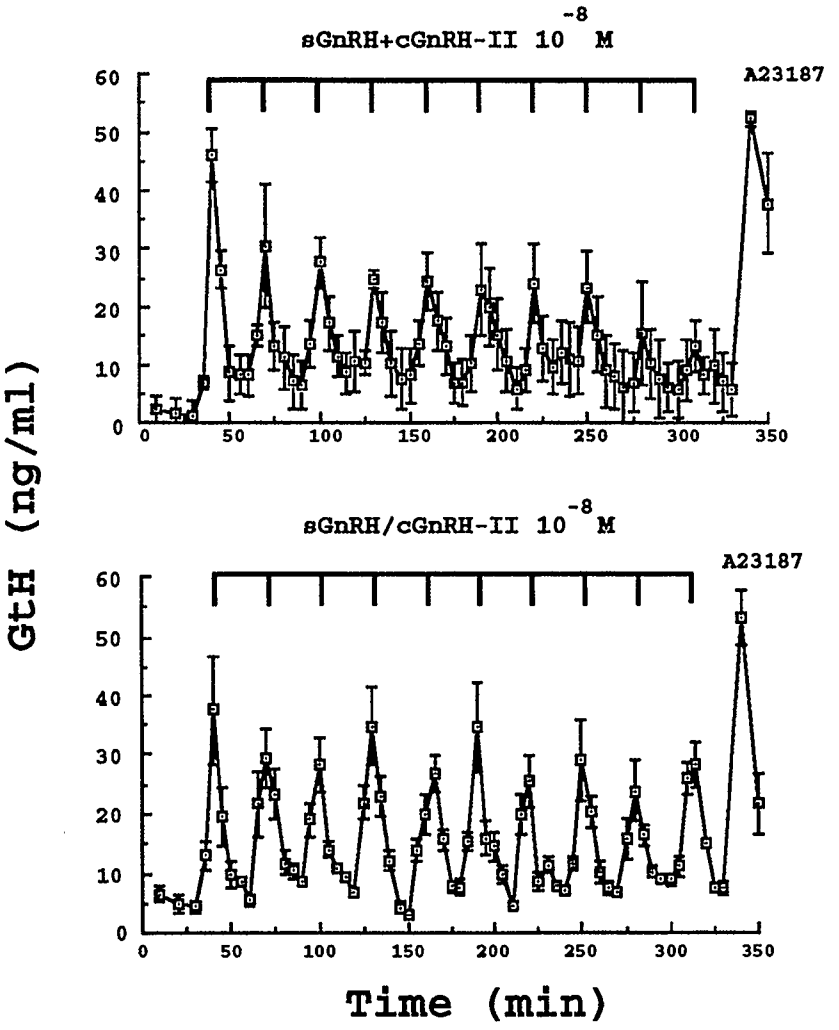



**Figure 2.5** Gonadatropin (GtH-II) release from superfused goldfish pituitary fragments following heterologous pulsatile treatment at 10-8 M (3 min pulses every 30 minutes). Heterologous treatments included a combination of peptides ( both peptides in the same treatment; s+cGnRH) or alternation of peptides (s/cGnRH) during the pulstaile treatment. At the end of the experiment, pituitary fragments were exposed to 3 min pulse of 0.1 mM calcium ionophore (A23187). Fractions were collected every five minutes for the first 20 minutes of each treatment and thereafter every 10 minutes. GtH-II concentration was measured by radioimmunoassay. Each value represents mean ± S.E. of 4 observations (4 different columns).

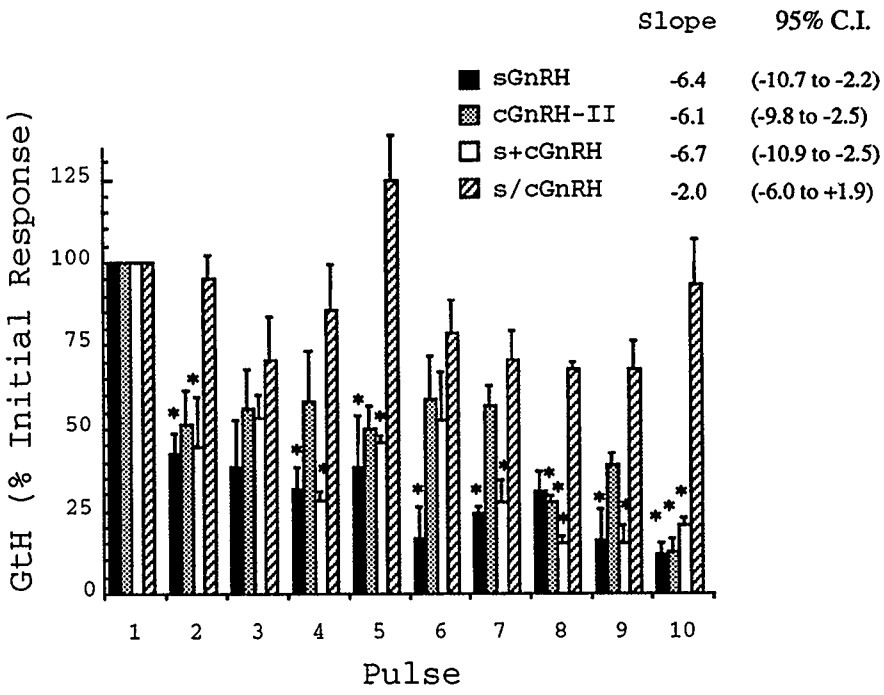



**Figure 2.6** GtH-II response to sGnRH and cGnRH-II pulses at 10-8 every 30 minutes was quantified for each treatment by determination of area under the curve. Values were expressed in terms of percentage control, taking the initial treatment as 100%. Values displaying (*) are significantly different from the initial 100% value. The slope values and 95% confidence intervalls were estimated by regression analysis.

administered for 60 minutes at a given concentration. The heterologous treatments were carried out at three different concentrations of $10^{-7}$, $10^{-8}$, and $10^{-9}$ M. It should be noted that switching the media during continuous treatments did not effect the GtH-II release; see experiments involving several changes of media (different doses or change back to normal media) (Figures 2.7-2.9).

Homologous continuous treatments with increasing doses of either sGnRH or cGnRH-II resulted in significant desensitization of GtH-II release as shown in Fig. 2.7. However, heterologous continuous treatments resulted in lower degrees of desensitization compared to homologous treatments (at the same concentrations $10^{-7}$, $10^{-8}$, or $10^{-9}$ M) (Figs. 2.8, 2.9). It should be noted that for heterologous treatments, the degree of desensitization was reduced at lower concentrations of the peptides. For all continuous treatments, a biphasic GtH-II release was observed involving initial rapid increase in GtH-II secretion (phase 1) followed by sustained plateau release above the baseline (phase 2) as described previously (Habibi, 1991a,b). For all continuous treatments, fractions were collected every 2 minutes for the initial 20 minutes of a treatment followed by 10 minute fraction until the next

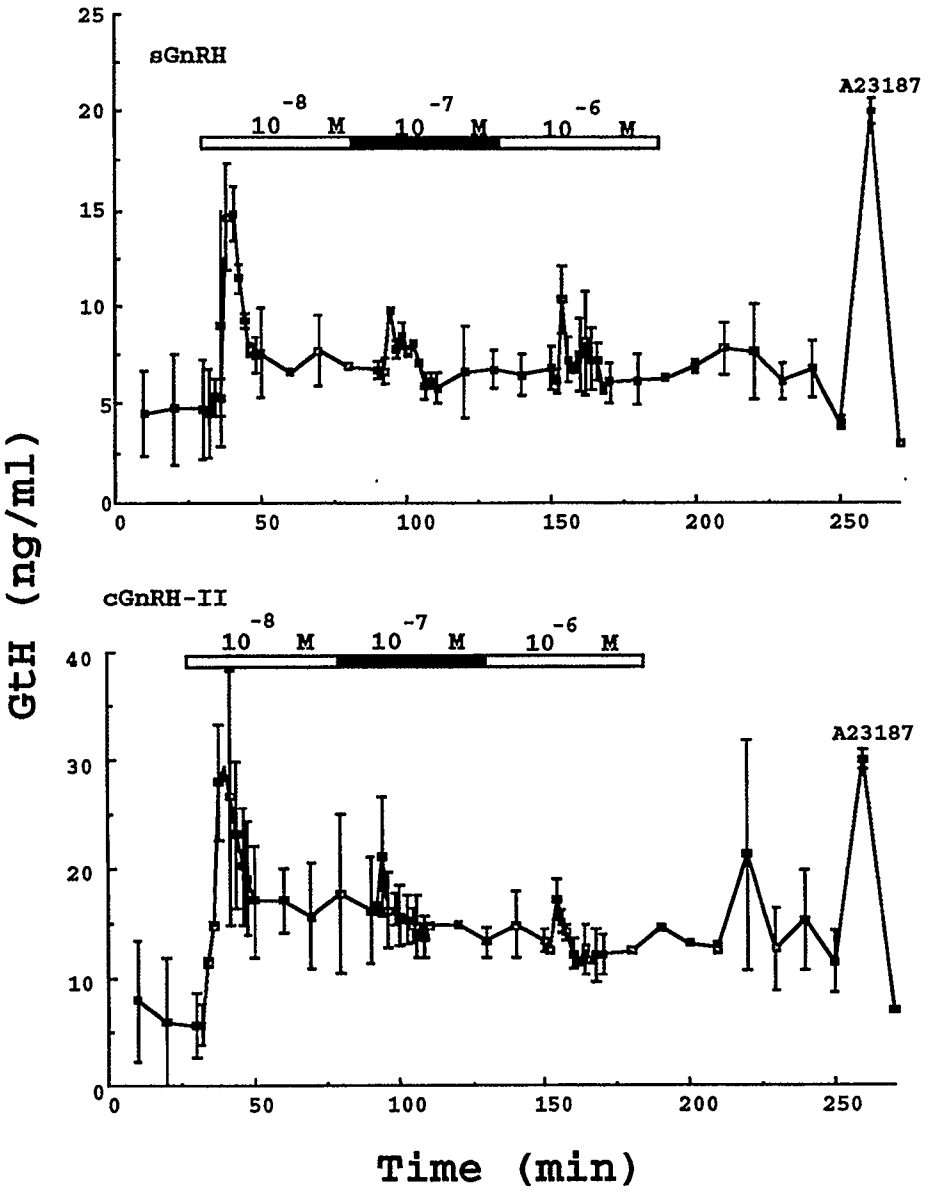



**Figure 2.7** Gonadotropin (GtH-II) release from superfused pituitary fragments following homologous continuous treatment with increasing concentrations of sGnRH or cGnRH-II. The duration of the treatments are indicated by the bars. At the end of the experiment, a 3-minute pulse of 0.1 mM calcium ionophore (A23187) was delivered. Fractions were collected every 2 or 10 minutes as shown, and GtH-II concentration was measured by radioimmunoassay. Each profile represents the mean ± of S.E. 4 observations (4 different columns).

