Teleost GnRHs and gonadotropins: Cloning, expression, localization, promoter motif analysis, interaction with sex steroids and thyroid hormones

THESIS SUBMITTED FOR THE DEGREE OF

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To my grandparents,
parents and teachers

University of Hyderabad
(Central University established in 1974 by act of parliament)
HYDERABAD – 500 046, INDIA

CERTIFICATE

This is to certify that **Ms. I. Swapna** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend her thesis "**Teleost GnRHs and gonadotropins: Cloning, expression, localization, promoter motif analysis, interaction with sex steroids and thyroid hormones**" for submission for the degree of Doctor of Philosophy of this University.

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I hereby declare that the work embodied in this thesis entitled **"Teleost GnRHs and gonadotropins: Cloning, expression, localization, promoter motif analysis, interaction with sex steroids and thyroid hormones"** has been carried out by me under the supervision of Dr. B. Senthilkumaran and this has not been submitted for any degree or diploma of any other university earlier.

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✍ *Swapna Immani*

Contents

General Introduction

Complex organisms require diverse integrating mechanisms for the efficient functioning of different organ systems and coordination of response to varied internal and external environmental cues. It is well established that the nervous system and endocrine systems act as the two main integrating systems coordinating the functioning of multiple organ systems in complex organisms. The early ideas that the two systems may be intertwined and that the brain might somehow be involved in the control of pituitary (the master gland influencing other endocrine glands) secretions, evolved by early 1930's. The initial concept of this 'neuroendocrine' integration was centered on the anatomical identification of a complex system of capillary networks in mammals connecting the hypothalamus and pituitary. It was hypothesized that these capillary networks might be conduit to substances produced by hypothalamus which upon action on the pituitary parenchyma could function as releasers and/or drive synthesis of all or each of the pituitary hormones. Initial ideas that the hypothalamus might contain secretary cells resembling endocrine cells was soon dispelled by the identification that certain neuronal soma and axons in hypothalamus were rich in secretory material released into hypothalamic-hypophyseal portal system. However, it was not until 1969 that the concept was finally validated by the isolation and identification of thyrotropin releasing factor (TRF) from the extracts of the ventral hypothalamus. Later several other hypothalamic releasing factors were identified in hypothalamic extracts and blood circulation (Table 1).

The neuroendocrine system comprise of the parvocellular and magnocellular neurons in the hypothalamus that send axons to the stalk of the pituitary gland. Magnocellular axons end in the posterior lobe where they release vasopressin and

oxytocin into systemic circulation. The parvicellular neurons release stimulatory or inhibitory factors into the portal system in the median eminance in case of mammals for transport to the anterior pituitary or directly innervate the anterior pituitary in case of pisces.

Table 1: The Hypothalamic Releasing and Inhibiting Hormones/factors

Neuropeptide Hormone	Target Cell Type	Action
Gonadotropin-Releasing Hormone (GnRH)	Gonadotrophs	Stimulation of Follicle stimulating hormone, Luteinizing Hormone (LH) release.
Corticotropin-Releasing hormone (CRH)	Corticotrophs	Stimulation of Adrenocorticotropin releasing hormone (ACTH) release
Growth Hormone-Releasing Hormone (GHRH)	Somatotrophs	Stimulation of Growth hormone (GH) release
Thyrotropin-Releasing Hormone (TRH)	Thyrotrophs	Stimulation of thyrotropin release
Somatotropin-release inhibiting factor (Somatostatin)	Somatotrophs	Inhibition of somatotropin release
Prolactin-inhibiting factor	Lactotrophs	Inhibition of prolactin release

Gonadotropin-Releasing Hormone (GnRH):

GnRH is the hypothalamic decapeptide central to the initiation of a cascade of events leading to sexual maturation and seasonal reproductive cycles, coordinated in response to a variety of physiological, social and environmental cues. The hormone derives its name from its key function- the ability to induce release of gonadotropins (GTH) i.e follicle-stimulating hormone (FSH or GTH-I) and luteinizing hormone (LH or GTH-II) from gonadotrophs in pituitary (Matsuo *et al.*, 1971; Burgus *et al.*, 1972; see Guillemin, 2005). The nomenclature, Luteinizing hormone-releasing hormone (LHRH) is also used indicating that it induces LH release (first identified function of GnRH) and a separate factor was believed to be responsible for release of FSH (Guillemin, 2005). However, in the absence of any known FSH releasing factor identified so far and in cue with the general observation that GnRH influences both LH, FSH levels, the term GnRH is more widely accepted. GnRH regulates the hypophyseal release of FSH and LH vis à vis gonadal maturation, oogenesis, spermatogenesis and sex steroid production (Fink, 1988). The central role of GnRH in reproductive functions is illustrated by the observation that a lack of its expression or mutations in the gene encoding GnRH receptor produces sterility (Mason *et al.*, 1986; MacColl *et al.*, 2002). The implications of a non functional GnRH system are similar in all vertebrate classes: lampreys (Sower and Kawauchi, 2001), cartilaginous fishes (Demski *et al.*, 1997), teleost fishes (Holland *et al.*, 2001), amphibians (Andersen *et al.,* 1992), reptiles (Licht *et al.*, 1987; Tsai and Licht, 1993), birds (Sharp *et al.*, 1998), and mammals (Fink, 1988). GnRH secretion is in turn influenced by complex interactions between numerous neurotransmitters, neuropeptides, sex steroids involving several negative and positive feedback loops.

The neuroendocrine regulation of reproduction involving the hypothalamo-hypophyseal-gonadal axis is well conserved among vertebrates. Teleosts are particularly suitable models to study mechanisms of neuroendocrine control of reproduction because of their relatively simple neuroendocrine organization (Blazquez *et al.*, 1998). They are unique among vertebrates in that they lack the specialized well-defined hypothalamo-hypophyseal portal system that delivers brain regulators to specific areas of pituitary in higher vertebrates. Instead, releasing hormones and neurotransmitter neurons directly innervate the pituitary gland in teleosts (Fig 1). In addition, hormone producing cells in the pituitary show a markedly distinct distribution pattern that facilitates studies on their innervation and neurotransmitter control.

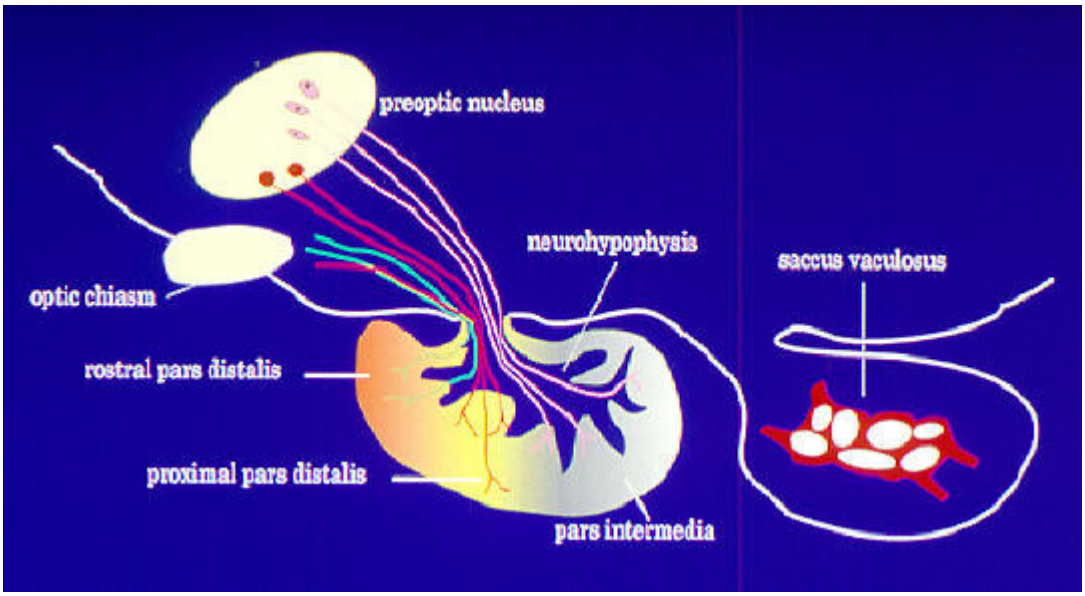

Fig 1*: Direct innervation of teleost pituitary by stimulatory and inhibitory pathways originating from preoptic anterior hypothalamic area (courtesy: http://www.utmsi.utexas.edu/staff/khan/research.htm)*

Teleosts are an especially interesting model for studies on GnRH as multiple forms of GnRH exist in the same species (Powell *et al.*, 1994). The multiple forms vary in their anatomical locations and specific function. It is now increasingly recognized that GnRH not only acts as an endocrine mediator of sexual maturation and reproduction but also functions as a neuromodulator (Soga *et al.*, 2005). Owing to the direct innervation of pre-optic neurons into target cell types, teleosts offer an exciting avenue for delineating the function and effector target cell type for the multiple GnRH variants in a species.

Multiple GnRH variants and their evolution:

The first GnRH molecule to be identified and isolated was the mammalian form (mGnRH, Matsuo *et al.*, 1971; Burgus *et al.*, 1972). Later several other variants were identified first in teleosts then in protochordates and invertebrates. To-date presence of 16 GnRH variants has been established, 14 from vertebrates and 2 from invertebrates (Table 2). Recently few more have been identified in invertebrates taking up the count to 24 GnRH variants (Gorbman and Sober, 2003). All the variants are essentially decapeptides with a C terminal amidation and N terminal pyroglutamate residue. Most of the amino acid variations are confined to positions 5-8 (Table 2).

GnRH Form	Amino acid sequence	Reference
Teleosts		
Mammalian GnRH (mGnRH)	pGlu His Trp Ser Tyr Gly Leu Arg Pro Gly-NH2	Matsuo *et al.*, 1971
Chicken GnRH – II (cGnRH-II)	_ _ _ _ His _ Trp Tyr _ _	Yu *et al.*, 1988
Perjery GnRH	_ _ _ _ Phe _ _ Ser _ _	Montaner *et al.*, 2001
Seabream GnRH (sbGnRH)	_ _ _ _ _ _ _ Ser _ _	Powell *et al.*, 1994

Salmon GnRH (sGnRH)	_ _ _ _ _ _ Trp Leu _ _	Sherwood *et al.*, 1993
Catfish GnRH (cfGnRH)	_ _ _ _ His _ _ Asn _ _	Bogerd *et al.*, 1994
Herring GnRH (hgGnRH)	_ _ _ _ His _ _ Ser _ _	Carolsfeld *et al.*, 2000
Whitefield GnRh (wfGnRH)	_ _ _ _ _ _ Met Asn _ _	Adams *et al.*, 2002
Other Vertebrates		
Mammalian GnRH (mGnRH)	pGlu His Trp Ser Tyr Gly Leu Arg Pro Gly-NH2	Matsuo *et al.*, 1971
Frof GnRH	_ _ _ _ _ _ _ Trp _ _	Yoo *et al.*, 2000
Lamprey – I (LI)	_ _ Try _ Leu Gln Trp Lys _ _	Sower *et al.*, 1993
Lamprey – III (LIII)	_ _ _ _ His Asp Trp Lys _ _	Sower *et al.*, 1993
Dogfish	_ _ _ _ His _ Trp Leu _ _	Lovejoy *et al.*, 1992
Guinea Pig	_ Tyr _ _ _ _ Val _ _ _	Jim´enez-Li˜nan *et al.*, 1997
Chicken GnRH – I (cGnRH-I)	_ _ _ _ _ _ _ Gln _ _	Miyamoto *et al.*, 1983
Invertebrates	_ _ _ _ Asp Tyr Phe Lys _ _	
Tunicate – I	_ _ _ _ Leu Cys His Ala _ _	Powell *et al.*, 1996
Tunicate – II		Powell *et al.*, 1996

***Table 2**: Primary structure of different GnRH variants identified so far. Only the amino acids varying from that of mGnRH have been indicated. The variants are named after the species from which they were first isolated*

Existence of two GnRH variants in same species is characteristic of teleosts as is the case with all vertebrates (identified later). Multiple GnRH in a single species were first identified in teleosts (Powell *et al.*, 1994; Okubo *et al.*, 2000a, b) and later in all vertebrate species. Three GnRH variants have also been identified for the first time in teleosts. Presence of three GnRH variants was thought to be a characteristic of evolved fish species. But however this notion lost impetus with the identification of a third form of GnRH - hgGnRH in the Pacific herring (*Clupea harengus*; Carolsfeld *et al.,* 2000) a primitive teleost and wfGnRH in whitefish (*Coregonus clupeaformis*; Adams *et al.*,

2002), a basal salmonid (Fig 2). Different GnRH variants in a species vary in their anatomical distribution. A comparison of the distribution of different GnRH variants in different orders of teleosts shows that a clear correlation cannot be obtained from the presence of either two or three variants and the position of a given species in the teleost lineage (Fig 2). cGnRH-II seems to have evolved early and is detected in cartilaginous fishes while mGnRH seems to have evolved little later and is detectable from bony fishes onwards in the animal kingdom. GnRH cell populations exist most prominently in the olfactory bulb plus telencephalon, preoptic area and midbrain tegmentum. In teleosts with only two forms of GnRH, cGnRH-II is mainly in the midbrain, and the second form is in both the olfactory region/terminal nerve (OLF/TN) and ventral telencephalic/preoptic (VT/PO) region. In species with three GnRH forms, cGnRH-II is in the midbrain, and the other two GnRH forms maybe in separate locations (with species specific form in the telencephalon/ preoptic area) or their anatomical locations may overlap to a minor extent with the species specific form mainly distributed in preoptic area- hypothalamus (POA-H; Parhar, 1997; Stefano *et al.*, 2000; Okubo *et al.*, 2000b; Pandolfi *et al.*, 2002; Tiwari *et al*., 2002; Dubious *et al.*, 2002; Amano *et al.*, 2004). This was further clarified by measurement of three GnRH forms in various brain regions using specific radioimmunoassay (Senthilkumaran *et al.*, 1999). A 12 aminoacid GnRH peptide has been recently identified in octopus indicating that GnRH peptides evolved even prior to protochordates (Iwakoshi *et al.*, 2002). The NH_2-terminal sequence of GnRH peptides (pGlu-His-Trp-Ser) and the COOH-terminal amino acids (Pro-Gly.NH_2) have been conserved over about 600 million years of chordate evolution, with the exception of two

conservative Tyr substitutions in frog and guinea pig at position 3 and 2 (Table 2). The conserved length of the peptide (10 amino acids), the NH_2 terminus and COOH terminus (Pro-Gly.NH2) indicates that these features are critically important for receptor binding and activation. Octopus homolog which is the most ancient form of GnRH also exhibits the characteristic pGlu and Pro9Gly10.NH_2 but has an additional two amino acids inserted in the middle region of the molecule is capable of stimulating LH release from quail pituitary cells (Iwakoshi *et al.*, 2002). All GnRHs are processed from a large precursor which includes a signal peptide (around 20–25 residues), the biologically active GnRH sequence, a processing tripeptide (Gly–Lys–Arg) and the GnRH associated peptide (GAP; around 40–50 residues).