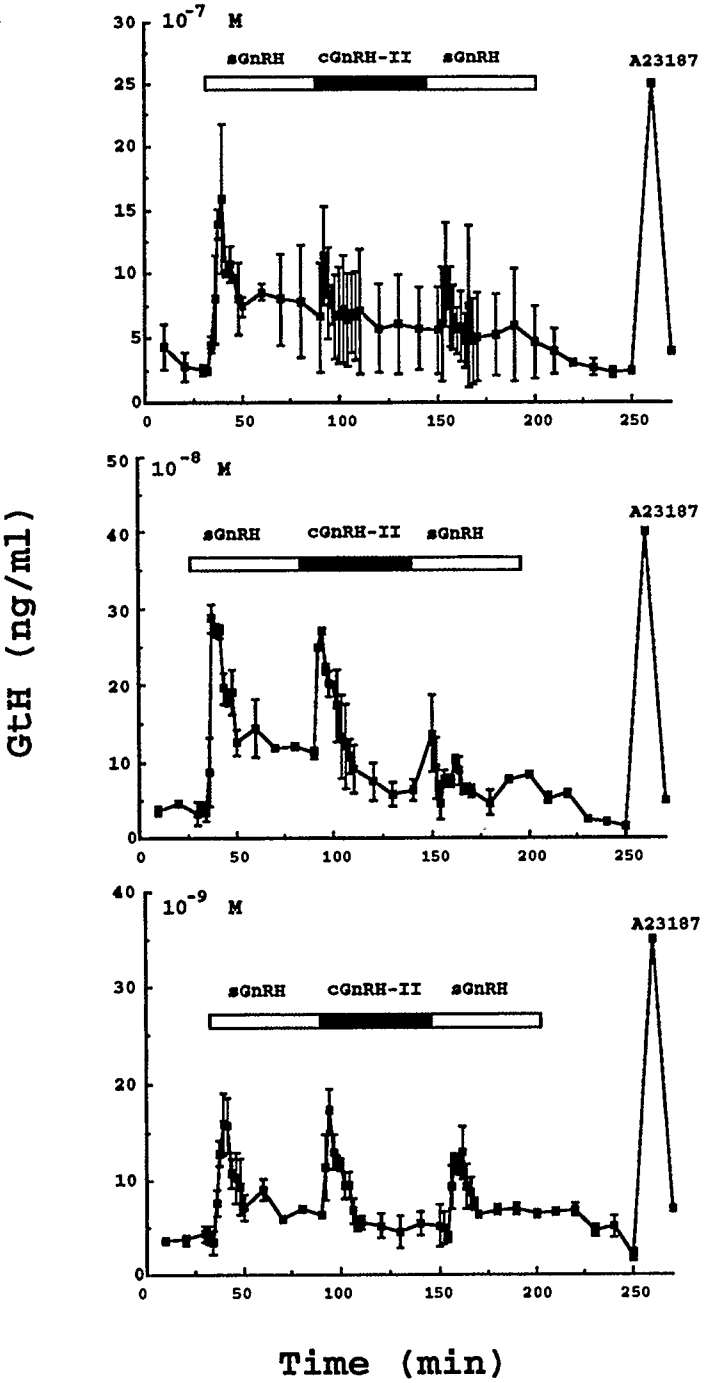



**Figure 2.8** Gonadotropin (GtH-II) release from superfused pituitary fragments following continuous treatment with alternate administration of sGnRH and cGnRH-II (sGnRH/cGnRH-II/sGnRH) at various doses. The duration of the treatments is indicated by the bar. At the end of the experiment, a 3-minute pulse of 0.1 mM calcium ionophore (A23187) was administered. Fractions were collected every 2 or 10 minutes as shown, and the GtH-II concentration was measured by radioimmunoassay. Each value in the profile represents the mean ± of S.E. 4 observations (4 different columns).

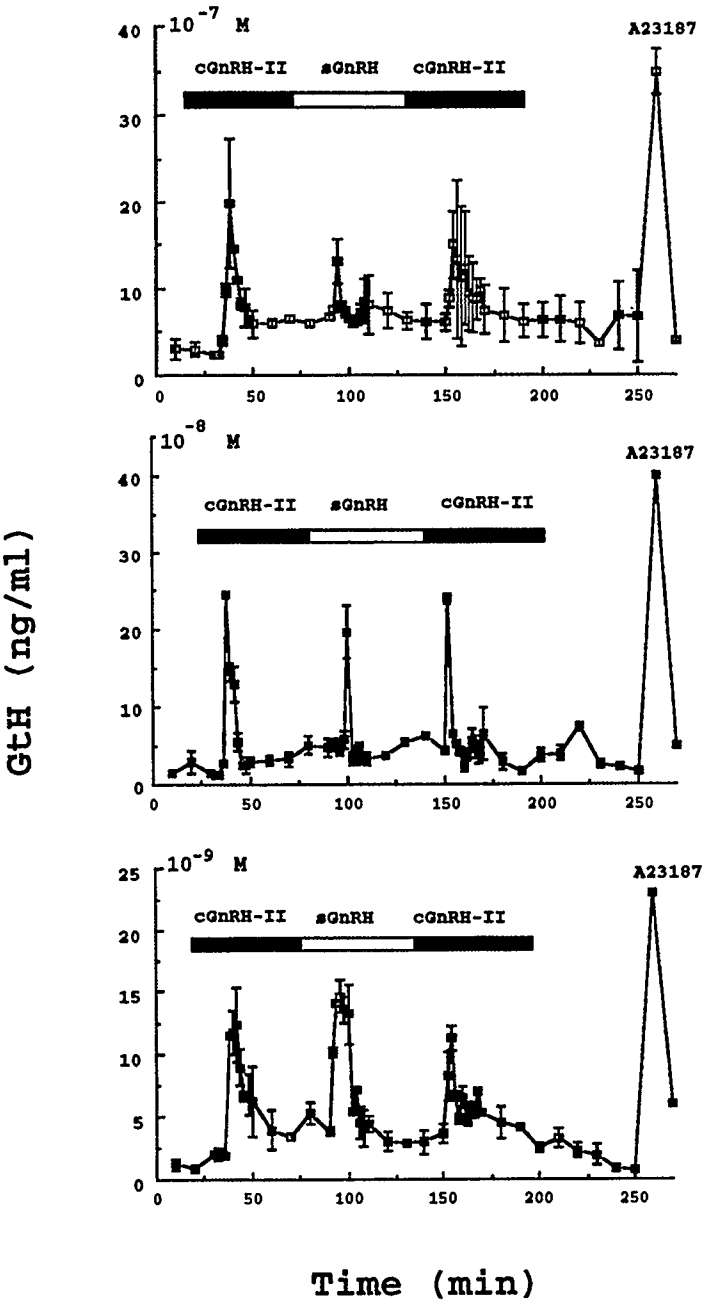



**Figure 2.9** Gonadotropin (GtH-II) release from superfused pituitary fragments following continuous treatment with alternate administration of sGnRH and cGnRH-II (cGnRH-II/sGnRH/cGnRH-II) at various doses. The duration of the treatments is indicated by the bar. At the end of the experiment, a 3-minute pulse of 0.1 mM calcium ionophore (A23187) was administered. Fractions were collected every 2 or 10 minutes as shown, and the GtH-II concentration was measured by radioimmunoassay. Each value in the profile represents the mean ± of S.E. 4 observations (4 different columns).

treatment. In this regard, the results of homologous continuous treatment shown in Fig.2.7 provide a control for the heterologous continuous treatments.

At the end of all pulsatile and continuous experiments, calcium ionophore (A23187) at 0.1 mM, was administered as a three minute pulse to ensure that desensitization of GtH-II release was not due to depletion of GtH stores in the gonadotropes.

### 2.3.3 *Gonadotropin Subunit mRNA Production*

Experiments were carried out to study the accumulation of GtH-II-β and GtH-II-α mRNA levels following *in vivo* treatments with sGnRH and cGnRH-II. Treatment of sexually regressed goldfish with sGnRH (at 1 and 4 μg/fish) significantly ($P<0.05$) increased levels of pituitary GtH-II-β and GtH-II-α mRNA levels, while similar treatment with cGnRH-II was without effect (Fig. 2.10); difference between sGnRH- and cGnRH-II-induced GtH-IIα levels at 4 μg/fish treatment was not statistically significant. This experiment was repeated four times and total RNA for each treatment group in each experiment was obtained from 7-10 regressed goldfish pituitaries. The quantified data as shown in the Fig. 2.10 represents mean ± S.E. of 4 observations (relative

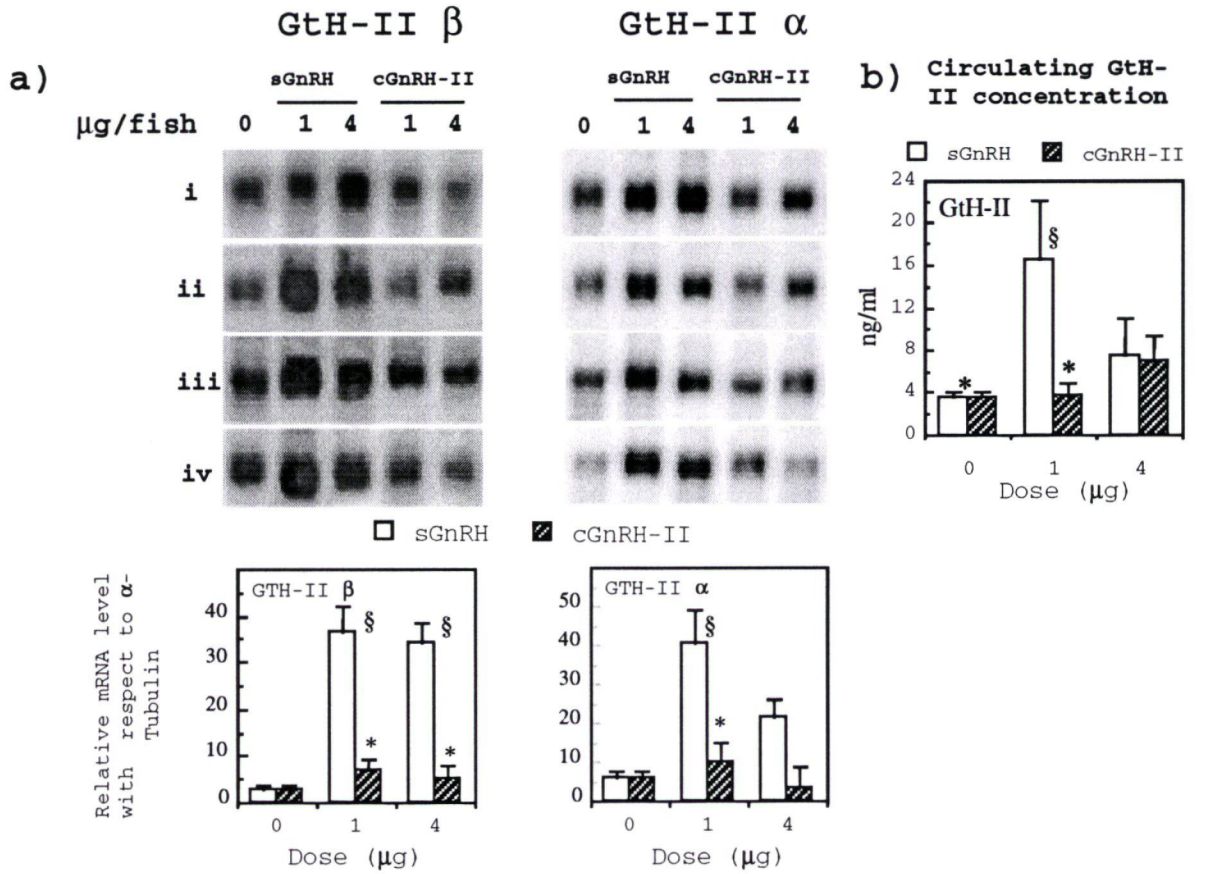



**Figure 2.10** GtH-II α and β mRNA levels following treatments with sGnRH and cGnRH-II at 1 and 4 µg/fish in sexually regressed goldfish. In this experiment, 7-10 animals were used per treatment. a) Total RNA was extracted 12 hours post-injection and 5 µg was loaded per lane for Northern analysis. Data were quantified using a computerized densitometer with respect to α-tubulin. The quantified data represent mean ± S.E. of 4 observations. For each dose group, values displaying § and * are significantly ($P<0.05$) different from one another. b) Circulating levels of GtH-II were determined in the same fish used to estimate mRNA levels. The values in terms of ng/ml represent mean ± of 7-10 animals per experimental group. Values displaying § and * are significantly ($P<0.05$) different from one another.

mRNA levels with respect to α-tubulin). Circulating levels of GtH-II were also measured in two of the above experiments (the same fish used to determine mRNA levels). The results indicate a significantly ($P<0.05$) greater level of circulating GtH-II in regressed goldfish injected with 1 μg of sGnRH than those injected with cGnRH-II (Fig. 2.10). No stimulation of GtH-II release was apparent in the groups injected with 4 μg of sGnRH or cGnRH-II.

Experiments were also carried out to study the effects of sGnRH and cGnRH-II on GtH-II mRNA levels in sexually mature fish (containing gonads at late stages of recrudescence). In these experiments, goldfish (7-10 fish per treatment group) were injected with 4μg/fish of sGnRH, cGnRH-II, or vehicle (control) and sacrificed 6, 12 and 24 hours after treatment. Both sGnRH and cGnRH-II treatments significantly ($P<0.05$) increased GtH II-β and GtH II-α mRNA levels in a time-related manner compared to control (Fig. 2.11). However, in the sexually mature animals, cGnRH-II was significantly ($P<0.05$) more effective than sGnRH in stimulating accumulation of GtH-II α and β mRNA levels after 12 and 24 hours of injection (Fig. 2.11). The experiment was repeated three times in the same manner, and the quantified mRNA level shown in Fig. 2.11 represents mean ± S.E. of 3

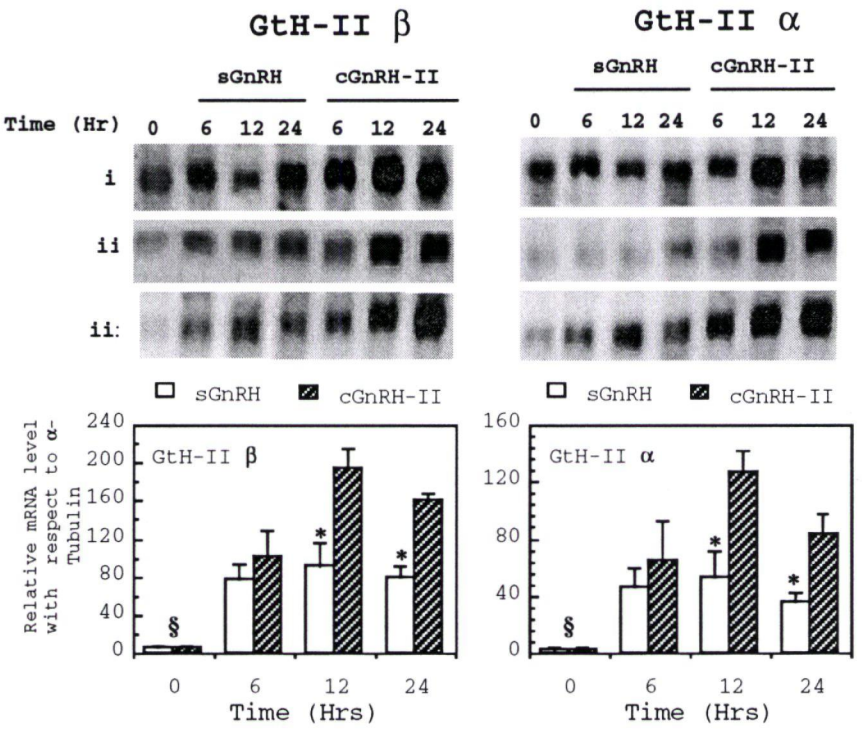



**Figure 2.11** Autoraiogram of GtH-II β and GtH-II α mRNA levels in sexually mature goldfish in response to 4 µg/fish injection (i.p.) of sGnRH or cGnRH-II. Total RNA was extracted 6, 12, and 24 hours post-injection. 5 µg RNA was loaded per lane on a formaldehyde gel electrophoresis for Northern analysis. Data was quantified using computerized densitometer with respect to α-tubulin as shown. The quantified data represents mean ± S.E. of 3 observations. For each time group, sGnRH-treated values displaying (*) are significantly different from cGnRH-II-treated group. Furthermore, the control values displaying (§) are significantly different from all other values shown.

observations (relative mRNA level with respect to α-tubulin). It should be noted that we determined α-tubulin level for all treatment groups as an internal marker for correcting variations due to RNA loading error (results not shown). In all cases, within each experiment α-tubulin levels were not significantly different within treatment groups.

## 2.4 *Discussion*

In this study we used GnRH desensitization as an experimental tool to study possible existence of receptor-effector mechanisms coupled to GtH-II release with specificity for sGnRH or cGnRH-II in the goldfish pituitary. The experimental approach was to study the differences between desensitization induced by sGnRH and cGnRH-II administered in homologous and heterologous fashion. Pulsatile alternate treatments with sGnRH and cGnRH-II (i.e. sGnRH/cGnRH-II or cGnRH-II/sGnRH) at $10^{-8}$ M (administered every 30 minutes), resulted in a significantly lower degree of desensitization compared to homologous treatments with either sGnRH or cGnRH-II (sGnRH/sGnRH or cGnRH-II/cGnRH-II), or when combined together (sGnRH+cGnRH-II). These observations suggest the presence of two receptor-effector mechanisms coupled to the GtH-II release with preferential specificity for sGnRH and cGnRH-II. It should be noted that a difference between homologous and heterologous desensitization could only be obtained when sGnRH and cGnRH-II are present at concentrations close to their respective ED50 values (sGnRH: 14.6±4.3 nM and cGnRH-II: 3.5±1.5) (Habibi, 1991a), and there appear to be a spillover of

specificity at concentration of $10^{-7}$ M or greater. In this context, at $10^{-7}$ M the observed differences between homologous and heterologous desensitizations were reduced significantly. It should also be noted that experiments involving administration of the peptides at 5 x $10^{-9}$ and $10^{-8}$ M (every 60 min) were found to be ineffective, since combination of low concentration and low frequency failed to desensitize pituitary GtH-II release to a sufficient degree to reveal any differences between homologous and heterologous desensitizations. Further support for this postulate is provided by the results of the experiments involving continuous treatments with sGnRH and cGnRH-II. These differences are also apparent to a greater extent at lower concentrations, providing additional support for possible cross-reactivity between sGnRH and cGnRH-II receptor-effector systems when administered at higher concentrations.

The present study also demonstrates that sGnRH and cGnRH-II differentially regulate GtH-II-$\beta$ and GtH-II-$\alpha$ mRNA levels, depending upon the stage of gonadal recrudescence in goldfish. In sexually regressed animals, sGnRH alone was able to increase accumulation of GtH-II-$\beta$ and GtH-II-$\alpha$ mRNA; cGnRH-II receptor-effector system coupled to production of

GtH-II subunit mRNA in regressed goldfish was either silent or absent. In sexually mature animals, however, both sGnRH and cGnRH-II were able to increase the GtH-II-β and GtH-II-α mRNA levels. Furthermore, in the sexually mature goldfish cGnRH-II exerted a significantly greater stimulatory effect than sGnRH on the mRNA levels of GtH-II subunits which is in accord with previous observations on GnRH-induced GtH-II release in the sexually mature goldfish (Habibi, 1991a; Chang et al., 1990; Habibi et al., 1991). The observed increase in GtH-II subunit mRNA levels could either be explained in terms of increased transcription induced by sGnRH or cGnRH-II, or increased stabilizing effect on the mRNA by these peptides.

It appears from the present and former studies that GtH-II subunit synthesis is correlated to some extent with GtH-II release in the goldfish pituitary. In mature goldfish, cGnRH-II has a greater stimulatory effect on GtH-II subunit synthesis than sGnRH which correlates with its greater GtH-II release potency in vitro and greater affinity for high affinity GnRH receptors in the goldfish pituitary (Habibi, 1991a). In sexually regressed goldfish, however, the circulating levels of GtH-II was found to be higher in the sGnRH-injected group (1μg/fish) compared to those injected

with cGnRH-II (1 or 4μg/fish) (present study). No significant increase in GtH-II level was observed following sGnRH treatment at 4 μg, and at present we are uncertain about the reason for this effect. However, it should be noted that GtH-II release in goldfish is negatively controlled by dopamine (Peter et al., 1986), and as a result a study of temporal relationship between in vivo GtH-II release and synthesis is difficult in goldfish in the absence of dopamine antagonists.

The present results are also consistent with recent findings indicating that sGnRH and cGnRH-II stimulate GtH-II release through different post-receptor signalling mechanisms in the goldfish pituitary (Chang et al., 1994; Jobin and Chang, 1992a,b; Chang et al., 1991). Incubation of goldfish pituitary cells in $Ca^{2+}$-deficient media revealed that sGnRH-induced GtH-II release was independent of extracellular $Ca^{2+}$ concentration while cGnRH-II-induced release was not (Jobin and Chang, 1992a,b). Furthermore, blockade of arachidonic acid (AA) metabolism reduced sGnRH-induced GtH-II release, but had no effect on cGnRH-II-induced release (Chang et al., 1991); the lack of AA involvement in cGnRH-II-induced GtH-II release was suggested to account for the greater sensitivity of cGnRH-II treatment

to DA agonists and changes in extracellular $Ca^{2+}$. In addition to these studies, there is evidence that cGnRH-II down regulates GnRH receptors to a greater extent and is more desensitizing than sGnRH (Habibi, 1991a). Moreover, with respect to biphasic GtH-II secretion in goldfish pituitary, the second phase of GtH-II release was found to be more pronounced for the sGnRH- treated group than the cGnRH-II-treated group (Habibi, 1991b). Significant differences were also observed between GtH-II release induced by sGnRH and cGnRH-II in terms of extracellular $Ca^{2+}$ concentration, pulse frequency, and dose response (Habibi, 1991b; Habibi et al., 1991).

In summary, the observations based on 1) lower degree of desensitization following heterologous treatments (sGnRH/cGnRH-II or cGnRH-II/sGnRH) compared to homologous treatments (sGnRH/sGnRH or cGnRH-II/cGnRH-II), 2) differential regulation of GtH-II-$\beta$ and GtH II-$\alpha$ mRNA levels by sGnRH and cGnRH-II in the sexually mature and regressed animals, 3) previously observed differences between sGnRH and cGnRH-II induced desensitization (Habibi, 1991a,b; Habibi et al., 1991), and 4) differences observed between post-receptor mechanisms for sGnRH and cGnRH-II in terms of GtH-II release (Jobin and Chang, 1992a,b; Chang et al.,

1991) collectively provide strong support for the existence of two receptor-effector systems with preferential specificity for sGnRH and cGnRH-II coupled to GtH-II synthesis and release in the goldfish pituitary. However, we are not able at this stage to specify if the observed differences between sGnRH and cGnRH-II is resulted from interaction with different receptor molecules or receptor-effector coupling systems for the two native peptides. It is interesting to note that a recent study in rat luteal cells provides evidence for the existence of GnRH receptor subtypes coupled independently to phospholipase C or $A_2$ (Watanabe et al.,1990). In this regard, most vertebrates possess more than one form of GnRH in the brain. However, it is not known if these GnRH forms work through the same or different receptor-effector systems. Hence these findings provide a framework for future studies in other vertebrate systems and may have a widespread implications.

# Chapter 3

## The Effects of Gonadal Steroids on the Maturational Gonadotropin-II (GtH-II) Subunit Gene Expression in the Goldfish Pituitary.

## 3.1 *Introduction*

Gonadotropins are glycoprotein horones and include the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). In fish, gonadotropins are referred to as the vitellogenic GtH (FSH-like; GtH-I) and maturational GtH (LH-like; GtH-II). All members of this family are heterodimers consisting of two different subunits, $\alpha$ and $\beta$, that are non-covalently associated. Within a given species, the $\alpha$-subunit is identical in all pituitary glycoprotein hormones (LH, FSH, and thyroid-stimulating hormone), while the $\beta$-subunit is unique and confers the biological activity (Pierce and Parsons, 1981).