GnRH like peptides have also been identified in Cnidaria, (Anctill, 2000) considered to be one of the first classes in the animal kingdom with neurons and true synapses. This indicates that GnRH family of peptides must have originated very early in vertebrate evolution almost 600 million years back. However, GnRH like peptides have not been identified in other invertebrates probably because many of these ancestral GnRH forms are hard to identify owing to lack of immunoreactivity with available GnRH antibodies. Fernald and White (1999) proposed that three different GnRH lineages can be distinguished based on the distinct brain areas of localization in vertebrates: (1) the conserved cGnRH-II or mesencephalic lineage; (2) the hypothalamic or "releasing" lineage whose primary structure has diverged by point mutations (mGnRH and its orthologous forms: hrGnRH, wfGnRH, cfGnRH, sbGnRH, and pjGnRH); and (3) the telencephalic sGnRH form (Dubois *et al.*, 2002; Fernald and White, 1999; Okubo and

Aida, 2003). This hypothesis does not take into consideration the lamprey and tunicate GnRH and has not been proven, though it has supporting evidence (Montaner *et al.*, 1999; Quambeck *et al.*, 1997; Yahalom *et al.*, 1999).

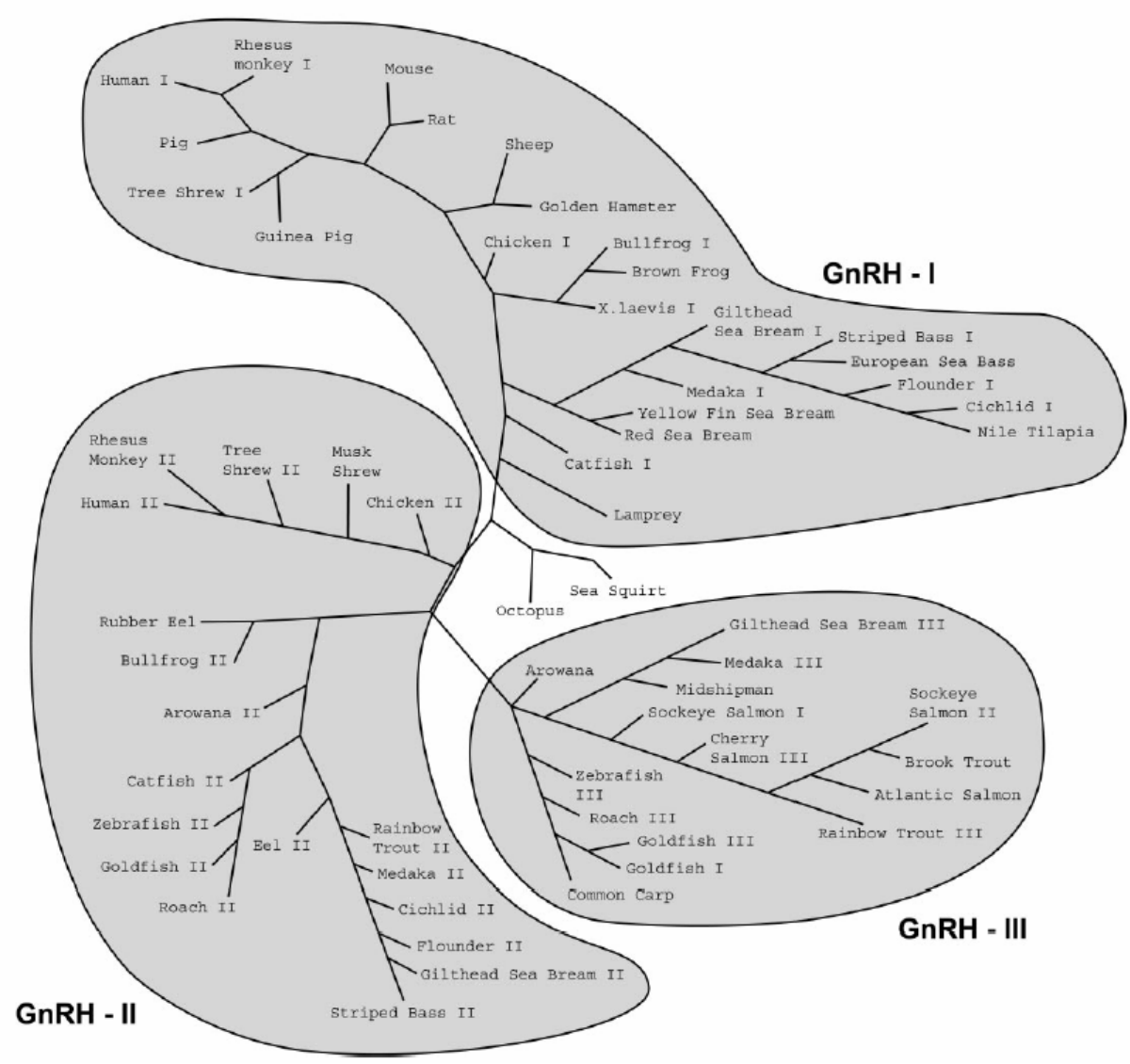

***Fig 2**: Phylogenetic tree constructed from GnRH primary amino acid structure obtained from several species. (King and Millar, 1995)*

GnRH Nomenclature:

GnRH molecules are now commonly designated by the name of the species in which they were first isolated with the exception of mGnRH. This continues to be the most prevalent and well used nomenclature for reference to different GnRH variants. However, alternative nomenclatures have been proposed and are occasionally used.

1) Nomenclature based on primary structure of the GnRH peptide: This nomenclature uses the mGnRH sequence as standard GnRH sequence, and all variants are named according to the amino acid alteration in a determined position. For example, cGnRH-II is named [His5,Trp7,Tyr8]GnRHand gpGnRH is known as [Tyr2,Val7] GnRH.

2) Nomenclature based on anatomical distribution of the GnRH variant: According to this nomenclature the hypothalamic- preoptic variant is referred to as GnRH1, the mesencephalic-midbrain variant- GnRH2 (cGnRH-II), the third form in case present is referred as GnRH3. This nomenclature however suffers from several disadvantages that it does not distinguish between the different variants found at the hypothalamus of different species i.e mGnRH, sbGnRH, cfGnRH, hrGnRH, wfGnRH, and pjGnRH are named with the same terminology: GnRH1. Further more, in case of salmonids and cyprinids, sGnRH can be referred to as GnRH3 or GnRH1 depending on its neuroanatomical location.

GnRH – Endocrine, Paracrine, Autocrine and Neuromodulatory functions:

GnRH plays a pivotal role in the regulation of reproduction by stimulating the release of gonadotropins GTH-I and GTH-II from the proximal pars distalis. GTH-I which is

analogous to FSH in mammals has only recently been isolated in fish and has important roles in gonadal steroidogenesis (Schulz *et al*., 2001; Swanson *et al*, 1991) however, there is very little information on its neuroendocrine control. However, extensive data is available for fish GTH-II which is analogous to LH in mammals. It stimulates gonadal steroidogenesis, gametogenesis and sperm release/ovulation (Xiong *et al*., 1994). The asynchronous variation in levels of GTH-I and II in response to GnRH surge remained an enigma for quite sometime. This paradigm is now considered to be atleast partially solved by the recognition that the pulse frequency of POA-H GnRH release determines the ratio of LH and FSH (Belchetz *et al*., 1978; Marshall *et al*., 1986; 1991). Slow, low frequency pulses favor transcription of FSHβ gene while rapid, high frequency pulses favor transcription of LHβ and gonadotropin α (GTHα) gene and LH secretion (Papavasilio *et al.*, 1986; Haisenleder *et al.*, 1990; Shupnik *et al.*, 1990; Kaiser *et al.*, 1997).

LH and FSH are crucial for oogenesis, ovulation, spermatogenesis, spermiation and sex steroid production, in turn influencing development of secondary sexual characters and sex specific behavior. Differential regulation of LH and FSH secretion is also believed to be influenced by the different half lives of the two peptides in circulation. Gonadal steroids, inhibins, activin, follistatin and leptin exert specific feedback actions on the gonadotropes to augment LH and/or inhibit FSH secretion (Ge *et al.*, 1992; Ge and Peter 1994; see Trudeau, 1997; Senthilkumaran and Joy 1996) in addition to local autocrine, paracrine action at gonadal level (Wang and Ge, 2003; 2004). Progesterone has been shown to mediate a decrease in GnRH pulse frequency and this is augmented in the presence of estradiol ($E_{2;}$ see Trudeau, 1997; Smith and Jennnes, 2001). Role of

neurotransmitters like 5-HT, GABA (Somozo and Peter, 1991; Yu et al., 1991; Senthilkumaran and Joy 1994, 1995a, 1995b; Senthilkumaran *et al*., 2001), Glutamate (Roberts *et al.*, 2006) and neuropeptides like NPY (Peng *et al.*, 1990; Senthilkumaran *et al.*, 2001) have been proposed in regulation of GnRH, GTH release and GnRH pulse frequency. However the mechanisms regulating GnRH pulse frequency and the relative role of neurotransmitters, neuropeptides, sex steroids in this process are not completely understood. Maintenance of pulsatile pattern of GnRH secretion is crucial for stimulation of gonadotrops and its been demonstrated that maintenance of continuous high levels of GnRH causes inhibition of gonadotropin secretion mediated by receptor desensitization (see Guillemin, 2005).

The identification of GnRH neurons in regions not involved in gonadotropin regulation lead to the hypothesis that GnRH may be acting as a neuromodulator in these regions. The mid brain GnRH is thought to be mainly involved in neuromodulation (Soga *et al.*, 2005) while the olfactory bulb GnRH in pheromonal response (Stacey *et al.*, 1994) and sexual behaviour. GnRH in gonads is believed to have paracrine, autocrine effects on Granulosa and Leydig cells influencing oocyte meiotic maturation and apoptosis respectively (Habibi and Pati, 1993).

Origin of GnRH neurons and mechanism of neuronal migration:

GnRH neurons originate in the nasal compartment, migrate along olfactory nerves and then diverge from the olfactory tract progressing caudally to the forebrain to reach their final destination in the POA-H (Tobet *et al.*, 1997; Wray, 2001; Amano *et al.*, 2004;

Wierman *et al.*, 2004). This is in contrast to other hypothalamic-releasing factors, such as thyrotropin-releasing hormone or corticotropin-releasing hormone, whose neurons originate in the endocrine hypothalamus. In addition to GnRH the peptides molluscan cardioexcitatory tetrapeptide (FRMFamide) in fish (White and Meredith, 1995), and somatostatin (Murakami and Arai, 1994) and neuropeptide Y (Hilal *et al.*, 1996) in chickens had been identified in cells migrating through the olfactory system. However, these peptides have not been observed in the olfactory migration routes of mammalian species. The dipeptide carnosine has been found in migrating cells in mammals, however, its role in neurotransmission is not known (Tarozzo *et al.*, 1994). However, none of these include known hypothalamic releasing factors.

GnRH neurons originate in the olfactory placode and are identified by the presence of GnRH immunoreactivity and *in situ* hybridization with GnRH mRNA. The progenitor cells of these neurons and their origin are not identified as the only distinguishing feature of the GnRH neurons is their GnRH immunoreactivity hence limiting traceability to after these neurons start GnRH production. Several molecules including the cell adhesion molecules containing terminal galactose residues (NCAM, laminin, endogenous lectin-galactin-1, tenacin, phosphocan), neurotransmitters (GABA, glutamate), classic axon guidance molecules like netrin-1, matrix metalloproteases and tissue inhibitors of matrix metalloproteases are thought to be involved in neuronal migration of GnRH neurons (see Tobet and Shwarting, 2006). Research on individuals with Kallman's disorder, an X linked inherited condition characterized by defective migration of GnRH and olfactory tract neurons, has shed light on pathways important in GnRH neuronal migration (Franco

et al., 1991; Gonzalez- martinez *et al.*, 2004; Okubo *et al.*, 2006). The disorder affects many vertebrates including humans. KAL1 encodes anosmin-1, a protein with extracellular matrix protein features, consisting of an N-terminal cystein-rich domain, followed by a WAP domain, four fibronectin (FNIII) domains and a C-terminal histidine-rich region (Soussi-Yanicostas *et al.*, 1996, 1998). The WAP domain is similar to other protease inhibitors and the FNIII regions are similar to neural cell adhesion molecules (Soussi-Yanicostas *et al.*, 2001). Anosmin-1 has been shown to promote axon outgrowth from the olfactory bulb and to stimulate branching from axons into the olfactory cortex (Soussi -Yanicostas et al., 1996). An autosomal dominant form of Kallmann's syndrome in humans is associated with a deletion of chromosome 8p11–12, resulting in defective production of the fibroblast growth factor (FGF) receptor 1 (FGFR1) (Dode *et al.*, 2003) i.e haploinsufficiency for FGFR1. X-linked Kallmann's and families with the autosomal dominant disorder exhibit similar clinical features leading to the assumption that anosmin-1 might be a crucial component of normal FGF signaling. Anosmin-1 can bind to heparan sulfate proteoglycans, which are important in the FGF–FGFR complex. FGFR1 and anosmin-1 are co-expressed across development (Dode *et al.*, 2003).

Recently, Protein kinase C (PKC) signaling has been proposed to be involved in migration of GnRH neuronal cells by driving cytoskeletal changes and neurite outgrowth (Choe *et al.*, 2003). PKCα was shown to be important in membrane ruffling and cytoskeletal reorganization (Choe *et al.*, 2003). By contrast, PKCγ has been shown to inhibit these alterations. Downstream of PKCα, p130Cas stimulation of focal adhesion kinase and a role for ERK, mitogen-activated protein kinase (MAPK), but not

p38MAPK, have also been implicated in cytoskeletal changes and motility (Choe *et al.*, 2003). Cytoskeletal modulation and neuronal migration is also thought to involve mediation of Rho family proteins and Gas6/ Ark pathway. Apart from promotion of GnRH neuronal migration the gas6/Ark pathway is also thought to promote GnRH expression. However, most of these studies were carried out in GnRH neuronal cultures and the endogenous activators/relevance of these pathways in GnRH neurons *in-vivo* remains unknown.

GnRH receptors

GnRH receptors are members of a superfamily of G protein coupled heptahelical membrane spanning receptors (GPCRs). The amino acid sequence of the GnRH receptor was first deduced for the mouse receptor cloned from the pituitary T_3 gonadotrope cell line (Tsutsumi *et al.*, 1992). Later pituitary receptors were cloned from the rat (Kaiser *et al.*, 1992), human (Millar *et al.*, 1999), sheep (Illing *et al*., 1993)), cow (Kakar *et al.*, 1993), rat (Eidne *et al.*, 1992) and pig (Weesner and Matteri, 1994) They share over 80% amino acid identity. Homologs of the mammalian GnRH receptors have also been cloned from a marsupial (possum; King *et al.*, 2000), catfish, two forms from goldfish (Illing *et al.*, 1999), bullfrog (Acharjee *et al.*, 2002), brown frog (Seong *et al.*, 2003), clawed toad, chicken, medaka (Okubo *et al.*, 2001), striped bass (Alok *et al.*, 2000), rainbow trout (Madigou *et al.*, 2000), masu salmon (Jodo *et al.*, 2003), the Japanese eel (Okubo *et al.*, 2000a), amberjack (Pubmed Accesion No. CAB 65407), rubber eel (Pubmed Accesion No. AD 49750), and an ascidian (Kusakabe *et al.*, 2003). The non-mammalian receptors

exhibiting greatest homology to the mammalian pituitary receptors show 42–47% amino acid identity with the mammalian receptors while the non-mammalian receptors share 58– 67% identity among each other. GnRH receptors have a typical structure of GPCRs with heptahelical transmembrane domains joined by intracellular and extracellular loops. The NH_2 terminal region is located extracellularly and has glycosylation sites while the COO^- terminal lies intracellular (Pfelger *et al.*, 2002; Seong *et al.*, 2003; Wang *et al.*, 2004).