In vertebrates, gonadotropin-releasing hormone (GnRH) is an important regulator of LH synthesis and release (Papavasiliou et al., 1986; Haisenleder et al., 1990, 1991,; Weiss et al., 1990). In addition, gonadal steroids are known to regulate LH synthesis and secretion from the pituitary. However, the effects of steroid on LH synthesis were found to be different depending on the experimental condtions. This may be attributed to difficulty in dissociating direct effects of steroids on the pituitary from those mediated through other factors. A number of investigators have demonstrated supressive effects of steroids through

castration studies. Removal of gonads was shown to result in increased serum LH and FSH concentrations as well as LH and FSH subunit mRNA levels (Papavasiliou et al., 1986; Corbani et al.,1984; Abbot et al., 1985; Gharib et al., 1986, 1990). There is also a report on the stimulatory effect of E-2 on LH-β mRNA production in isolated pituitaries of ovariectomized rats with no effect on the FSH-β and the α-subunit mRNA levels (Shupnik et al., 1989b). In anestrous ewes, *in vivo* studies demonstrated differential regulation of the LH and FSH subunits in response to E-2 treatment. It was observed that α-subunit mRNA levels increased, while the FSH-β mRNA levels decreased and LH-β mRNA levels only slightly increased (Landefeld et al., 1989). Treatment of orchidectomized rats with T increased FSH content of pituitary, while decreasing the LH content and the LH-β and α-subunit mRNA levels. The FSH-β mRNA levels were unchanged (Kitahara et al., 1991; Wierman et al., 1988). From the above studies, it appears that steroids differentially regulate the gonadotropin secretion and synthesis in mammals.

In teleosts, gonadectomy increases circulating GtH-II levels in rainbow trout (Bommelaer et al., 1981), African catfish (Habibi et al., 1989), and goldfish (Kobayashi and

Stacey, 1990). The observed increase in GtH-II levels were suppressed by treatment with E-2 and/or T. In sexually immature teleosts, however, sex steroids appear to predominately exert a positive feedback effect. In juvenile rainbow trout, T treatment resulted in increased pituitary GtH-II content (Crim and Evans, 1979). In addition, prolonged T treatment of immature trout also resulted in initiation of gonadal development (Crim and Evans, 1983). In European silver eels, E-2 was found to increase pituitary GtH-II (Dufour et al., 1983) and brain GnRH (Dufour et al., 1985). In Japanese silver eels, both E-2 and T stimulated pituitary GtH-II content and serum GtH-II levels (Lin et al., 1990). In common carp and Chinese loach, T treatment increased responsiveness to LHRH-A (Trudeau et al., 1991). More recently, in goldfish, it has been demonstrated that *in vivo* treatment with E-2 and T increases responsiveness to GnRH-induced GtH-II release *in vitro*. In addition, T has been shown to have a direct effect at the level of pituitary to increase GnRH responsiveness (Trudeau et al., 1993b). However, our information on the steroidal regulation of GtH subunit gene expression in teleosts and lower vertebrates, in general, is rather limited. To this end, both T and E-2 have been shown to stimulate GtH-II $\beta$ gene expression in

pituitary cells from juvenile rainbow trout (Trinh et al., 1986). T and E-2 have also exerted strong stimulatory effect on the GtH-II β mRNA levels in the European eel at the silver stage (Querat et al,. 1991).

The present study investigates the effects of E-2 and T on GtH-II subunit gene expression in the goldfish pituitary. In this study, *in vitro* and *in vivo* effects of androgens and estrogens were investigated in terms of GtH-II subunit mRNA production. In addition, the effect of a non-aromatizable androgen, 11-β hydroxyandrosterone, was tested to determine if aromatization of androgens to estrogens is a factor for the observed effects.

## 3.2 *Materials and Methods*

### 3.2.1 *Animals*

Goldfish, *Carassius auratus*, of mixed sex (ranging from 8-10 cm in length), were purchased from aquatic imports (Calgary, Alberta). They were maintained in a semi-recirculating aquarium (1500 L) at 17 C on a 16-h light and 8-h dark photoperiod. They were fed a commercial fish diet.

### 3.2.2 *Hormones and chemicals*

Steroids (17β-estradiol, testosterone, and 11β-hydroxyandrosterone) were obtained from Sigma (St. Louis, MO). The carp GtH-II-β cDNA fragment (0.7-0.8 kB in length) and GtH-II-α cDNA fragment (0.8-0.9 kB in length) were provided by Dr. F. L. Huang (Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan). The trout α-tubulin cDNA fragment (1.5 kB) was provided by Dr. G. Dixon (Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada).

### 3.2.3 *In vivo steroid treament*

Goldfish, (mixed sex; ~30 g each) most of them inlate gonadal recrudescent stage, were treated with various doses

of 17β-estradiol (E-2), testosterone (T), or 11β-hydroxyandrosterone (11β-HA) through intraperitoneal (ip) injections (6-8 goldfish per group). At various time intervals after treatment, fish were anesthesized and sacrificed. The pituitaries were removed and total RNA was extracted.

### 3.2.4 *In vitro steroid treatment*

Pituitary GtH-II subunit gene expression was determined in response to treatment with (E-2), (T), and (11β-HA) using a superfusion system as described previously (Khakoo et al., 1994). Briefly, pituitary fragments (8 pituitaries per column) were equilibriated in M199 for 2 hours before treatment. The fragments were then treated continuously for 15 hours (as indicated) with steroids. The pituitary fragments were removed from the columns after treatment and total RNA was extracted.

### 3.2.5 *Determination of GtH-II subunit mRNA*

RNA extraction and Northern analysis were performed as described previously by Khakoo et al. (1994). Briefly, total RNA was extracted from pituitaries using guanidine thiocyanate-phenol-chloroform extraction method (Chomczyski

and Sacchi, 1987). Sample purity was determined from ratio of sample absorbance at 260:280 nm. The ratio ranged between 1.7-2.0. RNA was separated and resolved on a 1.2% agarose/formaldehyde gel and transferred onto a Hybond-$N^+$ membrane (Amersham, Arlington Heights, IL) in 20X SSPE transfer buffer using capillary transfer method. Purified cDNA fragments were labeled using random primer method with [$\alpha$-$^{32}$P] deoxycytidine 5'-triphosphate (dCTP) (~3000 Ci/mmol, Amersham, Arlington Heights, IL). The membranes were prehybridized and hybridized for 2 hours in 10 ml of Rapid Hybridization Buffer (Amersham, Arlington Heights, IL). The membranes were subsequently washed in a series of high stringency washes up to 0.1X SSPE in the presence of 0.1% sodium dodecyl sulfate (SDS). The membranes were initially hybridized with cGtH-II-$\beta$, stripped, and subsequently hybridized with cGtH-II-$\alpha$ and $\alpha$-tubulin. The $\alpha$-tubulin was used as an internal control for expression. In addition, the gel was stained with ethidium bromide to verify equal loading of total RNA in all lanes.

The autoradiograms were scanned using a computerized densitometer scanner, gelscan, and quantified using the Image program provided by NIH (Bethesda, MD). The program computes the area and average density of given selection.

The values were transferred to a spreadsheet and statistical analysis program for further analysis of the data. The quantified GtH-II-β and -α mRNA levels were expressed with respect to α-tubulin levels of the corresponding lanes. The quantified values were analyzed statistically by one-way ANOVA followed by multiple comparison of the means using the Tukey's test with $P<0.05$ degree of significance.

## 3.3 *Results*

### 3.3.1 *Time-related effects of gonadal steroids in vivo*

Time-course studies involved i.p. treatment with E-2, T, or 11β-HA (20 μg/fish), followed by RNA extraction after 12, 24, 48, 72, and 96 hours of treatment.

Treatment with T resulted in a biphasic change in GtH-II subunit mRNA levels (Fig. 3.1). The GtH-II α and GtH-II β mRNA levels were reduced initially during shorter time intervals (12-48 hours), followed by increased mRNA levels after 72 and 96 hours of treatment. A similar experiment was carried out to study the effect of a non-aromatizable androgen, 11β-HA, on GtH-II subunit gene expression *in vivo* (Fig. 3.2). Treatment with 11β-HA also resulted in an initial (12-24 hours) reduction in GtH-II α nd β mRNA levels, followed by significant increase in GtH-II subunit mRNA levels after 48-96 hours.

Treatment with E-2 resulted in a time-related increase in GtH-II α and GtH-II β mRNA levels, with the maximum effect after 72 hours of treatment (Fig. 3.3). At 96 hours following treatment, GtH-II α and GtH-II β mRNA levels decreased to the control levels (Fig. 3.3).

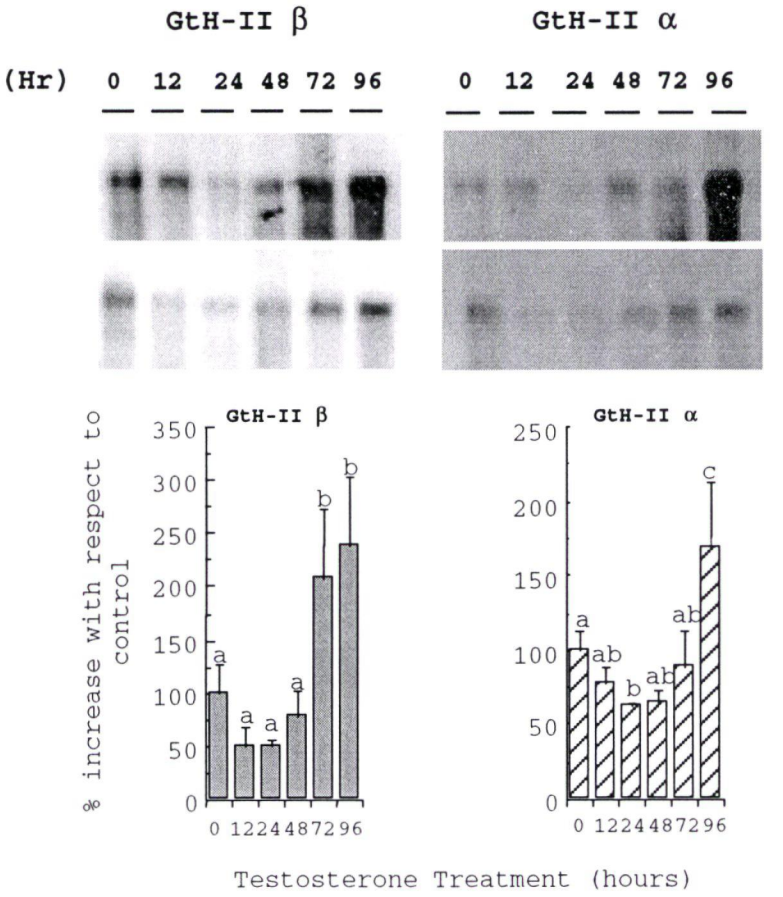



**Figure 3.1** GtH-II α and β mRNA levels following testosterone time-course treatment (20 µg/fish) *in vivo*. Most of the goldfish were in late gonadal recrudescence. Total RNA was extracted 12, 24, 48, 72, and 96 hours after treatment and 5 µg RNA was loaded per lane for Northern analysis. Data was quantified using computerized densitometer. The values were corrected relative to α-tubulin and are expressed as percent increase with respect to control. Bars with dissimilar letters are signficantly different ($P<0.05$) from eachother. Quantified data represents mean ± S.E. of 2 observations. Each experimental group represented 5-8 animals.

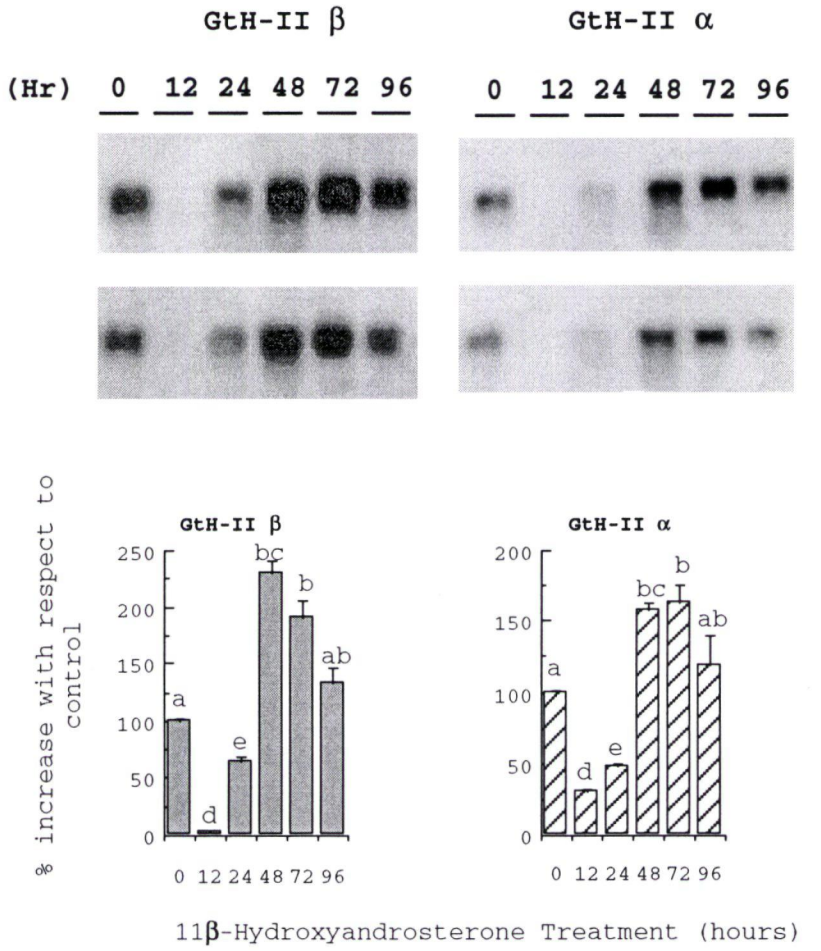



**Figure 3.2** GtH-II α and β mRNA levels following 11β-hydroxyandrosterone time-course treatment (20 μg/fish) *in vivo*. Most of the goldfish were in late gonadal recrudescence. Total RNA was extracted 12, 24, 48, 72, and 96 hours after treatment and 5 μg RNA was loaded per lane for Northern analysis. Data was quantified using computerized densitometer. The values were corrected relative to α-tubulin and are expressed as percent increase with respect to control. Quantified data represents mean ± S.E. of 2 observations. Bars with dissimilar letters are significantly different ($P<0.05$). Each experimental group represented 5-8 animals.

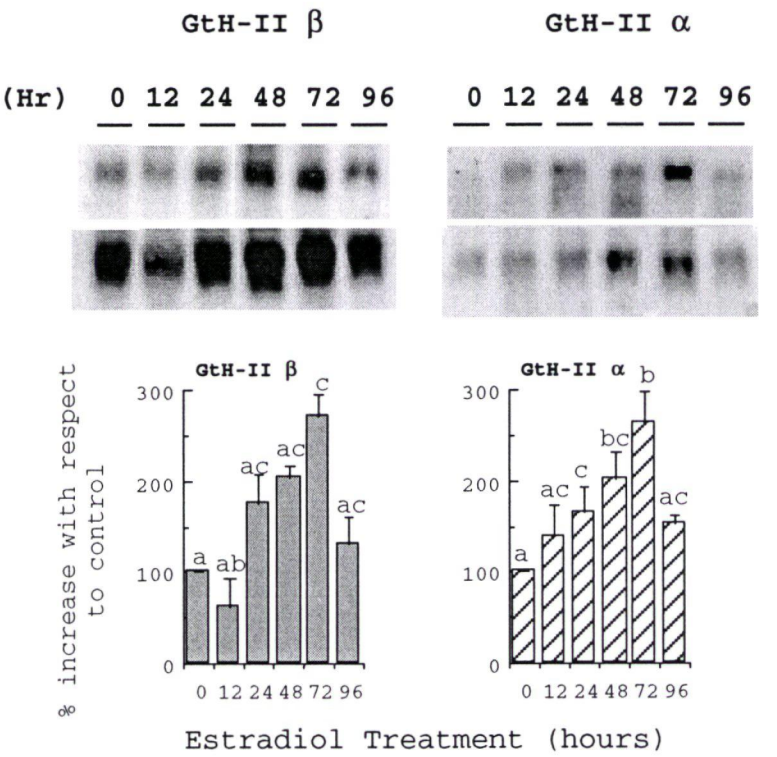



**Figure 3.3** GtH-II α and β mRNA levels following estradiol time-course treatment (20 μg/fish) *in vivo*. Most of the goldfish were in late gonadal recrudescence. Total RNA was extracted 12, 24, 48, 72, and 96 hours after treatment and 5 μg RNA was loaded per lane for Northern analysis. Data was quantified using computerized densitometer. The values were corrected relative to α-tubulin and are expressed as percent increase with respect to control. Bars with dissimilar letters are significantly different from eachother. Quantified data represents mean ± S.E. of 2 observations. Each experimental group represented 5-8 animals.

### 3.3.2 *Dose-related effects of gonadal steroids in vivo*

Goldfish were injected i.p. for 24 hours with T, 11β-HA, or E-2 at three separate doses of 0.2, 2, and 20 μg/fish. Treatment with T resulted in a biphasic change in GtH-II subunit mRNA levels (Fig. 3.4). At lower doses (0.2 and 2 μg/fish), T injection significantly stimulated GtH-II α and GtH-II β subunit mRNA levels after 24 hours. At the highest dose (20 μg/fish), however, T treatment was without effect on GtH-II α and GtH-II β mRNA levels. Similarly, treatment with the the lowest concentration (0.2 μg/fish), of 11β-HA significantly increased GtH-II-β and GtH-II-α subunit mRNA levels. However, at the higher doses (2 and 20 μg/fish) the GtH-II α and GtH-II β subunit mRNA levels decreased below the control levels (Fig. 3.5).

Treatment with E-2 for 24 hours resulted in a dose-related increase in GtH-II α and GtH-II β mRNA levels, reaching maximum levels at 2 μg/fish (Fig. 3.6). At 20 μg/fish, E-2 injection was found to be less stimulatory compared to lower doses, but did not cause inibition observed following androgen treatment.

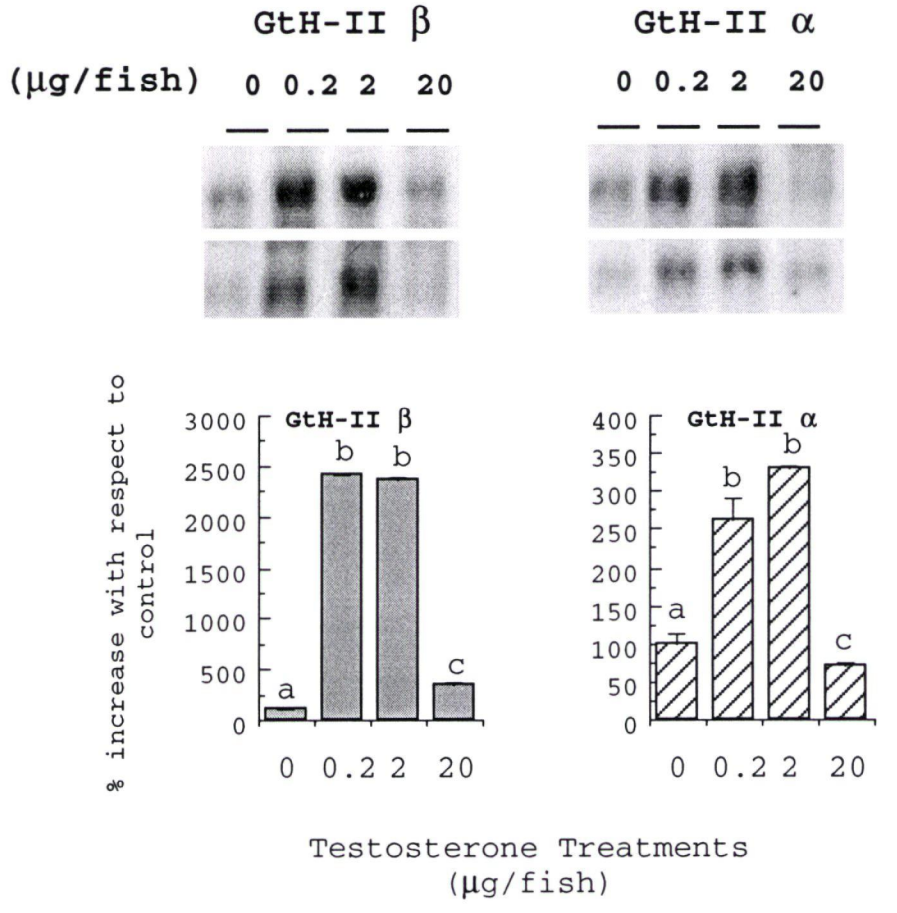



**Figure 3.4** GtH-II a and b mRNA levels in response to various doses of testosterone (T). Goldfish were treated (i.p.) with 0.2, 2, and 20 μg/fish of T for 15 hours. Most of the goldfish were in the late gonadal recrudescence. Total RNA was extracted and 5 μg was loaded per lane for Northern analysis. Data was quantified using a computerized densitometer. The values are expressed with respect to a-tubulin and are percent increase relative to control. Bars with dissimilar letters are significantly different ($P<0.05$) from eachother. Quantified data represents mean ± S.E. of two observations. Each experimental group had 5-8 animals.

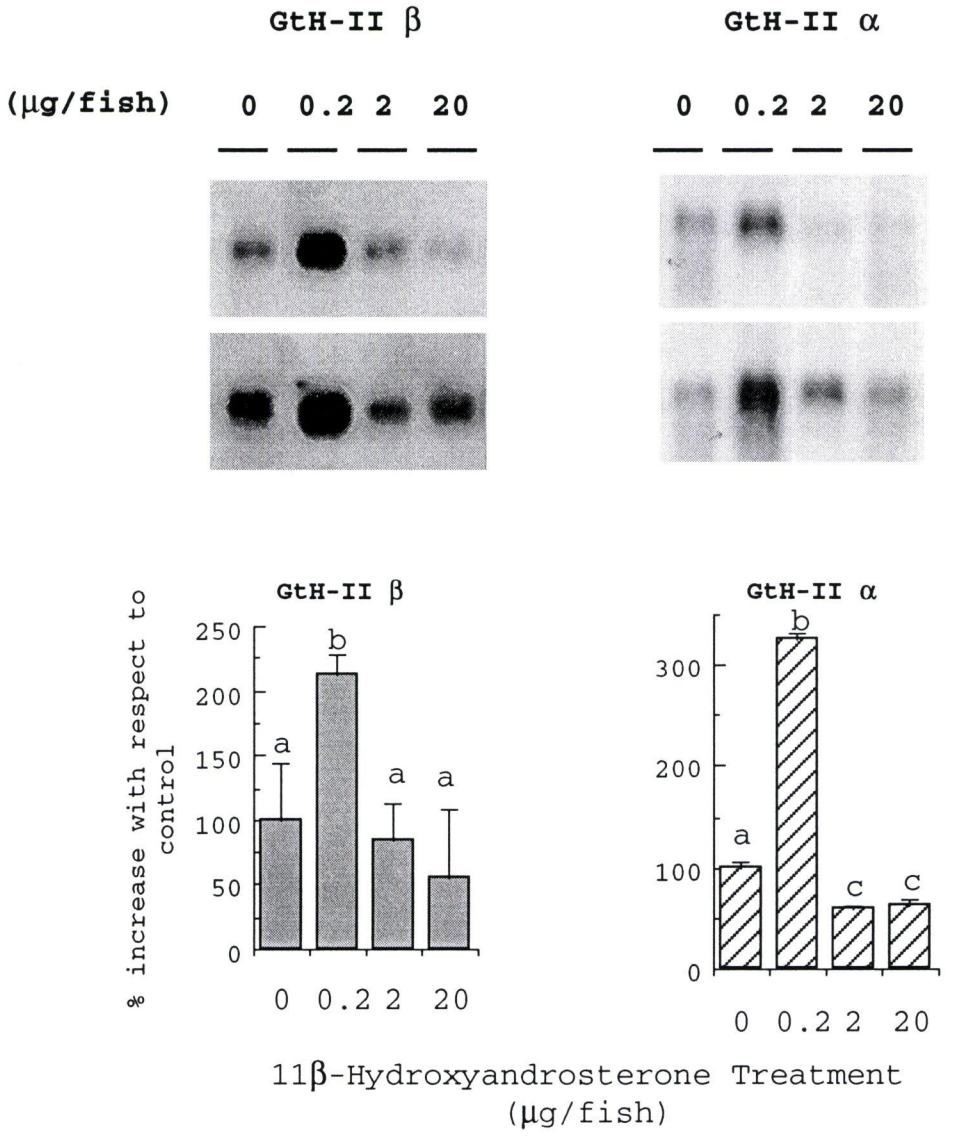



**Figure 3.5** GtH-II α and β mRNA levels in response to dose-dependent studies with 11β-hydroxyandrosterone. Goldfish were treated (i.p.) with 0.2, 2, and 20 μg/fish of 11β-HA for 15 hours. Most of the goldfish were in late gonadal recrudescence. Total RNA was extracted and 5 μg was loaded per lane for Northern analysis. Data was quantified using a computerized densitometer. The values are expressed with respect to α-tubulin and are percent increase relative to control. Bars with dissimilar letters are significantly different ($P<0.05$) from eachother. Quantified data represents mean ± S.E. of two observations. Each experimental group had 5-8 animals.