In lieu with the presence of multiple forms of GnRH, it might be expected that multiple forms of GnRH receptors be present in a given species. In fact two or three types of GnRH receptors have been identified in several teleosts species. In the *Morone saxalis* (Alok *et al.*, 2000) goldfish (Troskie *et al.*, 1998, Illing *et al.*, 1999; Peter *et al.*, 2003), zebra fish (Troskie *et al.*, 1998), catfish (Bogerd *et al.*, 2002; Vischer and Bogerd 2003), and salmon there are two isoforms (type Ia and type Ib) that have 70% amino acid identity (Fig 2). In the goldfish, type Ia has a putative SH3 binding domain (poly proline sequence) in the carboxyl-terminal tail, which potentially conveys the possibility of coupling to MAPKs. Type II GnRH receptor have also been cloned from bullfrog (Wang *et al.*, 2001), clawed toad, marmoset, macaque, green monkey and a type III GnRH receptor have been obtained from bull frog (Wang *et al.*, 2001). These findings suggest an early evolution of the three GnRH receptor subtypes in vertebrates which parallels that of the GnRH ligands. However, the GnRH receptor type II cloned from humans has a frame-shift and an internal stop codon rendering the transcripts incapable of being translated to a full-length GPCR. This apparent silencing of the type II receptor is very

paradoxical given the extraordinary conservation of the cognate GnRH II ligand from teleosts to humans. However, it is possible that a partial receptor is formed and is functional or the frame shift and internal stop codon are post translationally taken care of producing a full length functional receptor. The human type I GnRH receptor lacks a COO^- terminal tail which is unique to GPCRs. As the evolutionar time separating amphibians and mammals is similar to that separating amphibians and bony fish, the poor conservation of sequence of the mammalian type I GnRH receptor with the non-mammalian receptors implies a sudden acceleration in evolutionary change in the mammals.

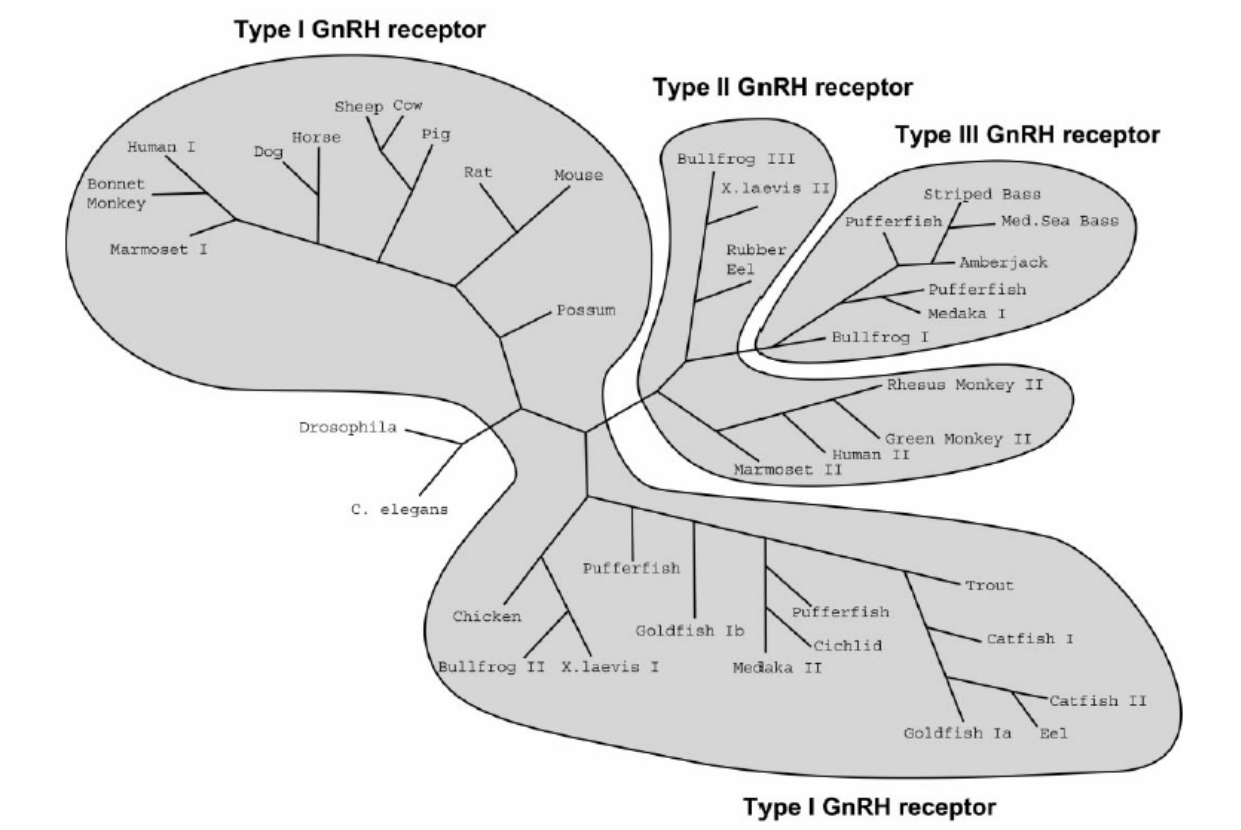

***Fig 2**: Phylogenetic tree constructed from GnRH receptor primary amino acid structure obtained from several species. (King and Millar, 1995)*

GnRH mediated cell signaling:

GnRH ligand binding to the receptor results in the activation of phospholipase Cβ through the Gaq/11 G proteins (Hseih and Martin, 1992; Liu *et al.*, 2002a) which in turn catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to (Asaoka *et al.*, 1992) inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 mobilizes intracellular calcium which activates the PKC isoforms, of which α and βII have been identified in gonadotrope cell lines (Johnson *et al.*, 1993; Junoy *et al.*, 2002). The generation of DAG is likely to lead to the late activation of novel PKC isoforms like PKCε present in gonadotrophs (Liu *et al.*, 2002b). The initial phase of calcium surge results from

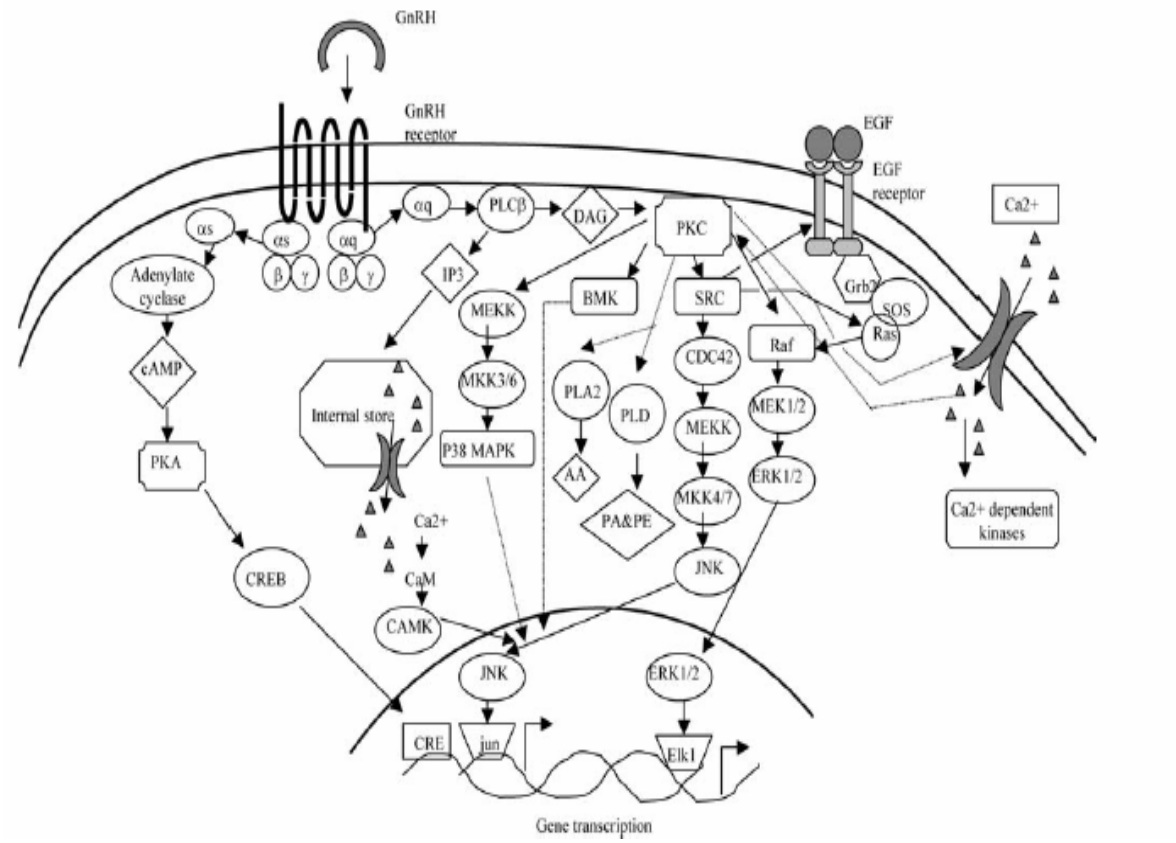

***Fig 4**: GnRH modulated signaling networks (Ruf et al., 2003)*

intracellular whereas the plateau phase depends on external calcium entry predominantly via L-type voltage-sensitive Ca^{2+} channels (Merelli *et al.*, 1992). Phospholipase D and phospholipase A2 are sequentially activated by GnRH receptor signaling probably via PKC. GnRH receptor activation leads to stimulation of several MAPK cascades that mediate gene induction (Naor *et al.*, 2000; Roberson *et al.*, 1995). The MAPK cascade activation by GnRH involves dependent and independent pathways (Naor *et al.*, 2000). Four kinase cascades are activated by GnRH receptor signaling, leading to activation of ERK (Bernard *et al.*, 2001), JNK (Mulvaney and Roberson, 2000; Yokoi *et al.*, 2000),

p38MK (Roberson *et al.*, 1999), and BMK (Naor *et al.*, 2000) (Fig 4). Most of the cells signaling pathways were elucidated using αT3 cell. The active MAPKs translocate to the nucleus where they activate transcription factors, such as Ets-family proteins, and thereby modulate the promoter activity of various genes (Weck *et al.*, 2000; Wolfe and Call, 1999).

GnRH desensitization and regulation of the transcription of GTHα, LHβ, FSHβ subunit genes:

The GTH α-subunit responds to constant exposure or high pulse frequency (_q 10 min), whereas the LHβ subunit promoter responds best to lower frequency pulses (_q 30–60 min) and FSHβ to even lower frequencies. It was initially proposed that the frequency decoder of the pituitary gonadotrophs resides in the differential desensitization of GnRH receptor with pulse frequency variations. However unlike many heptahelical receptors, it was observed that the GnRH receptor shows minimal desensitization (Forrest-Owen *et al.*, 1999; Mcardle *et al.*, 1999) and a mechanism of frequency sensitivity based on signaling components common to induction of all gonadotropin subunits would not explain the different frequency response characteristics of the various subunits. It is now believed that the positioning of the individual genes in the genetic network, the control mechanisms and promoter structures may contribute to the differential regulation of gonadotropin subunits in response to GnRH frequencies. A role for ERK, ELK-1, MRK, PKC and IP3 induced

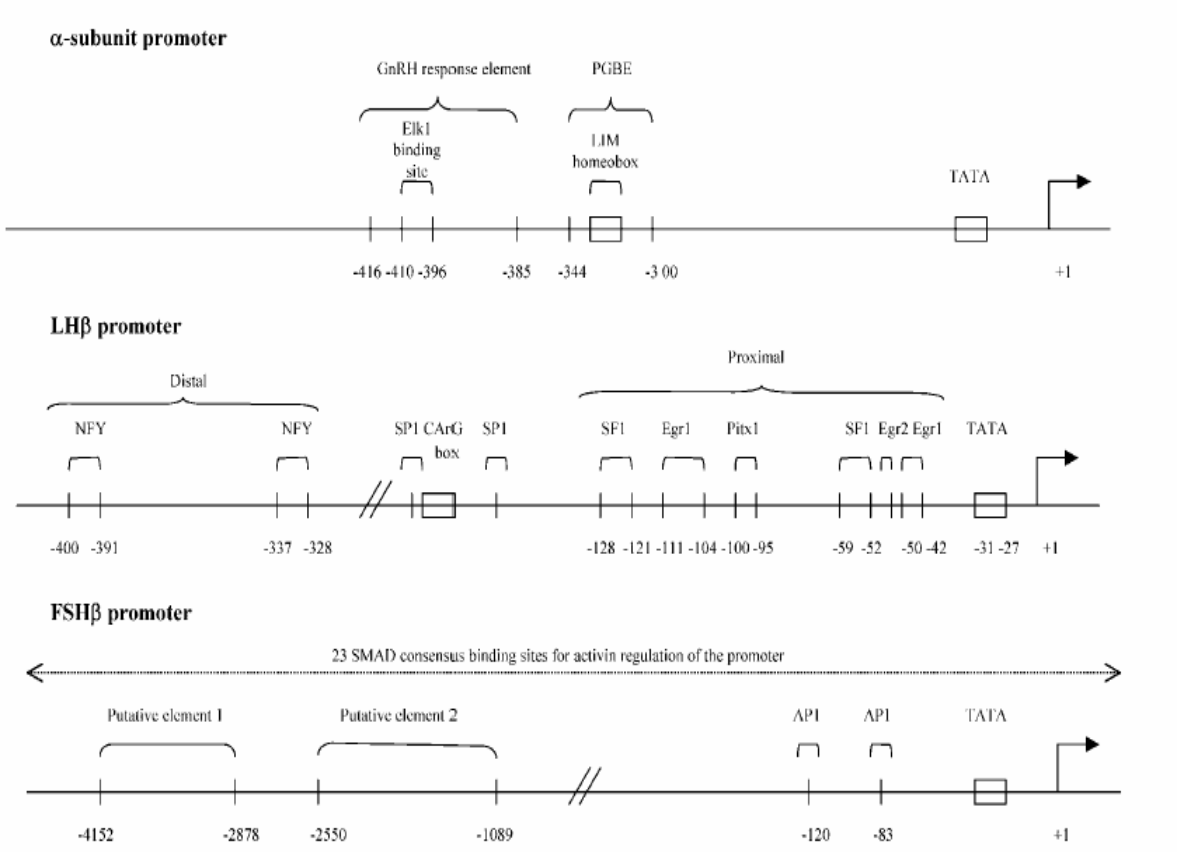

***Fig 5**: Promoter elements of Gonadotropin Subunits showing distinct GnRH response elements or binding sites for transcription factors induced by GnRH (Ruf et al., 2003)*

intracellular calcium rise have been suggested in GTHα subunit regulation. The GTHα promoter (elucidated in rats) has the pituitary glycoprotein basal element (PGBE) and the GnRH response element (GnRH-RE) with interaction sites for LIM-homeobox and Ets family transcription factors, respectively (Fig 5). The PGBE region, in conjunction with other promoter sites, influences the basal expression of the common gonadotropin subunit transcript, whereas the GnRH-RE mediates GnRH stimulated induction.

The LHβ promoter differs from the GTHα promoter in its concomitant requirement of both newly synthesized, preformed transcription factors for its activation and an absolute requirement for the induction of at least one newly synthesized transcription factor, Egr1. LHβ induction by GnRH is also believed to likely involve phosphorylation of transcription factors. The LHβ promoter can be divided into two distinct proximal and distal domains, that lie within 500 bp of the transcription start site and believed to act together as a composite GnRH-response element. Cis-Acting elements are located 150 bp upstream from the start site. They comprise the tandem gonadotrope specific elements (GSE) that bind SF-1 (Quirck *et al.*, 2001, Kaiser *et al.*, 2000). The proximal domain also contains a pair of regulatory elements that bind Egr-1 (Kaiser *et al.*, 2000, Quirk *et al.*, 2001; Weck *et al.*, 2000). Located between the pairs of Egr-1 and SF-1 binding elements in the proximal domain is a single binding site for Pitx-1 (Quirk *et al.*, 2001). MAPK induced SF-1 phosphorylation and PKC, Protein kinase A (PKA) mediated phosphorylation of Erg 1 have been proposed to be important in LHβ transcription. The LHβ promoter shows much variation among species. Effect of GnRH on FSHβ transcription and secretion is believed to be involve activation of TNFα. PKC, MAPK and calcium signaling.

Desensitization of GnRH receptors:

Longstanding exposure to GnRH causes desensitisation of GnRH-stimulated gonadotrophin secretion probably via receptor down-regulation and internalisation of

GnRH-Rs via coated pits (Jennes *et al.* 1985, 1986, Conn *et al.*, 1987). GnRH binding to

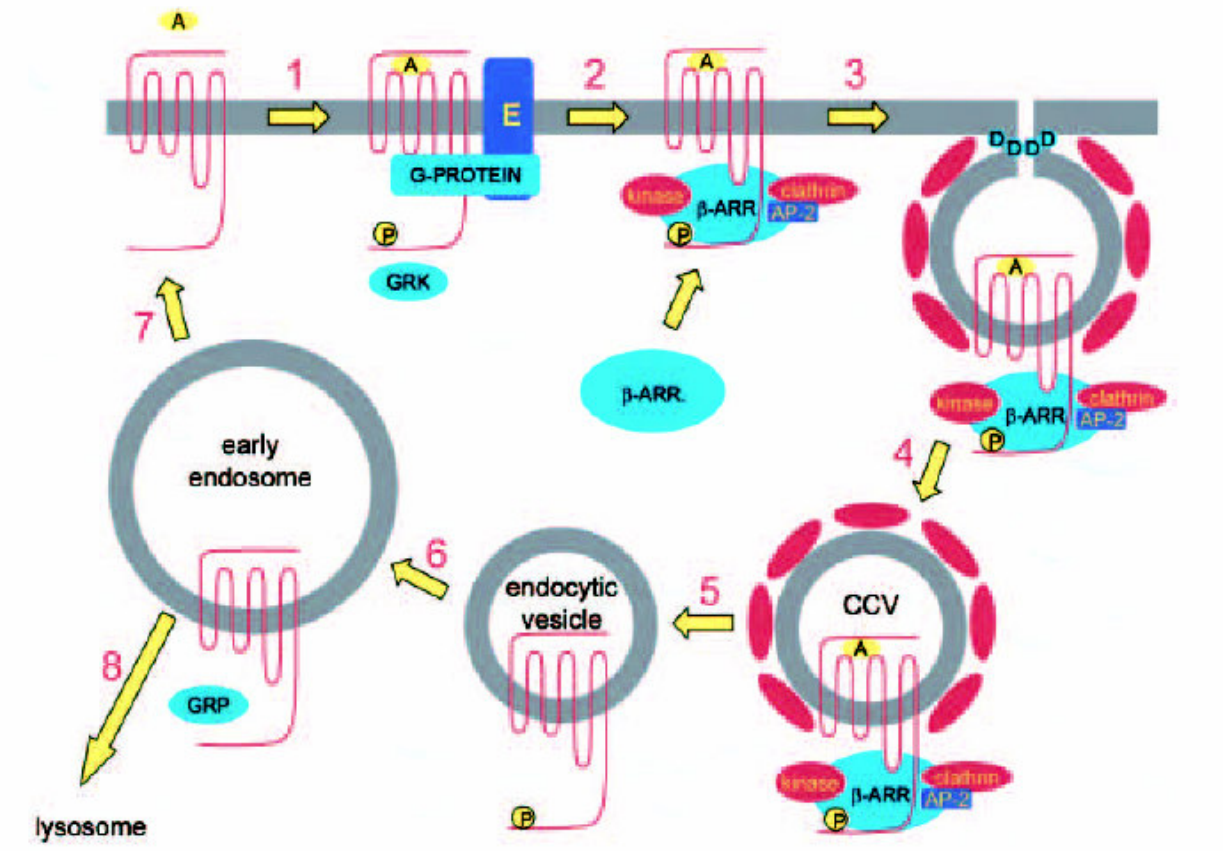

Fig 6: *Rapid homologous desensitisation and cycling of G-protein-coupled receptors (GPCRs) – general model (A-agonist, E- effecter, D- Dynamin, GRK- G protein coupled receptor kinases, GRP- G protein coupled receptor phosphatases)Mc Ardle et al., 2002*

the GPCR's receptors drives rapid phosphorylation of C terminal region mediated by specific G-protein receptor kinases (GRKs), by second messenger-regulated kinases (e.g. PKC or PKA), or by casein kinases (Tobin *et al.* 1997, Hanyaloglu *et al.*, 2001). This phosphorylation stabilizes interaction with barrestin which sterically hinders G protein binding and prevents receptor activity. Arrestin also acts as an adapter, targeting desensitized GPCRs for internalisation (Goodman *et al.* 1996, Ferguson 2001). GPCRs

are internalised from the cell surface by endocytosis, most often via clathrin-coated vesicles. The formation of these vesicles is typically controlled by a dynamin collar (Mc Ardle *et al.*, 1999). After internalization, the receptors are either recycled to the cell surface or targeted to lysosomes for degradation. Most of the GPCRs follow an analogous mechanism of receptor desensitization (Fig 6). In lieu with the observation that receptor desensitization is based on COO^- terminal region of GnRH receptors it has been observed that the GnRH type I receptors that lack the COO^- terminal region do not exhibit receptor desensitization.

Regulation of GnRH by gonadal factors, steroids, monoamines and GnIH:

Multiple factors have been identified to regulate GnRH, GTH secretion and release from the POA-H and adenohypophysis respectively. The chief of these factors include sex steroids (see Trudeau, 1997; Smith and Jennnes, 2001), monoamines (Peter *et al.*, 1986; Goos 1987; Yu *et al.*, 1991; Senthilkumaran *et al.*, 2001), gonadal factors like inhibin and activin (Ge *et al.*, 1992; Ge and Peter 1994; see Trudeau, 1997), neurotransmitters like glutamate (Roberts *et al.*, 2006) and neuropeptides like NPY (Senthilkumaran *et al.* 2001) (Fig 7). Recently a peptide has been isolated from quail hypothalamus showing inhibitory effect on GnRH secretion. This peptide has been referred to as the

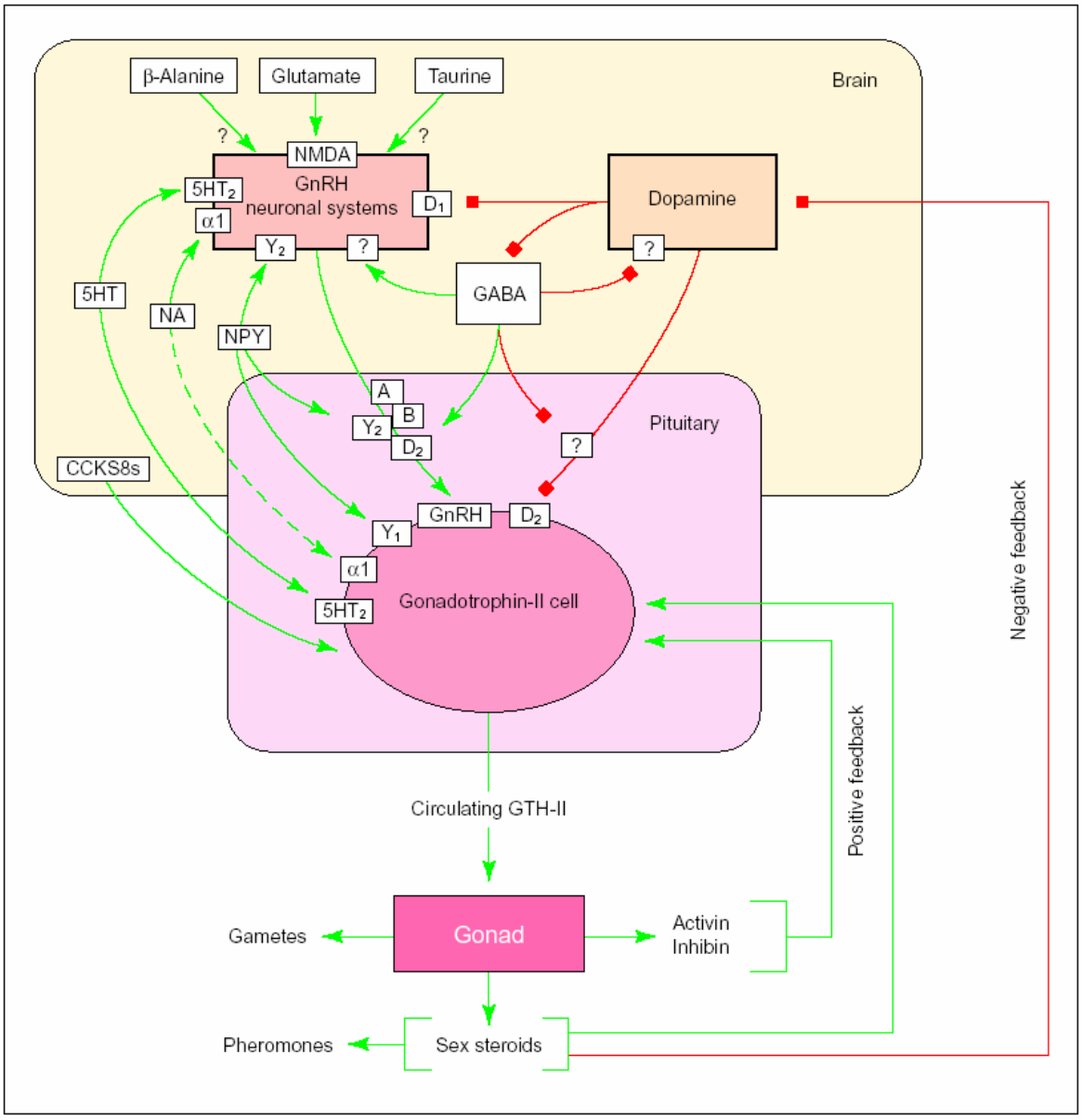

***Fig 7**: Multifactorial control of GnRH and gonadotrophin II (GTH-II) release in goldfish. Adopted from: Trudeau 1997* ➤: stimulation; ■: inhibition. *Recently Y1 NPY receptors are also believed to be involved (Senthilkumaran et al., 2001)*

gonadotropin inhibitory peptide (GnIH). It has been observed by immunocytochemistry that these axons project to cerebral areas where GnRH neurons are distributed indicating that they may have direct contact with GnRH neurons. The GnIH neurons posses

estrogen receptors and estrogen can induce GnIH production which has inhibitory effect on GnRH production negatively effecting gonadotropin secretion. This peptide is yet to be identified in teleosts and mammals.

Though the mechanism of GnRH synthesis, prepro hormone processing, GnRH receptor ligand interactions, regulation of gonadotropin subunits, sex steroid interactions, GnRH mediated intracellular signaling and receptor desensitization have been extensively studied over the past four decades since its first identification from bovine hypothalamic extracts, there remain more questions to be answered then ever.

Role of GnRH as the central regulator of sexual maturation has been established (Somoza *et al.*, 2002). But GnRH and GTH peptides have been detected early in brain even before fully differentiated gonads are observed raising questions as to its possible functions in early development of gonadal and cerebral sex differences. Possible clues to this may be obtained by following the ontogeny of GnRH and GTH peptides in male and female fish during early development. But this cannot be done in a wild mixed sex population as there exist no histological or morphological markers for distinguishing between male and female fish prior to gonadal differentiation and development. Hence, specially developed mono sex population is required for this purpose.

Thyroid hormones have been known to influence reproduction and gonadal function (Blanton and Specker, 2007). The effect of thyroid hormones on female reproduction and during development in juveniles has been extensively studied in both mammals and pisces but their role in male reproduction remains ambiguous. GnRH, GTH and sex steroids are the chief regulators of sexual maturation and seasonal gonadal development.

Limited literature exists as to the effect of thyroid hormones on GnRH, GTH and sex steroid levels vis à vis the interaction of hypothalamo-hypophyseal-gonadal and hypothalamo-hypophyseal-thyroid axis. Another important parameter to be considered in terms of this interaction is the effect of thyroid hormones on monoamines in POA-H as cathecholamines provide the chief stimulatory and inhibitory input for GnRH and GTH release.

An intriguing aspect of GnRH functioning is observation of GnRH transcription in gonads (Habibi and Pati, 1993). Compounding this intrigue is the observation that GTH subunits are produced in gonads. Given that GnRH and GTH are the main regulators of sex steroid production and contribute the mechanism behind endocrine regulation of sex steroid and reproductive stages, their production in gonads is confounding. Factors controlling GnRH expression in POA-H in pisces are largely unexplored as are the genetic basis for the pulsatile secretion pattern of GnRH, the tissue specific, region specific expression and photoperiod, seasonal and stress regulation. Isolation and analysis of the promoter elements involved in regulation of GnRH expression may provide important insights and understanding into these aspects.

In the backdrop of this existing state of knowledge, the present thesis is an effort in understanding the role of GnRH in early sexual differentiation, sex steroids, monoamine, thyroid hormone, GTH-GnRH interactions and implications of extra hypothalamic/pituitary GnRH-GTH production. Owing to the above mentioned relative advantages of studying neuroendocrine regulation in Piscine models, perciforms *Oreochromis niloticus* (the Nile tilapia), *Channa striatus* (snakehead

murrel), and a siluriform *Clarias gariepinus* (African or air-breathing catfish) have been utilized for the current studies.