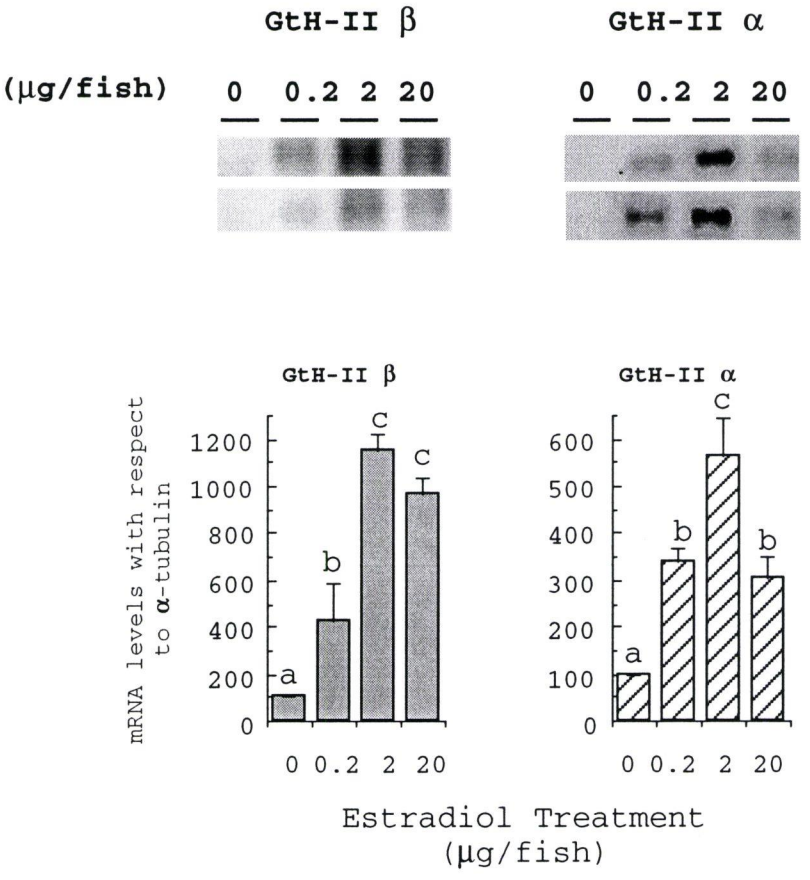



**Figure 3.6** Autoradiogram of GtH-II α and β mRNA levels in response to various doses of estradiol. Goldfish were treated (i.p.) with 0.2, 2, and 20 μg/fish of E-2 for 15 hours. Most of the goldfish were late gonadal recrudescence. Total RNA was extracted and 5 μg was loaded per lane for Northern analysis. Data was quantified using a computerized densitometer. The values are expressed with respect to α-tubulin and are percent increase relative to control. Bars with dissimilar letters are significantly different ($P<0.05$) from eachother. Quantified data represents mean ± S.E. of two observations. Each experimental group had 5-8 animals.

### 3.3.3. *The effects of gonadal steroids in vitro*

In order to study the effect of steroids at the level of pituitary, pituitary fragments were treated with 20 ng/ml of E-2 or T for 15 hours (Fig. 3.7). Treatments with either T or E-2 increased GtH-II $\alpha$ and GtH-II $\beta$ mRNA levels above the control levels. Overall, T was found to be more potent than E-2 in stimulating GtH-II subunit synthesis at this dose level.

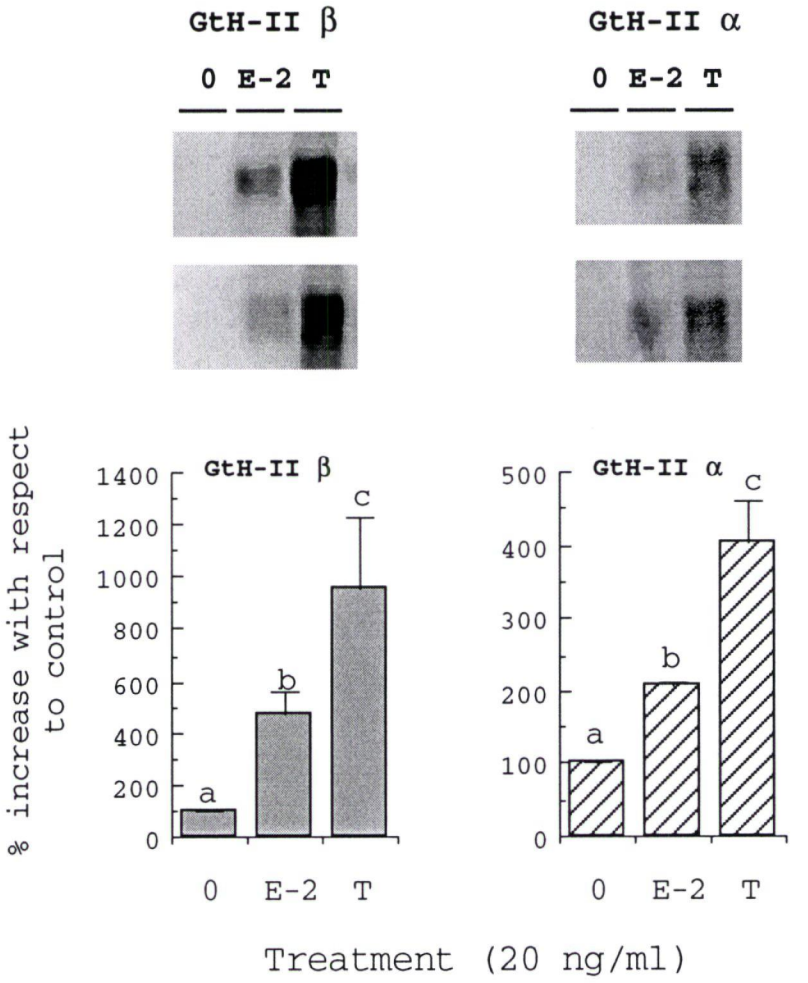



**Figure 3.7** GtH-II subunit mRNA levels in response to *in vitro* steroid treatment. Pituitary fragments were treated with 20 ng/ml of T or E-2 for 15 hours. Total RNA was extracted and 5 μg was loaded per lane for Northern analysis. Data was quantified using computerized densitometer. The values are expressed with respect to α-tubulin and are percent increase relative to control. Bars with dissimilar letters are significantly different ($P<0.05$) from eachother. Quantified data represents mean ± S.E. of 2 observations. Each experimental group represented 8-10 animals. Values dissimilar letters are significantly ($P<0.05$) different from eachother.

## 3.4 *Discussion*

The present findings demonstrate that androgens exert a biphasic effect on the GtH-II subunit gene expression in the goldfish pituitary depending on the concentration. Time-course experiments with T and 11β-HA demonstrated an initial decrease in GtH-II subunit mRNA production followed by increases at longer time intervals (72 and 96 h). Dose-response studies indicate that the observed biphasic response is the result of differences in concentration of the steroids. At the earlier time intervals, the circulating concentration of the steroids was high (20 μg/fish), but at longer time intervals the hormone concentrations were reduced due to physiological degradation and clearance. The dose-response studies clearly supported this hypothesis. During the period that inhibitory effect was observed in the time-course studies, lower doses of both T and 11β-HA were highly stimulatory, whereas higher doses inhibited GtH-II subunit gene expression.

The effect of E-2 was found to be different from T and 11β-HA. Treatment with E-2 resulted in a dose-related increase in GtH-II mRNA levels, with only a slight reduction in expression at the highest dose. This indicates that the

observed inhibition at high doses is predominately androgenic. This postulate is supported by the results obtained following treatment with a non-aromatizable androgen, 11β-HA, which is not converted to estrogens.

The observed biphasic regulation by androgenic steroids may have physiological significance. Goldfish is a seasonal spawner and undergoes annual reproductive cycles in response to environmental cues (Lam and Monro, 1987). In teleosts, the concentration of gonadal steroids fluctuate in close correlation with the circulating concentrations of GtH throughout the year with higher levels during the reproductive seasons (Kobayashi et al.,1986; Schoonen, 1987; Breton et al., 1983). Previous studies in goldfish have demonstrated that circulating concentration of T reaches approximately 20 ng/ml shortly before the ovulatory surge of GtH-II (Kobayashi et al., 1987). These findings indicate that T may be an important factor in stimulating circulating GtH-II surge in goldfish. Concentration of E-2 also increases progressively during the period of vitellogenesis presumably in response to higher levels of GtH (Kobayashi et al., 1987). The rising levels of E-2 which remain below 20 ng/ml may help to sustain basal synthesis of GtH subunits in the goldfish pituitary. The effects of E-2 and T are likely

to result in the ovulatory surge of GtH-II release which is required for stimulation of gametogenesis and steroidogenesis. The increased steroid levels, however, could act negatively to bring GtH-II levels back to basal levels through inhibition of GtH-II subunit mRNA synthesis. This pattern of GtH and steroid change occurs in a number of teleosts. In sexually immature and regressing salmonids, the pituitary GtH content is low and reaches its peak during spawning (Billard et al., 1978). In sexually mature salmonids, the sex steroids levels are high and a negative feedback effect on the hypothalamo-hypophysial axis was shown in response to T treatment (Billard, 1978). Pituitaries from sexually regressed and recrudescent goldfish demonstrated increased sGnRH-induced and cGnRH-II-induced GtH release following treatment with T and E-2 (Trudeau et al., 1993b). However, pituitaries from post-spawning goldfish treated with T demonstrated positive effect for cGnRH-II-induced but not sGnRH-induced GtH release. E-2 had no effect on GnRH responsiveness in these fish (Trudeau et al.,1993b).