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Chapter 1

Sex related differences in sbGnRH immunoreactivity in brain and pituitary of the Nile tilapia, Oreochromis niloticus during early development

Abstract:

In the present study, we investigated the variations in ontogeny of sbGnRH, the chief preoptic area-hypothalamus (POA-H) form of GnRH involved in sexual maturation between sexes to understand its impending role during sex differentiation. For this, the differences in immunocytochemical localization of sbGnRH in genetically male (XY) and female (XX) fish were studied from 1 day after hatching (dah), through the critical period of sex differentiation (7-21 dah) to 50 dah and in immature and mature Nile tilapia. SbGnRH Specific antisera against sbGnRH were used for immunolocalization. sbGnRH immunoreactive (ir-) neurons were observed in POA-H as early as 5 dah and 15 dah in XY fish and XX fish respectively. Higher immunoreactivity was detected in the POA-H of XY tilapia compared to XX population. There was a qualitative drop in sbGnRH ir- in POA-H around 20 dah till 30 dah in XY population compared to other durations. sbGnRH ir- cells were detected in pituitary of XX fish by 15 dah and in XY fish around 10 dah but seemed to drop down by 20 dah in XY while it continued to remain steady in XX fish. The sbGnRH ir- in XY fish showed a rise from 35 dah and thence till 50 dah. This study revealed subtle differences in POA-H and pituitary sbGnRH ir- during early development between male and female fish with possible implications in sex differentiation.

Introduction

Gonadotropin-releasing hormone (GnRH) is a central neuropeptide involved in regulation of vertebrate reproduction. It stimulates the synthesis of gonadotropins (GTH) from the adenohypophysis (Breton *et al.*, 1971), which in turn effect gonadal

maturation, and secretion of sex steroids. The hypothalamo-hypophyseal axis is subject to feedback influence of the sex steroids thus maintaining the loop (Kalra 1993). In addition to the well-referred role in GTH synthesis and release there also exists many reports supporting a neuromodulatory role for GnRH with implications in modulation of sexual behavior (White *et al.*, 1995; Soga *et al.*, 2005). Fourteen distinct GnRH variants have been characterized to date in vertebrates and all variants are decapeptides with sequence variations commonly being noted (occurring) in 5-8 amino acid positions (Sherwood *et al.*, 1997; Lethimonier *et al.*, 2004).

Most vertebrate species possess at least two different GnRH systems. Existence of multiple GnRH system in a single species was first identified in pisces (Powell *et al.*, 1994) and later established to be true in all vertebrate species. They vary in embryonic origin, neuroanatomical location and function (Parhar, 1997). Most vertebrate species have species specific septo-preoptic system and highly conserved midbrain (chicken GnRH-II [cGnRH-II]) system. Early teleosts like catfish and salmonids express a similar pattern i.e. they have a septo-Preoptic catfish GnRH (cfGnRH-II) or salmon GnRH (sGnRH) respectively and midbrain cGnRH-II system. However, some of the more evolved teleosts like seabreams and other perciforms, posses three different GnRH variants in one species (Powell *et al.*, 1994; White *et al.*, 1995; Amano *et al.*, 1997). *Oreochromis niloticus* (the Nile Tilapia) belongs to the class perciforms and has three distinct GnRH variants (Senthilkumaran *et al.*, 1999). The sGnRH localized in the terminal nerve ganglion is believed to be involved in modulation of sexual behavior while cGnRH-II localized in the midbrain tegmentum, is involved in neuromodulation. The seabream GnRH (sbGnRH) which is the species specific form is localized in the POA-H. Though *in vitro* all three GnRH variants are capable of

stimulating GTH secretion from pituitary, under physiological conditions the POA-H form is the main form involved in GTH release (Senthilkumaran et al., 1999). Teleosts lack a hypothalamic- hypophyseal portal system and the POA-H GnRH neurons directly innervate the proximal pars digitalis where they stimulate GTH secretion via controlled GnRH release as evident from *in- situ* studies and immuno localization (Suzuki *et al.*, 1992; Gothif *et al.*, 1996; Amano *et al.*, 2002). Although cGnRH-II and sGnRH are widely distributed in brain their absence or very low levels in POA-H and pituitary rule out their role in gonadotropin secretion under physiological conditions (Senthilkumaran *et al.*, 1999). *In vitro* studies depict that sbGnRH is the main form of GnRH released from POA-H slices on monoaminergic stimulation (Senthilkumaran *et al.*, 2001). Further seasonal variations in sbGnRH but not sGnRH and cGnRH-II coincide with the changes in gonado-somatic index and reproductive stage of gonad (Senthilkumaran *et al.*, 1999).

Sex differentiation in tilapia is largely determined by genetic factors but is susceptible to manipulation by treatments that alter sex steroids or by administering sex steroids and their analogs like methyltestosterone and ethnylestradiol, during a critical window of 7-14dah (Nagahama, 2000; Kwon *et al.*, 2000; Kobayashi *et al*., 2003). This period corresponds to the crucial period of sex differentiation in Tilapia. The POA-H GnRH system is critical for sexual maturation, sexual behavior and for synchronization of the gonadal cycle (Senthilkumaran *et al.*, 1999). The appearance of GnRH neurons in POA-H region coincides with period of sex differentiation (Soga *et al*., 2005). In light of the above cited literature and reports that sbGnRH is the chief form of GnRH involved in sexual maturation and reproduction, we chose to study variations in sbGnRH ontogeny between XX and XY Nile tilapia using immuno localization

techniques and their possible relevance to sex differentiation. This could have implications in the process of sex differentiation. Corroborating this line of thought are reports in sex changing fish and hermophrodites (Lee *et al.*, 2001; Du *et al.*, 2005), quoting variations in levels of POA-GnRH during crucial stages of sex reversal. Further, subtle variations in GnRH levels and localization may also influence differences in sexual behavior. The present study is the first of its kind in lower vertebrates using specific sbGnRH antibodies and genetic sex population.

Materials and methods:

Fish and sampling protocol: All XX and XY population of the Nile tilapia were obtained as described by (Wang *et al.*, 2002). The hatchlings were collected at various intervals post hatching (1 day, 3 day, 5 day, 7 day, 10 day and thence up to 45 days at 5 day intervals each). Immature (three months) and mature tilapia (both sexes) were reared in aquarium tanks with constant aeration and 12 hr-light dark conditions. Brain and pituitary were dissected out from adult fish and fixed while the entire larvae were fixed as such in Bouin's fluid and then cast into paraplast blocks for sectioning. Serial sagittal and cross sections of 6 µm thickness were cut in a rotatory microtome (Leitz, Germany) and spread on Mayer's albumin coated glass slides. The sections were then used for immunolocalization.

Immunocytochemistry : Immunocytochemistry (ICC) was carried out using the avidin-biotin peroxidase method. Sections were deparafinnised in xylene, rehydrated in successively lower graded concentrations of ethanol and then treated with 0.1% H_2O_2 to prevent endogenous peroxidase reaction. The sections were washed twice in

0.1M phosphate buffered saline (PBS) -1% Tween 20 and then blocked with 10% normal goat serum in 0.1M PBS for 10 minutes at room temperature. The primary antibody in PBS-Tween-BSA was applied to the sections and incubated for 48 hrs at 4^0C in a humid chamber. The primary antibody used in the present study was raised against synthetic sbGnRH peptide and showed very high specificity and very low cross reactivity with other forms of GnRH (Senthilkumaran *et al.*, 1999). Following incubation with primary antibody, sections were washed twice with PBS to remove excess unbound antibody and incubated with biotin conjugated secondary antibody at room temperature for 1 hr. Following thorough washes the sections were incubated with streptavidin labeled horse radish peroxidase (HRP) conjugate for 30 min at room temperature. The sections were washed and color was developed using commercially supplied 3' 3' diaminobenzidine (DAB) substrate for HRP. The sections were incubated for optimal color development in DAB for 15 min following which the sections were washed, dehydrated in graded ethanol, cleared in xylene and mounted using DPX mountant. All reagents and secondary antibodies for performing ICC were obtained from Bangalore Genei Pvt Ltd, Bangalore, India.

Results:

Figure 1 gives a schematic representation of the distribution of sGnRH, sbGnRH and cGnRH-II in sagital section of teleost brain. cGnRH-II ir- cell bodies were observed around mid brain tegmentum (MT) while sGnRH ir- cell bodies were most abundant in the olfactory bulb region and ventromedial olfactory bulb (VOB), terminal nerve ganglion (TNG).

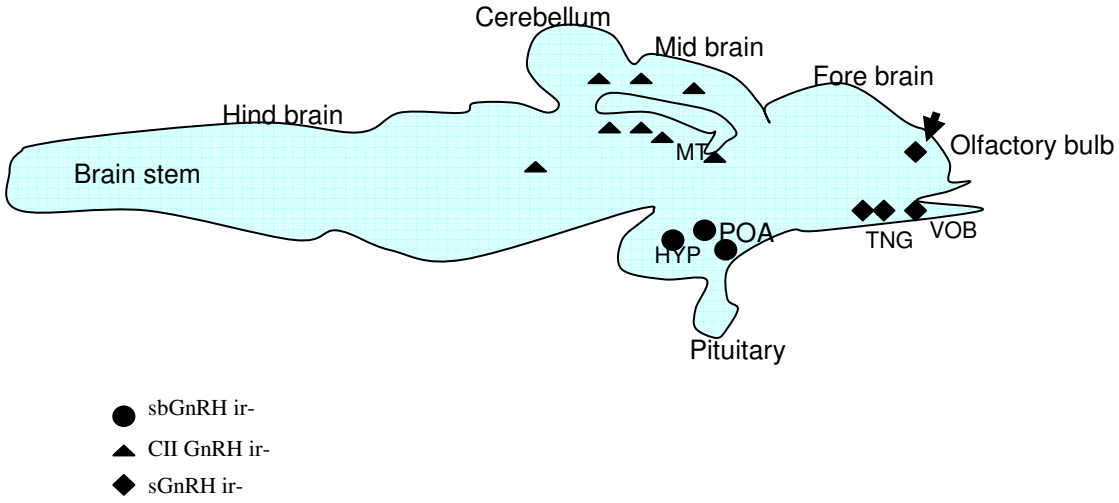


Fig 1: *Schematic representation of the distribution of sGnRH, cGnRH-II and sbGnRH immunoreactive cell bodies in different regions of teleost brain. MT midbrain tegmentum, VOB Ventromedial olfactory bulb, TNG terminal nerve ganglion, POA pre optic area, HYP hypothalamus.*

cGnRH ir- cell bodies in MT and sGnRH in VOB are shown in Fig 2. sbGnRH ir- cell bodies were evident in the POA-H, nucleus preopticus, optic chiasma (OC) and in the anterior pituitary (Fig 1, Fig 2C). sbGnRH ir- was detectable in POA-H of XY tilapia

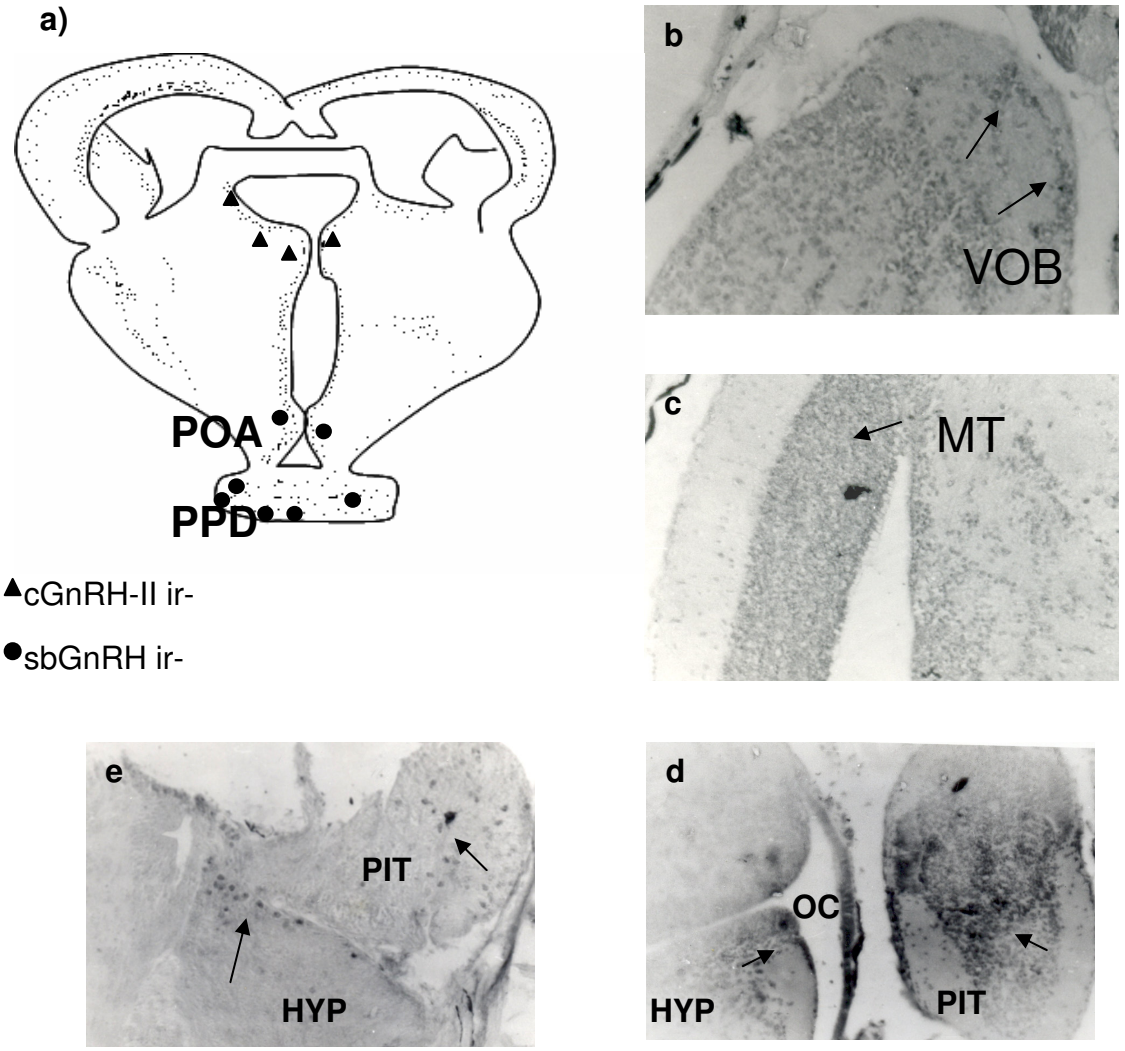
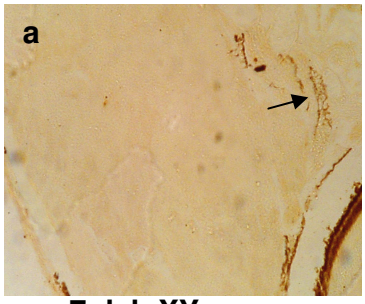
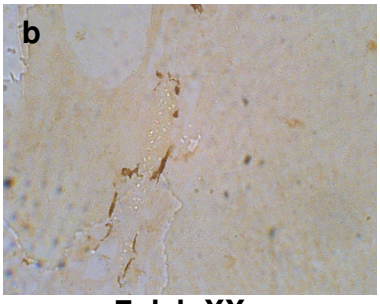


Fig 2: *sbGnRH immunoreactivity in cross section of POA-H and Pituitary of female (a)Schematic representation of distribution of cGnRH-II ir- and sbGnRH in cross section of tilapia brain and pituitary. (b) sGnRH ir- cell bodies in VOB (for schematic representation refer Fig 1, (c) cGnRH-II ir- cell bodies in Midbrain tegmentum (MT) (d) sbGnRH ir- in POA-H and pituitary of adult male and female (e) brain. Fig b, c, d, e – 50X magnification and f- 200X magnification. Arrows indicate positive ir-*

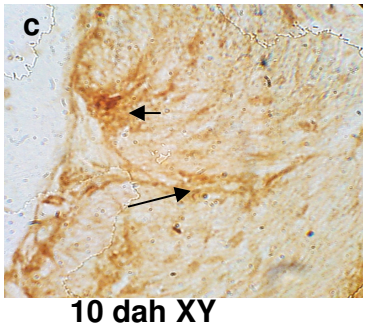
from 5 days after hatching (dah) onwards (Fig 3a and b). It increased progressively from 5 to 15 dah in XY. During this period XX tilapia did not express any detectable sbGnRH ir- in POA-H.



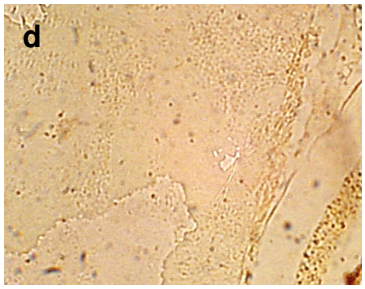
7 dah XY



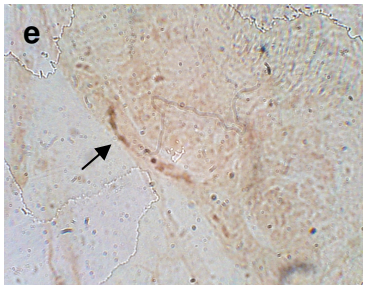
7 dah XX



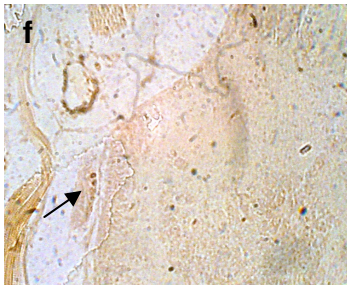
10 dah XY



10 dah XX



15 dah XX



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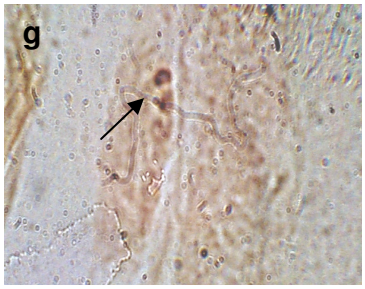
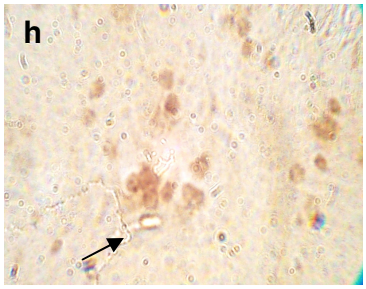
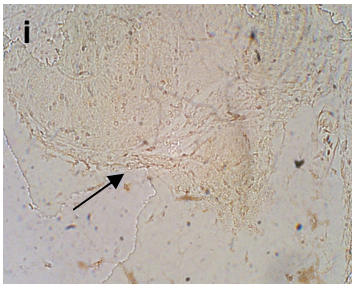


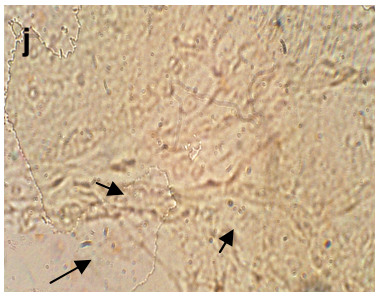
15 dah XX



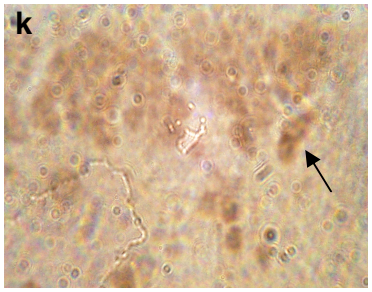
15 dah XY



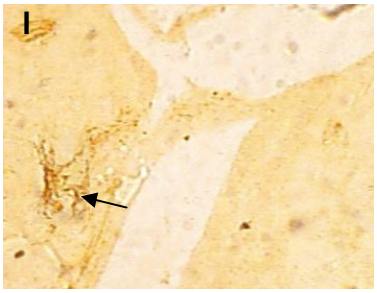
20 dah XY



20 dah XY



20 dah XX



30 dah XX

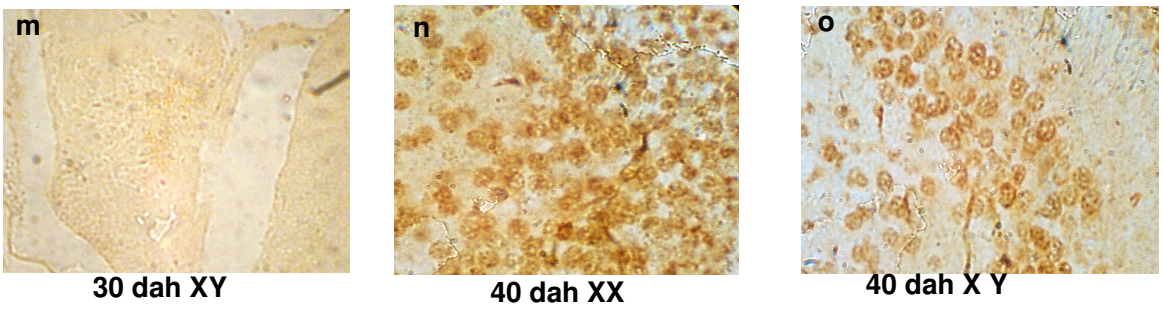


Fig 3: *sbGnRH immunoreactivity in saggital sections of XX and XY brain at various intervals post hatching. n, o 50X magnification; a, b, e, f, I, m – 100X magnification; c, d, g, h, j, k – 200X magnification. Arrows indicate positive ir-*

sbGnRH ir- was observed in pituitary of XY tilapia by 10 dah (Fig 3 c and d). SbGnRH ir- was detectable in POA-H of XX tilapia by 15 dah and showed a progressive increase in ir- till 30 dah. However, XY tilapia showed a decrease in sbGnRH ir- from 20 dah

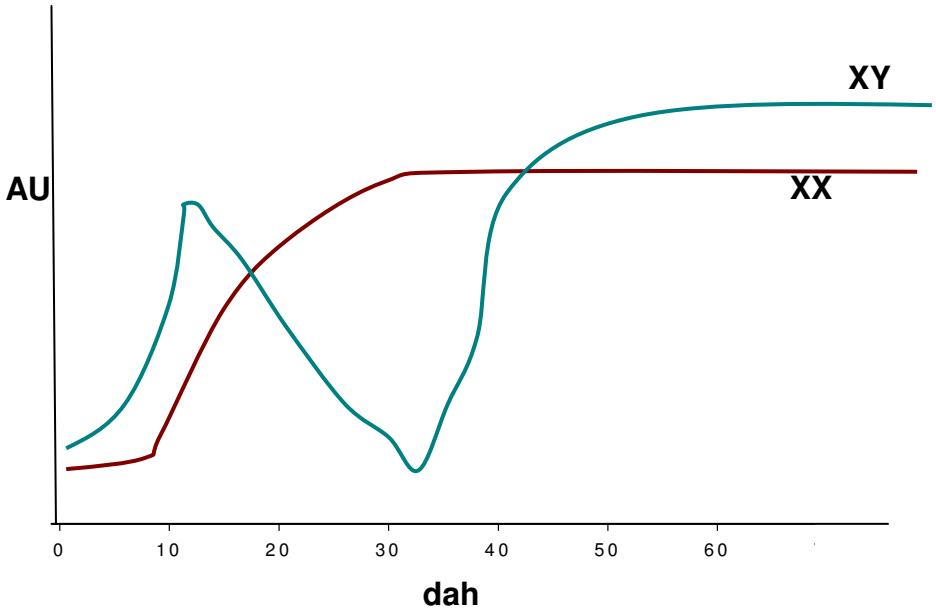


Fig 4: *Schematic graphical representation of the intensity of sbGnRH ir- in POA-H of XX and XY fish at different dah.*

(Fig 3e, f, g, i, j, k) and by 30 dah it was almost undetectable in POA-H. By 15 dah only few sbGnRH ir- cell bodies were detected in POA-H of XY fish (Fig 3h). sbGnRH ir- was observed in pituitary of XX tilapia by 30 dah during which period sbGnRH ir- was absent in pituitary of XY fish(Fig 3l and m). sbGnRH ir- remained more or less similar from 30 to 50 dah (maximum duration studied) in XX tilapia while in XY tilapia sbGnRH ir- increased from undetectable levels at 30 dah to levels comparable to XX tilapia by 50 dah. sbGnRH ir- was much higher in POA-H and pituitary of adult males compared to females (Fig 3). Fig 4 presents a schematic representation of the sbGnRH levels in POA-H based on the intensity of ir-.

Discussion:

The present results corroborate the early appearance of GnRH ir- in POA-H even prior to or at the time of onset of gonadal sex differentiation (i.e. 7 to 14 dah). sbGnRH ir- in POA-H showed a distinct pattern of expression in XX, XY fish during this critical period of sex differentiation. Specificity of the polyclonal antisera for different GnRH forms used in the present study for localization of sbGnRH, cGnRH-II and sGnRH antisera was evident from the different patterns of ir- corroborating with earlier reports of varied pattern of their cerebral distribution (Parhar, 1997; Senthilkumaran *et al.*, 1999). This was the same antibody used for radioimmnoassay (RIA) studies by Senthilkumaran *et al.* (1999) wherein a detailed account of cross reactivity with different forms of GnRH was reported (Senthilkumaran *et al.*, 1999). The specificity of the antibodies was therein validated by RIA in competitive binding

assays using sbGnRH, cGnRH-II and sGnRH from different perciform species including the Nile tilapia. Specific RIA systems for detection and quantitation of sbGnRH, cGnRH-II and sGnRH were also developed with these antibodies and a cross reactivity of less than 0.01% with other forms of GnRH was observed (Senthilkumaran *et al.*, 1999) i.e. antisera against sbGnRH showed less than 0.01% cross reactivity with cGnRH-II and sGnRH like wise for other forms such as sGnRH and cGnRH-II. In other words, the GnRHs antisera are highly specific for the form for which it was generated.

GnRH neurons have been earlier reported to originate in the olfactory bulb region and later migrate to the respective positions as present in the adult (Tobet *et al.*, 1997; Amano *et al.*, 2002; Elizabeth *et al.*, 2005). However, in the present study no such migratory pattern was observed in the sbGnRH neurons with respect to both XX, XY fish. This is unlike in mammals but similar observations were made in some other cichlid fish like *Cichlasoma dimerus* (Pandolfi *et al.*, 2002). It appears that in these fish a population of POA-H neurons originates in the POA-H itself or else migrate from some other cerebral or extracerebral region and start synthesis of GnRH (i.e. become GnRH ir-) only after reaching the POA-H region. This however remains a difficult paradigm to be solved as the only distinguishing feature of GnRH neurons is synthesis of GnRH. Hence, GnRH neurons thus far cannot be traced prior to GnRH synthesis.

POA-H sbGnRH ir- appeared in XY fish much earlier than XX fish and reached peak intensity by 10 dah midway between the critical period of sex differentiation in tilapia. During this period GnRH ir- in POA-H of XX fish was negligible. This raises the question as to the possible role of POA-H GnRH during early gonadal sex

differentiation. Another important report supporting this view is the observation that POA-H GnRH levels exhibit a distinct pattern of variation in certain sex reversing fish during the critical period of sex reversal (Lee *et al.*, 2001; Du *et al.*, 2005). Studies in black porgy, a protandrous marine teleost have indicated that high levels of plasma luteinizing hormone (LH) and POA-H GnRH promote testicular development while low levels of LH and POA-H GnRH promote testicular regression and ovarian development (Du *et al.*, 2005). The testicular tissue has more number of GTH receptors and is more sensitive to GTHs than ovarian tissue (Kramer *et al.*, 1993). The observation that high levels of sbGnRH observed in POA-H and pituitary of XY fish compared to XX fish may be important for testicular differentiation is further emphasized by the observation that exogenous hCG, LH or GnRH induced gonadal sex change from female to male in two protogynous teleost species, *Thalassoma bifasciatum* and *Monoptrus albus* (Tang *et al.*, 1974; Koulish and Kramer, 1989; Kramer *et al.*, 1993) but not in protandrous species like black porgy (Du *et al.*, 2005). Results from our recent studies revealed that both LH and follicle stimulating hormone (FSH) levels are significantly higher in XY fish compared to the XX population during early development of the Nile tilapia (Sakai *et al.*, 2005).

Another important factor involved in early sex differentiation is aromatase and estradiol (E_2; Tsai *et al.*, 2001). Higher aromatase activity is present in male brain. But the correlation between these early changes in brain aromatase activity, E_2, LH and POA-H GnRH are yet to be established. A correlation between these factors could be obtained from estimation of steroids and plasma LH, FSH levels, but this was not feasible in the current study due to the inability to obtain serum from 1 day to 30 day hatchlings (i.e. during the critical period of sex differentiation). Whole body

homogenates could be used but this does not give us an idea of the steroid source. Finally it remains to be established whether the changes in POA-H GnRH ir- have any role to play in differential synthesis of sex steroids during gonadal sex differentiation or is merely secondary to the variation in sex steroid profiles in XX and XY during gonadal sex differentiation.

Though it has been difficult to establish the pattern of variation in sex steroids between sexes during early development due to limitations in sample size and plasma volume, steroidogenic enzyme genes and aromatase have been localized in gonads of XX fish during early sex differentiation i.e. by 7-14 dah (Nagahama, 2000; Nagahama, 2005). The presence of binding sites for sex dependent transcription factors like SOX-9, SOX-5 (Kitahashi *et al.*, 2005) in the sbGnRH promoter may suggest that the varied pattern of sbGnRH expression in XX and XY during early gonadal sex differentiation may be a primary effect driven by sex dependent factors rather than secondary to feedback by sex steroids. Neverthless, both mechanisms may act synergistically to induce this differential expression of sbGnRH in POA-H of XX, XY fish vis-à-vis gonadal sex differentiation. A similar differential pattern of GnRH ir- between male and female mice was also reported during early development (Gore *et al.*, 1999) indicating this mechanism is evolutionarily conserved from fishes to mammals implying important functional consequences to this phenomenon.

The present study for the first time establishes distinct differences in the ontogeny of POA-H sbGnRH ir- during critical period of sex differentiation in genetic sex (XX, XY) population. Previous studies on ontogeny of GnRH concentrated on distinct pattern of distribution and origin of various GnRH forms and did not take into consideration differences between genetic sex populations (see Parhar, 1997). The

mechanisms underlying this differential expression of POA-H sbGnRH during early gonadal differentiation and their impact on gonadal sex differentiation remains to be established. Studies are also underway in our laboratory to elucidate the possible role of SOX family of transcription factors and sex steroids in this differential expression of sbGnRH in POA-H between sexes.