Studies were carried out to investigate direct action of E-2 and T at the level of pituitary. Treatment with both E-2 and T, *in vitro*, resulted in increased GtH-II subunit mRNA

levels. A number of other studies also investigated the role of steroids *in vitro* at the level of pituitary and demonstrated stimulatory effects in terms of GtH release and synthesis. For example, treatment of pituitary cells from female rats with E-2 was found to stimulate basal FSH and LH secretion (Lagace et al., 1981; Miller and Wu, 1981). Treatment of pituitary cells from intact male and female rats with T was also found to increase FSH β mRNA levels (Gharib et al., 1990). A number of studies, in rats have demonstrated that *in vivo* treatments with steroids result in both stimulatory and inhibitory effect in terms of LH β subunit synthesis, whereas *in vitro* treatment by E-2 is always stimulatory (Shupnik et al., 1989; Shupnik and Rosenzweig, 1991). Studies in juvenile trout demonstrated an increase in GtH-II β mRNA levels in response to steroid treatment *in vivo* (Trinh et al., 1986) and *in vitro* (Xiong et al., 1994). Pituitary cells from sexually mature (pre-spawning) trout which actively synthesize GtH-II β mRNA, responded positively to steroid treatments. However steroid treatment of pituitary cells from the spawning fish was without effect on GtH-II β mRNA levels (Xiong et al., 1994). In goldfish, direct *in vitro* treatment with T was shown to increase responsiveness to sGnRH (Trudeau et al., 1993). In

the present study, the effects of T and E-2 were studied, *in vitro*, at a single dose, time point and stage of sexual maturation (late gonadal recrudescence). The results demonstrate a clear stimulation of both $\alpha$ and $\beta$ GtH-II subunit gene expression by T and E-2. However, we cannot rule out a negative effect of steroids at the level of pituitary in goldfish at other doses or time points. It would be desirable in future studies, to study the dose-related, time-related, and seasonal effects of steroids on GtH-II subunit gene expression to obtain a more clear picture of the role of gonadal steroids in regulation of reproduction in goldfish.

From the present findings it appears that gonadal steroids, administered *in vitro* at physiological concentrations, exert a predominately positive effect on GtH-II synthesis at the level of pituitary. This may be through modulation of GnRH receptor number, post-receptor mechanisms, or a direct effect at the gene level. Recently, Sealfon et al., (1990) and Wu et al., (1994) demonstrated that E-2 increased GnRH receptor mRNA levels in ovine pituitary cells. In addition, estrogen regulatory elements have been mapped in the rat LH $\beta$ gene (Shupnik et al., 1989) and salmon GtH-II $\beta$ gene (Xiong et al., 1993, 1994). These

elements have been demonstrated as the site of action for stimulatory steroid effect (Shupnik and Rosenzweig , 1991; Xiong et al., 1994). With respect to modulation at the post-receptor level, Liu and Jackson (1988,1990) have demonstrated E-2 enhances LH release from rat pituitary cells via interaction with $Ca^{2+}$ and phospholipase C mediated secretory mechanisms but not arachadonic acid pathway. Estradiol has also been shown to modulate protein kinase C activity (Audy et al., 1990).

The *in vivo* effects of steroids observed in this study may be occurring at multiple sites such as influencing GnRH secretion and pulsatility. It has been demonstrated that during the breeding season in ewes, E-2 inhibits the LH pulse amplitude by decreasing the GnRH pulse amplitude and pituitary response to GnRH. It however does not exert any inhibition on the LH and GnRH pulse frequency (Goodman and Karsh, 1980; Evans et al.,1992). Steroids may also exert their effect indirectly through modulation of neuronal factors, such as GABA and dopamine (DA), known to effect GtH synthesis and release. GABA stimulates GtH-II release in goldfish during early stages of gonadal recrudescence but not from sexually regressed or mature fish (Kah et al., 1992). It appears that during early stages of gonadal

recrudescence, goldfish respond to GABA and this response is potentiated by T (Trudeau et al., 1993). However, as the gonads develop, E-2 levels rise and this seems to reduce GABA responsiveness (Kah et al., 1992; Trudeau et al., 1993). In accordance with this, sex steroids have been demonstrated to modulate GABA receptor number in neural tissues (Lasaga et al., 1988; Schummacher et al., 1989). Trudeau et al. (1993) also demonstrated that sex steroids exert a negative feedback effect by increasing the DA turnover in the dopaminergic nerve terminals in the goldfish brain. Interestingly, a recent study in ewes demonstrated seasonal shifts in DA neurons activity in response to E-2 modulation which may be in response to steroidal regulation of the hypothalamo-hypophysial responsiveness (Havern et al.,1994).

With respect to *in vivo* GnRH receptor regulation, Habibi et al.(1989) demonstrated seasonal variation of GnRH binding capacity in goldfish. The number of binding sites significantly increased during the time of maximal gonadal recrudescence. However, in a recent study it was demonstrated that the stimulatory effect of T in goldfish GtH-II release is not mediated through changes in GnRH receptor number (Trudeau at al., 1993). Therefore, GtH-II

subunit mRNA production may be a very important factor in steroidogenic regulation of goldfish release and synthesis. Hence, it appears that the observed steroid effect may be occuring at multiple sites ranging from a direct effect at the level of pituitary to indirect regulation through neuronal factors within the brain.

In summary, the present findings demonstrate that gonadal steroids, in particular androgens, exert dose-dependent biphasic regulation of GtH-II subunit mRNA levels in the goldfish pituitary. At physiological concentrations, direct effect of these steroids at the level of pituitary is stimulatory in nature.

# Chapter 4.

## Construction of cDNA Librabries from Goldfish Pitutary, Brain, and Ovary.

## 4.1 *Introduction*

Gonadotropin-releasing hormone receptor (GnRH-R) plays a pivotal role in linking the neural and endocrine systems. GnRH stimulates the release and synthesis of gonadotropins through high affinity membrane receptors on the gonadotrophs. Until recently, most known characteristics of GnRH receptors came from GnRH binding studies, photoaffinity labeling and SDS-PAGE gel electrophoresis (for review see Habibi and Peter., 1991). Characterization of GnRH receptors in the goldfish pituitary has demonstrated the presence of two classes of binding sites, a high affinity/low capacity site and a low affinity/high capacity site (Habibi et al., 1987). Photoaffinity labeling and SDS-PAGE electrophoresis showed presence of two major bands with molecular weight of 71 and 51 kDa. Ligand displacement studies demonstrated 51 kDa band to be consistent with the high affinity binding sites involved in the GTH release (Habibi et al., 1989; 1990). In addition to pituitary, specific GnRH binding sites have also been demonstrated in the brain and ovary of goldfish (Habibi and Pati, 1993).

While a lot of progress has been made in the biochemistry and physiology of GnRH-R regulation and desensitization, study of GnRH-R gene has only recently become possible,

since it was cloned in mouse (Tsutsumi et al., 1992; Reinhart et al., 1992), rat (Reinhart et al., 1992; Perrin et al., 1993), and human pituitary (Kakar et al., 1992; Chi et al, 1993). It should now be possible to use information on mammalian GnRH-R sequence to clone GnRH receptor in the goldfish pituitary. Hence, the objective of the present study is to construct a cDNA library from the goldfish pituitary which will enable the cloning and characterization of GnRH receptor. Furthermore, cDNA libraries from the goldfish ovary and brain will also be constructed to allow cloning and characterization of receptors for GnRH in these tissues. In addition to GnRH-R, other neurohormones such as GnRH and GTH could also be cloned and characterized using the same cDNA libraries.

## 4.2 *Materials and Methods*

### 4.2.1 *Overview*

cDNA libraries represent genetic information present in the mRNA of a particular tissue. RNA is very susceptible to degradation and difficult to amplify, hence the information encoded by RNA needs to be transferred to a stable double-stranded DNA molecule. This DNA, which is complementary to RNA (cDNA), is then inserted in a self-replicating lamda vector. Once a cDNA library is constructed in this way, individual segments of genetic information can be isolated and examined. The two major steps in construction of a cDNA library are, first the isolation of highly purified sample of RNA from the tissue of interest followed by the actual construction of the library. Commercially available kits were used to carry out these steps. A brief outline of the procedures is provided below.

### 4.2.2 *Animals*

Goldfish, *Carassius auratus,* of mixed sex were purchased from Aquatic Imports (Calgary, Alberta). They were maintained in a semi-recirculating aquarium (1500 L) at 17 C on a 16-h light and 8-h dark photoperiod. They were fed a

commercial fish diet.

### 4.2.3 *mRNA Purification from pituitary, ovary, and brain*

QuickPrep *Micro* mRNA Purification kit from Pharmacia was used for mRNA isolation. Briefly, about 100 mg of tissue was homogenized in the extraction buffer provided. This buffer contained guanidinium thiocyanate (GTC) which protected RNA from degradation by inactivating endogenous RNases. The extract was then diluted three times with dilution buffer. This caused a number of proteins to precipitate thereby yeilding the intial purification. Following centrifugation, the supernatant was transferred to a tube containing Oligo(dT)-Cellulose. The mixture was allowed to stand for 10 min in order to bind the poly(A)$^+$ RNA to bind the Oligo(dT)-Cellulose. The tube was centrifuged and supernatant decanted, and the cellulose pellet was washed with high-salt and low-salt buffer. After the last wash with low-salt buffer, it was transferred to a microspin column, washed with low-salt buffer, and the poly-adenylated material was eluted with elution buffer. The yield of mRNA varied from 5-20 μg depending on the tissue. Following gel electrophoresis and Northern bloting, the mRNA was found to be free of DNA

and protein contamination.

### 4.2.4 *cDNA Synthesis*

ZAP-cDNA synthesis kit and Gigapack II Gold Packaging Extract kit from Stratagene was used to construct the cDNA library. For a summary flowchart of the procedures please see Figure 4.1. 5-7 μg of mRNA was used from each tissue to begin the first strand synthesis of cDNA, which served as a template. A 50 basepair oligo with a "GAGA" sequence to protect *Xho I* restriction enzyme site and 18 base of poly(dT) sequence was used as a primer. In this system, the *Xho I* restriction site enables the finished cDNA to be inserted into the Uni-ZAP XR vector in a sense orientation (*EcoR I- Xho I*). The poly(dT) region binds to the 3' poly (A) region of the mRNA and in the presence of Moloney-Murine leukemia virus reverse transcriptase (M-MuLVRT) and nucleotide mixture (dATP, dGTP, dTTP, and 5-methyl dCTP) the first strand of the cDNA is synthesized. The first strand has a methyl group on each cytosine base to protect it from degradation in subsequent steps. The second strand synthesis involved the presence of RNase H which nicked the RNA bound to the first strand into many fragments. These served as primers for DNA polymerase I to synthesize the second strand