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Chapter 1.1

Sex related differences in LH and FSH immunoreactivity during early development of the Nile tilapia, Oreochromis niloticus

Abstract:

Gonadal development and sex steroid synthesis in teleosts, is regulated by two gonadotropic hormones; luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Earlier studies in tilapia have shown that FSHβ and LHβ appear by 14 days after hatching (dah), results from the current study corroborate with these previous reports in tilapia. Here we demonstrate the appearance of LH in pituitary between 14 dah and 20 dah. In addition to this the present study primarily focuses on any possible differences in appearance of LHβ and FSHβ immunoreactivity (ir-) between XX and XY population of Nile tilapia. LH ir- was found to be lower in pituitary of XX fish when compared to XY fish. The development of FSHβ ir- in pituitary of the Nile tilapia is also presented. Overall, it remains to be established what significance these findings on the appearance of gonadotropins hold for sex differentiation in tilapia.

Introduction

In teleost fishes, like in other vertebrates, gonadal development is stimulated by two gonadotropic hormones; luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The pituitary gland synthesizes the gonadotropic hormones, which are heterodimers containing noncovalently linked α and β subunits. Within a species, the α subunits are identical, while the β subunits differ and confer the physiological specificity to the hormone. LH and FSH are synthesized and released from the anterior pituitary regulated by the hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH). Observations from earlier studies in our laboratory showed that the GnRH system in

fishes is established around the same time as gonadal differentiation. Further GnRH protein expression showed significant variation between XX and XY individuals in studies from rodents (Villalobos *et al.*, 1997; Takahashi *et al.*, 1988). Since, the main functional role of GnRH in vertebrate reproduction is the regulation of LH and FSH release, these observations with respect to GnRH may have implications for LH, FSH expression in XX and XY populations. Fan *et al.* (2003) reported expression of FSHβ subunits in pituitary from 0 days after hatching (dah) and also quote the expression of FSH and LH receptors from 5 dah i.e. much prior to sex differentiation, allowing for the assumption as to the possible role of LH and FSH in sex differentiation in fish. Reports on LHβ and FSHβ glycoprotein appearance in pituitary during development of teleosts with emphasis on the comparison between XX and XY fish are incomplete and sparse. Thus to achieve a better understanding of the role of gonadotropins in teleosts during the critical period of sex differentiation, we analyzed the presence of LHβ and FSHβ glycoproteins in pituitary over the period of development from 1 dah to 50 dah- the period corresponding to gonadal sex differentiation in XX and XY individuals of Nile tilapia.

Materials and Methods

Collection of Fish: Production of XX and XY population of the Nile tilapia was done as described by Wang *et al.* (2002). Tilapia XX and XY fry were collected from 1 dah onwards every 2 dah till 8 dah and at 5-day interval from 10 dah to 50 dah. Whole

animals were fixed in Bouin's fluid till 25 dah. From 30 dah to 50 dah head was dissected and fixed.

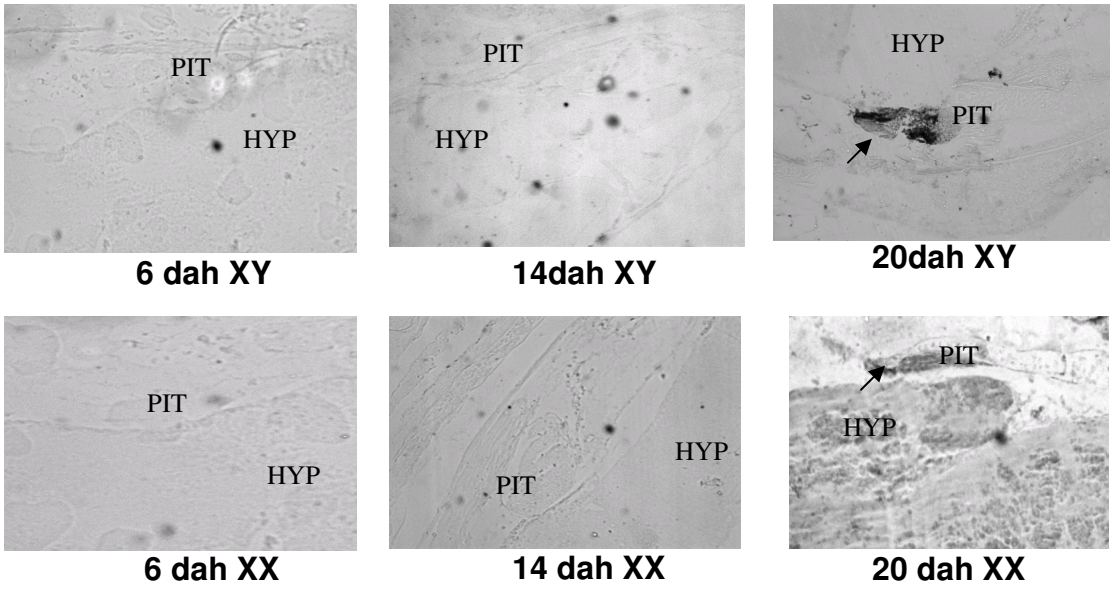
LH and FSH Immunocytochemistry: Tissues were fixed in freshly prepared Bouin's fluid for 1-2 h at room temperature, then processed with graded alcohol series and embedded in paraplast (Kendall, Mansfield, MA, USA). Sections of 6µ thickness were cut with a rotatory microtome (Leitz, Wetzlar, Germany) and immunohistochemistry (IHC) for LH and FSH was carried out according to the following procedure. The sections were deparaffinised and rehydrated in a gradient of decreasing concentrations of alcohol and finally in PBS containing 1% tween (pH 7.6). The sections were then treated with normal goat serum (1:10 dilution). Primary antibodies against LH and FSH at 1:2000 dilution were applied to the sections and incubated at 4°C for 48 h in a humid chamber. The sections were then washed 3 times with PBS at 5 min interval and incubated at room temperature for 1 h with biotin labeled anti-rabbit IgG. The unbound antibody was washed away with PBS and the sections were incubated at room temperature for 30 min with streptavidin horseradish peroxidase conjugate. The sections were then washed with phosphate buffer (PB) and incubated with 0.05% 3,3'diamino benzidine tetra hydrochloride with 0.01% H_2O_2 in 0.05M Tris-HCl (pH 7.6) for 10 min in a dark chamber at room temperature. The slides were then rinsed with PB, dehydrated through graded alcohol series, cleared in xylene and mounted in DPX. All the chemicals and secondary antibodies used for IHC were part of the kit components supplied by Bangalore GENEI Pvt. Ltd. (Bangalore, India).

Characterization and crossreactivity of the LH, FSH antibodies were earlier reported by Shimizu *et al.* (2003).

Results:

LH immunoreactivity (ir-) appeared in pituitary of XY fish by 20 dah. No LH ir- was observed at 6 dah and 14 dah. LH ir- was present consistently from 20 dah, 30 dah and 50 dah (Fig 1) in males. In XX fish LH ir- was observed in pituitary of 25 dah, 35 and 50 dah. LH ir- was not observed in pituitary of XX fish by 6 dah and 18 dah (Fig 1). FSH ir- was observed in XX fish by 29 dah and in XY fish also around 25 dah (Fig 2).

LH ir- was more diffusely distributed in pituitary while FSH ir- was localized in distal regions. LH ir- in XX fish was less intense in XX when compared to XY fish. FSH ir- appeared in pituitary of XY by 20 dah and only by 28 dah in pituitary of XX fish.



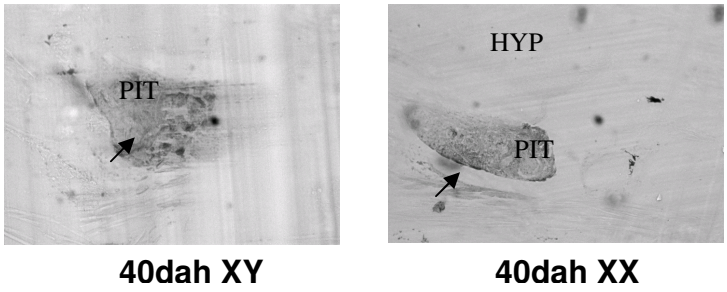


Fig 1: LH ir- in pituitary of XY and XX Nile Tilapia. Arrows indicate positive ir-. PIT indicates pituitary and HYP indicates preoptic area- hypothalamus. All images were taken at 20X magnification

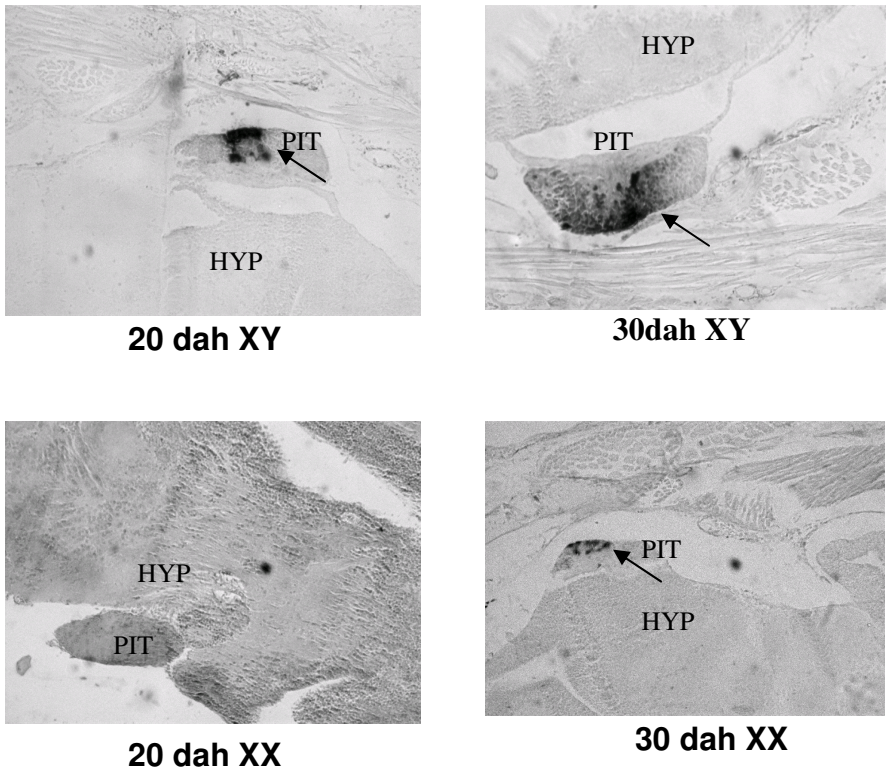


Figure 2: FSH ir- in pituitary of XY and XX Nile Tilapia. Arrows indicate positive ir-. PIT indicates pituitary and HYP indicates preoptic area- hypothalamus. All images were taken at 20X magnification.

Discussion:

The present study demonstrates the appearance of LHβ and FSHβ protein in pituitary by 18 to 25 dah in both XX, XY fish. This coincides with the critical stage of sex differentiation in tialpia, which lasts from 14 dah to 21 dah. Fan *et al*. (2003) reported for the presence of FSHβ mRNA in pituitary by 5 dah while FSH ir- was observed from 25dah onwards in the present study. This indicates that a translational delay occurs between transcriptional activation and protein expression of FSHβ. This may in part be attributed to a probable delayed expression of gonadotropin α (GTHα) subunit that is required for the formation of the complete FSH glycoprtein. GTHα expression during development of tilapia has not yet been reported. The appearance of GTHα might be an important determinant for the late appearance of FSH ir-, inspite of the presence of FSHβ transcript from 0 dah onwards. It is also conceivable to interpret a delay in translational activation of the FSHβ transcript the exact mechanism of which requires further explanation. Parhar *et al.* (2003) had earlier reported the simultaneous appearance of FSH and LH ir- in the brain and pituitary of tilapia by 14 days after fertilization. The appearance of FSH and LH ir- in the present study seems to be delayed. This difference of 4-5 days may be due to variations in rearing and growth conditions or because of the usage of specific XX and XY population. In addition, in the present study we used heterologous antibody raised against FSH and LH β subunits of red seabream *Pagrus major*, a related perciform. Furthermore, in contrast to the earlier study on similar topics the current study focuses on differences in LH and FSH ir- between XX and XY fish. LH ir- was found to be higher in XY fish compared to XX fish.

The LHβ ir- seems to be lower in XX fish, however gradually increasing from 25 dah to 40 dah while the pituitary from XY fish exhibit high ir- by 20 dah.

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Chapter 2

Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, Clarias gariepinus

Abstract

In the present study, thiourea-induced thyroid hormone depletion was used as a strategy to understand the influence of thyroid hormones on testicular recrudescence of the air-breathing catfish, *Clarias gariepinus*. Treatment with 0.03% thiourea via immersion for 21 days induced hypothyroidism (thyroid hormone depletion) as evidenced by significantly reduced serum T_3 levels. Thiourea-treated males had narrowed seminiferous lobules with fewer spermatozoa in testis, very little or no secretory fluid, reduced protein and sialic acid levels in seminal vesicles when compared to controls. The histological changes were accompanied by reduction in serum and tissue levels of testosterone (T) and 11-ketotestosterone (11-KT), a potent male specific androgen in fish. Qualitative changes in the localization of catfish gonadotropin-releasing hormone (cfGnRH) and luteinizing hormone (LH, heterologous system) revealed a reduction in the distribution of immunoreactive neuronal cells and fibers in thyroid depleted fish. Interestingly, thiourea-withdrawal group showed physiological and histological signs of recovery after 21 days such as reappearance of spermatozoa and partial restoration of 11-KT and T levels. These data demonstrate that thyroid hormones play a significant role in testicular function of catfish. The mechanism of action includes modulating sex steroids either directly or through the hypothalamo (GnRH)-hypophyseal (LH) axis.

Introduction

Thyroid hormones are essential endocrine mediators exerting tissue and development specific effects in almost all vertebrates studied to date (King and May 1984; Buchholz *et*

al., 2003). Studies in mammals demonstrated a critical role for thyroid hormones in reproduction and embryogenesis (Stradtman 1993; Jannini *et al.*, 1995; Teerds *et al.*, 1998) in addition to its inputs in cellular metabolism. Thyroid abnormalities are associated with irregularities in reproductive cycles and delayed onset of puberty (Jannini *et al.*, 1995; Maran, 2003). The role of thyroid on ovarian function is well known (Stradtman, 1993), while similar studies on male reproductive system present several conflicting observations. Weiss and Burns (1988) indicate little or no effect on testicular histology, spermatogenesis and serum testosterone (T) levels while Crissman *et al.* (2000) reported a decrease in serum T levels and sperm motility with no marked reduction in fertility. T_3 affects Sertoli cell proliferation and differentiation, the onset of the formation of adult-type Leydig cell population and their function during the neonatal-prepubertal period in rats (Teerds *et al.*, 1998; Ariyaratne *et al.*, 2000). Neonatal hypothyroidism in rats increases adult testis size and capacity for sperm production while neonatal hyperthyroidism shows an opposite effect. The timing of hypothyroidism around puberty affects testicular function (Anasti *et al.*, 1995). Hypothyroidism also affects biochemical constituents and functions of male accessory reproductive glands such as prostate and seminal vesicles in adult rodents (Buitrago and Diez, 1987; Senthilkumaran *et al.*, 1991; Maran, 2003).