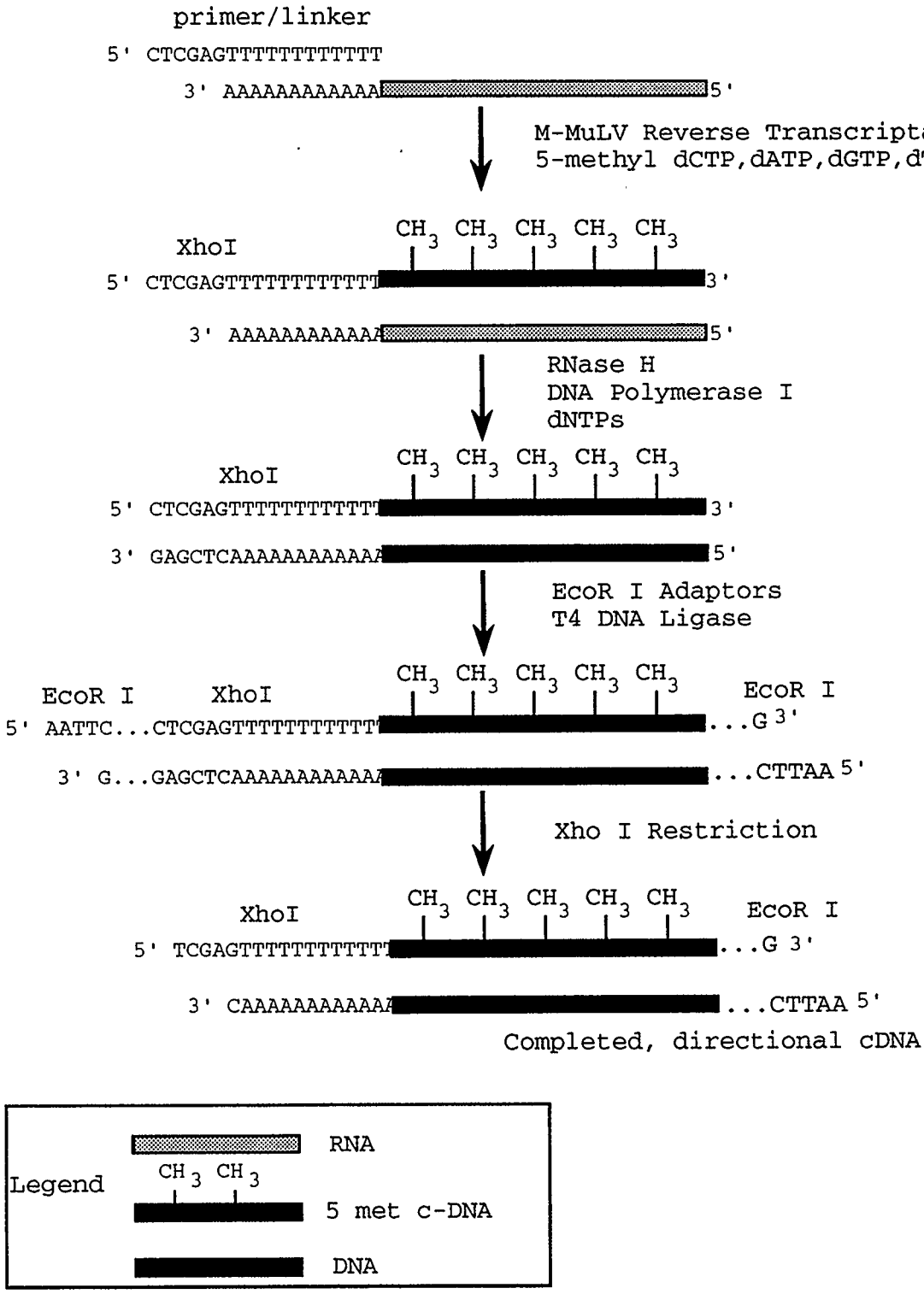



**Figure 4.1** cDNA synthesis summary flowchart.
from Staragene ZAP-cDNA synthesis kit instruction manual, 1993.

of cDNA in presence of nucleotide mixture (dATP, dGTP, dTTP, and dCTP). The uneven ends of cDNA were nibbled or filled with Klenow and *EcoR I* adapters ligated to the blunt ends. The *Xho I* digest then released the *EcoR I* adaptor and residual linker-primer from the 3′ end of the cDNA. These fragments were separated through a Sephacryl column. The size-fractionated cDNA was precipitated and ligated to Uni-ZAP XR vector arms. The library was then packaged in a Gigapack II Gold packaging extract. The primary libraries are unstable and amplification is therefore necessary. To amplify, aliquots of the packaged mixture were mixed with the host cells (*XL1 Blue MRF′*) to allow attachment of the phage to the host cells. The infected bacteria were left at 37 C overnight to allow for plaque formation. Subsequently, the plates were overlayed with SM buffer allowing the phage to diffuse into the buffer. The bacteriophage suspension was recovered after 24 hours, chloroform was added to a final concentration of 5% and the cell debris was removed by centrifugation. The final supernant was stored in 0.3% chloroform at 4 C as well aliqotes were stored at -80 C in 7% dimethylsulfoxide (DMSO).

## 4.3 *Results and Discussion*

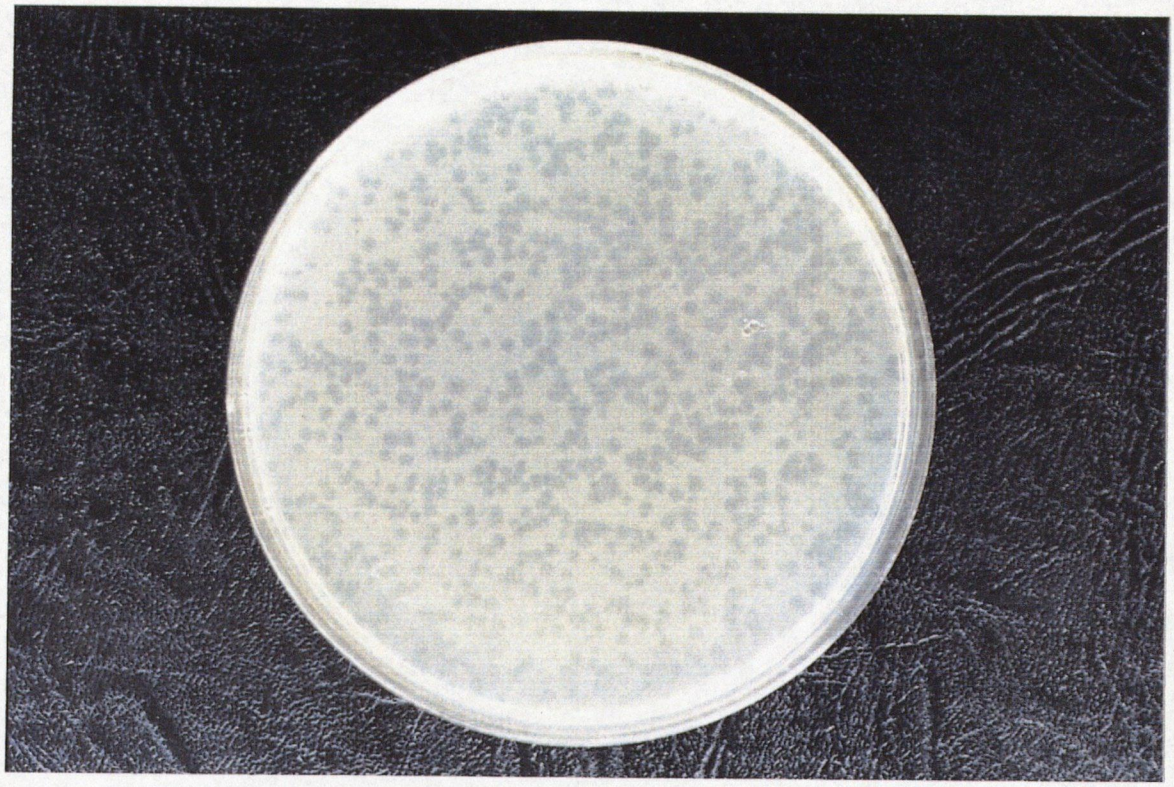
The cDNA libraries for pituitary, brain, and ovary were successfully constructed. Color selection by α-complementation with the Uni-ZAP vector in presence of IPTG (isopropyl β-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) was performed to determine the ratio of recombinants to nonrecombinants within a newly constructed library. The recombinant plaques are white and should be 10-100 fold above background (blue plaques). The plates for the primary cDNA libraries with IPTG-X-gal color selection from brain and pituitary are shown in Figure 4.2. Following the first round of amplification, the titre of the libraries amounted to:

| | |
|---|---|
| Ovary cDNA library | $5 \times 10^9$ *pfu/ml |
| Brain cDNA library | $6 \times 10^9$ pfu/ml |
| Pituitary cDNA library | $2 \times 10^{12}$ pfu/ml |

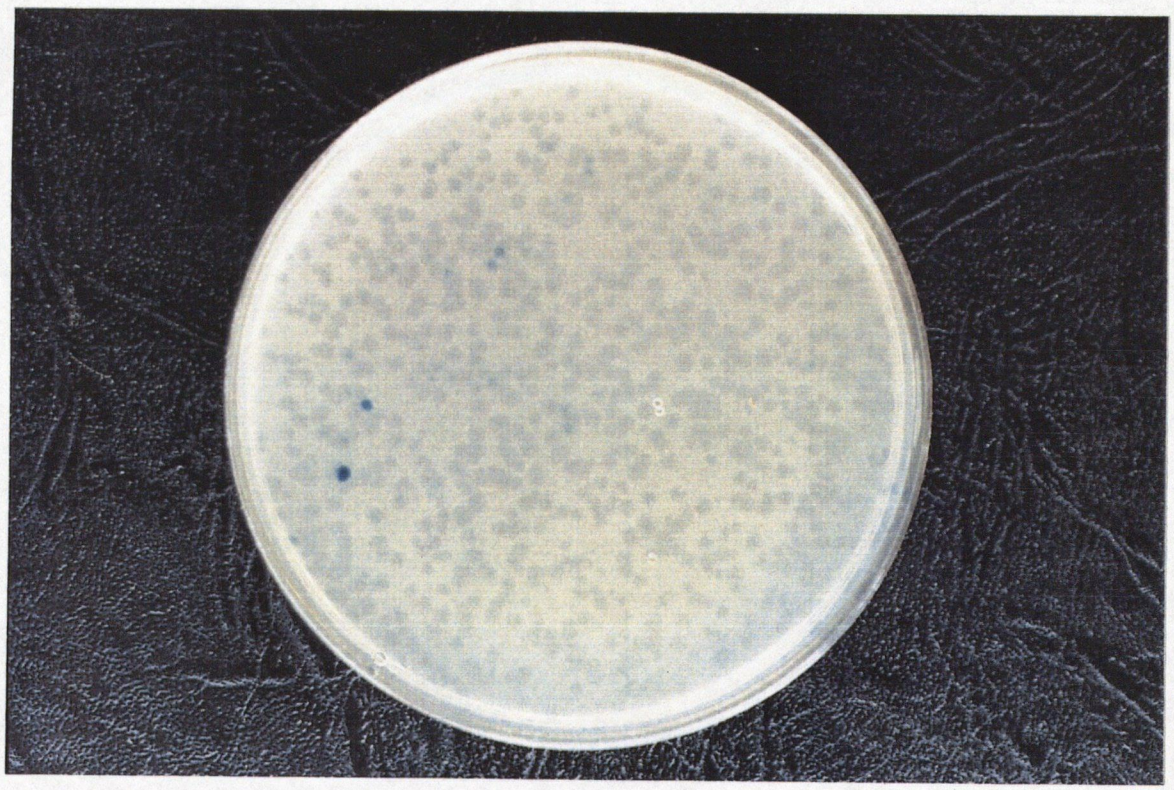
*; plaque forming units (pfu)

**Figure 4.2** Plates showing recombinant (white plaques) and non-recombinat plaques (blue plaques) following color selection by α-complementation. A) Brain cDNA library. B) Pituitary cDNA library

A)



B)



## 4.4 *Future Studies*

The cDNA libraries constructed in this study were found to have good titres (>$10^9$ pfu/ml) and reflect a good representation of both low and high trancription messages. Future studies on these libraries will likely provide valuable genomic and molecular information on the GnRH-R as well as other neurohormones in the goldfish pituitary. Future investigations may also focus on the regulation of GnRH-R gene transcription and synthesis. I anticipate that the cloning of GnRH-R will lead to a better understanding of the complex interaction between the hypothalamic, pituitary, and gonadal factors involved in the regulation of reproduction in goldfish.

## 5. *Final Concluding Remarks*

The findings provide a strong support for the existence of different receptor-effector mechanisms with preferential specificity for sGnRH and cGnRH-II in terms of GtH-II synthesis and release in the goldfish pituitary. Furthermore, the results indicate that GnRH receptor activity undergoes seasonal variation, with maxiaml activity during the reproductive period. In this regard, gonadal steroids were found to exert a dose-related biphasic effect on GtH-II mRNA levels *in vivo*. In addition, a direct stimulatory effect of these steroids was observed, at the level of pituitary, *in vitro*.

Two important questions need to be addressed in the future studies:

1) Is sGnRH- and cGnRH-II-induced GtH-II synthesis influenced by gonadal steroids ?

2) What are the structures of the receptor molecules for sGnRH and cGnRH-II in the goldfish pituitary?

Future studies should focus on the effects of gonadal steroids on GnRH-induced GtH-II synthesis in the goldfish pituitary. In addition, cDNA library constructed from goldfish pituitary should be screened with the objective to

characterize the molecular structure of sGnRH and cGnRH-II receptor molecules.

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