Studies in fish contributed relevant information to understand the role of thyroid in metamorphosis (de Jesus *et al.*, 1991), embryogenesis (Liu and Chan, 2002) and reproduction (Reine and Leatherland, 2003). Teleosts do not posses a compact thyroid gland as do other vertebrates; rather the thyroid follicles are diffusely dispersed. The

reproductive state during the course of the study assumes significance as thyroid hormones may exert a stage dependent effect. Fish have well defined seasonal reproductive cycles associated with a definite pattern of changes in thyroid hormone levels (Cyr *et al.*, 1988). Much of the earlier work in fish with regard to thyroid hormone on male reproduction was done in the spawning phase and/or concentrated only on few histological morphometric changes in interstitial cells (Misra and Pandey, 1985). The role of thyroid hormones in fish reproduction generated new insight after the recent cloning of thyroid stimulating hormone (TSH) receptor from testis of the African catfish, *Clarias gariepinus* (Vischer and Bogerd, 2003), gonad of the striped bass, *Morone saxatilis* (Kumar *et al.*, 2000) and the demonstration that the goitrogen 6-n-propyl-2-thiouracil given during testis development increases Sertoli and germ cell numbers per cyst in the Nile tilapia *Oreochromis niloticus* (Matta *et al.*, 2002). Recent evidences also indicated the association of thyroid with gonadotropin-releasing hormone (GnRH) and catecholaminergic systems in fish (Parhar *et al.*, 2000; Chaube and Joy, 2003), raising the possibility for thyroid hormone action on gonadal function via hypothalamo-hypophyseal axis.

The present study sought to investigate the effect of thyroid hormone depletion on the male reproductive system of the air-breathing catfish during testicular recrudescence (stage corresponding to renewed spermatogenesis), with special emphasis on changes in testicular and 'seminal vesicle' histology, serum and tissue levels of T and 11-ketotestosterone (11-KT). Qualitative changes in the localization of gonadotropin-releasing hormone (catfish form; cfGnRH) and luteinizing hormone (LH; heterologous

system) were also probed. Thiourea, a well-documented thyroid hormone depleting compound was used to induce hypothyroidism. It has been shown to act via inhibition of thyroid peroxidase (Davidson *et al.*, 1979).

Materials and Methods: Adult (early recrudescence/early preparatory phase) air-breathing catfish *C. gariepinus* (200-250g) caught from local (Hyderabad, India) fresh water ponds were purchased from fisherman at the end of January 2004 for experimentation. Male fish were selected and acclimated for 3 weeks by maintaining in aquarium tanks filled with filtered tap water under normal photoperiod and ambient temperature (26±2°C). Fishes were fed minced goat liver *ad libitum* during acclimation and experimentation. Commonly referred to as the African catfish, this species is also abundantly available in ponds and lakes in and around Hyderabad, and in some rivers of the north India. However, not much information is available with regard to the seasonal cycling patterns of this species in the south India. Our preliminary observations revealed that the local population exhibited a seasonal cycling pattern. Four broadly distinguishable phases, namely, early preparatory, prespawning, spawning phases and postspawning were seen in both sexes (Lehri, 1967; van Oordt *et al.*, 1987) as opposed to the laboratory maintained fish kept under constant photoperiod and temperature conditions that are ready for spawning throughout the year (van Oordt *et al.*, 1987). Wild caught fish are annual breeders and the spawning phase lasts for about three months from June to August coinciding with the south west monsoon in the region. However, sometimes the spawning season could extend up to late October. The preparatory phase

lasts from the mid February to May and fish caught around late November to January are usually in resting to early preparatory phase.

Thiourea treatment: The laboratory acclimated fish were divided into three groups of 10 fish each. The first group of fish (Group I) were sacrificed to examine status of testis prior to the start of treatment with thiourea. Unlike mammals, in fish the thyroid gland is not a single encapsulated organ, rather the thyroid follicles are diffusely dispersed within the connective tissue on the surface of the ventral aorta and other sub pharyngeal and para pharyngeal areas. This particular feature makes it difficult to induce thyroid hormone depletion in fish by routine thyroidectomy. Hence, the chosen route for thyroid hormone depletion in fish is by using thiourea, one of the known thyroid hormone depleting compounds. After pilot studies, thyroid hormone depletion was induced by adding thiourea (SRL, Mumbai, India) to the final concentration of 0.03% (Misra and Pandey, 1985; Chaube and Joy, 2003) to well-aerated aquarium tanks holding the catfish for 21 days. Group II fish were maintained in filtered water as parallel control. Group III fish were maintained in filtered water containing thiourea, which was replenished once a day. Five fish from Group III were not sacrificed after 21 days thiourea treatment. They were maintained for an additional 21 days period in absence of thiourea and then sacrificed to study effects of withdrawal of thiourea.

Sample collection and processing: After the duration of 21 days, fish from both groups were weighed and blood was obtained by caudal puncture. Serum was obtained by

centrifuging the blood after clotting at 1500xg and stored in -80°C freezer till hormone estimations were done. Testes and seminal vesicles were dissected out, weighed. 500mg was fixed in Bouin's for histological examination, and the remaining tissue was snap frozen in liquid nitrogen till processing for analysis of protein, sialic acid, 11-KT and T levels. Tissue processing for all biochemical estimations was done at 4°C. Testis and seminal vesicular tissues were homogenized in 0.1M phosphate buffered saline (PBS) and the resulting homogenate (10%) was lyophilized and stored briefly in -80°C freezer for the estimation of hormones.

For histological examination, tissues were fixed in freshly prepared Bouin's fluid for 1-2h at room temperature, then processed with graded alcohol series and embedded in paraplast (Kendall, Mansfield, MA, USA). Sections of 6μm thickness were cut with a rotatory microtome (Leitz, Wetzlar, Germany) and stained with hematoxylin-eosin. Microscopic examinations and photography was done with a Nikon EFD-3 microscope fitted with a Nikon NFX-35 automatic camera (Nikon, Tokyo, Japan). Higher magnification images were obtained using a Leica microscope (Buffalo, NY, USA) fitted with a Kodak (DX 7630) digital camera. Cells in different stages of spermatogenesis (spermatogonia, spermatocytes, spermatids/spermatozoa), were counted from twenty different fields in various regions of testis. The percentage of three different stages was then calculated for each field against the total number of cells present in that field; the mean of twenty fields was calculated for each category. Since primary and secondary spermatocytes could not be distinguished, they were grouped as spermatocytes. Likewise spermatids and mature spermatozoa were grouped as spermatozoa.

Estimation of proteins and sialic acid: Estimation of Protein was done by the method of Lowry *et al.* (1951). Tissue sialic acid levels were determined as per the method described in Senthilkumaran and Joy (1993). In brief the tissue was precipitated with 5% trichloroacetic acid. After overnight drying in a dessicator, the precipitated portion was hydrolyzed in 1ml of 0.1N H_2SO_4 for 1 hr at 80°C to liberate the bound sialic acid. The mixture was centrifuged at 1000xg and 0.3 ml of the supernatant was oxidized with sodium metaperiodate in concentrated phosphoric acid. The periodate-oxidation product was coupled with thiobarbituric acid and the resulting chromophores were extracted into cyclohexane. The pink coloured solution (end-point) was measured at 532 and 562 nm in a Shimadzu spectrophotometer (Japan).

Enzyme Immunoassay (EIA) for T_3, T and 11-KT: Serum T_3 levels were assayed using specific T_3 EIA (Medix Biochemica, Kauniainen, Finland) kit. The intra- and inter assay coefficients of variation [n=5, mean ± SEM] were 1.34 ± 0.13% and 2.78 ± 0.15% for T_3. The sensitivity for T_3 was 0.2 ng/ml using the Medix Biochemica kit. Serum and tissue 11-KT and T levels were estimated using specific EIA (Cayman, Ann Arbor, MI, USA) kits using manufacturer's protocol. Intra- and inter assay variations were within the limits specified in the manufacturer's protocol. T antisera of Cayman kit cross reacts with 5α-Dihydrotestosterone (5α-DHT; 27%), 5β-Dihydrotestosterone (5β-DHT; 19%), Androstenedione (4%), 11-KT (2%), 5-Androstenedione (0.51%), Epi-testosterone (0.2%), Androsterone (0.05%), Androsterone sulfate (0.04%), Testosterone sulfate (0.03%), Dihydroepiandrostenedione sulfate (0.02%). T and 11-KT are the major

androgens in catfish (Cavaco *et al.*, 2001). Presence of 5α-DHT and 5β-DHT have not been earlier reported in catfish (Cavaco *et al.*, 2001; Cavaco, 2005). 11-KT antisera supplied in the Cayman EIA kit showed cross reactivity with 4-Androsten-11β,17β-diol-3-one (0.01%), T (<0.01%), 5α-Androstan-17β-ol-3-one (<0.01%), 5α-Androsten-3β-diol (<0.01%). A recovery of 93-95% was obtained by the extraction procedure described in the manufacturer's manual for T and 11-KT (Cayman, USA).The dilutions of series of serum samples and/or tissue extracts from catfish with differing hormone concentrations were linear with standards of T and 11-KT, respectively. Intra- and inter assay coefficients of variation [n=5, mean ± SEM] were 1.02 ± 0.04% and 1.81 ± 0.15% for T and 0.81 ± 0.09% and 1.32 ± 0.11% for 11-KT. The sensitivity was 6 pg/ml using the Cayman T kit and 1.3 pg/ml with the Cayman 11-KT kit.

Immunocytochemistry for cfGnRH and LH: Immunocytochemistry (ICC) for cfGnRH was carried out as per the following procedure. The sections were deparaffinised and rehydrated in a gradient of decreasing concentrations of alcohol and finally in PBS containing 1% tween (pH 7.6). The sections were then treated with normal goat serum (1:10 dilution). Primary antibodies against cfGnRH at 1:2000 dilution were applied to the sections and incubated at 4°C for 48 h in a humid chamber. The sections were then washed with PBS thrice at 5 min interval and incubated at room temperature for 1 h with biotin labeled anti-rabbit IgG. The unbound antibody was washed away with PBS and the sections were incubated at room temperature for 30 min with streptavidin horseradish peroxidase conjugate. The sections were then washed with phosphate buffer (PB) and

then incubated with 0.05% 3,3'diamino benzidine tetra hydrochloride with 0.01% H_2O_2 in 0.05M Tris-HCl (pH 7.6) for 10 min in a dark chamber at room temperature. The slides were then rinsed with PB, dehydrated through graded alcohol series, cleared in xylene and mounted in DPX. All the chemicals and second antibodies used for ICC were part of the kit components supplied by Bangalore GENEI Pvt. Ltd. (Bangalore, India).

Similar ICC procedure was followed for LH using the antiserum (pmGTH-II) raised against red seabream (*Pagrus major*) LH. The antiserum was found to work efficiently at 1:2000 dilution in our (heterologous) system. For better clarity of staining, all the ICC analysis was done with photomicrographs of 40X magnification or higher. Lower magnification (10X) with full field view of section was not shown. Characterization and cross-reactivity of cfGnRH and LH antisera are as earlier reported by Dubois *et al.* (2001), and Kagawa *et al.* (see1998a, b), respectively.

Relative quantification of LH in control and experimental animals was done by Western blot analysis using the same LH primary antibody used in ICC and Anti-rabbit IgG Alkaline phosphatase secondary antibody conjugate (Banglore Genei) followed by scanning densitometry using UV-SCAN-IT gel, Version 5.1 (Silk scientific cooperation, USA).

Data analysis: All data are expressed as mean ± SEM (n=5). Pair wise comparisons were done by one-way ANOVA followed by Student's-Newman-Keuls' test using Sigma Stat software. The level of significance was $P<0.05$ for all comparisons.

Results

Fish caught from local water bodies showed an annual seasonal cycling pattern histologically and morphological distinguishable into four distinct testicular stages: preparatory phase, prespawning with the spawning season lasting between July to early October and post-spawning phase (Table 1). Morphological examination of testis showed a considerable increase in testicular volume from mid February (preparatory phase) to July (prespawning/spawning phase). All regions of testis showed a similar pattern of spermatogenesis.

Table 1:

	January (First week)	**March (First week)**	**May (First week)**	**July (First week)**	**Late November**
Spermatogonia	**3.98** ± 0.69	**3.48** ± 0.39	**0.123** ± 0.01	**Not detectable**	**11.7** ± 1.02
Spermatocytes (primary/secondary)	**31.56** ± 1.07	**24.39** ± 1.24	**1.59** ± 0.09	**0.249** ± 0.008	**58.8** ± 3.52
Spermatids	**65.89** ±	**75.64** ±	**97.95** ±	**99.4** ± 0.5	**29.41** ±

/Spermatozoa	2.23	2.69	4.51		4.21

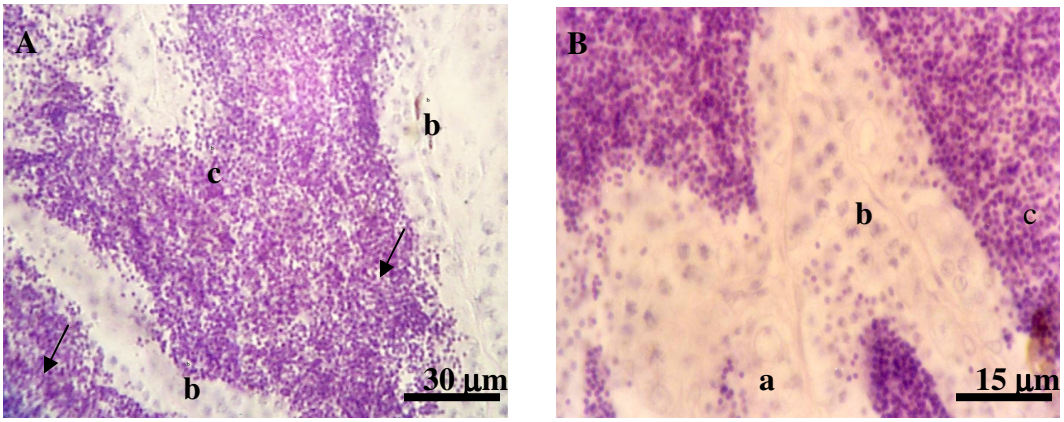
Percentage distribution of cells in different stages of spermatogenesis as observed in testis during various intervals in 2004 expressed as mean ± SEM (n=5).

T_3, protein, sialic acid levels: Treatment of thiourea significantly decreased (P<0.05) serum T_3 levels (0.290 ± 0.008 ng/ml) relative to parallel control group (0.701 ± 0.009 ng/ml). The reduction was more than 50% when compared to the parallel control group. Following thyroid hormone depletion, both total protein and sialic acid levels decreased significantly in testicular and seminal vesicular tissues, the effect being more pronounced in the latter (Table 2). Following withdrawal of thiourea treatment, the T_3 levels were restored (0.658 ± 0.017 ng/ml) to those observed in controls.

Table 2:

	Protein (µg/mg tissue)		Sialic acid (pmoles/mg tissue)	
	Parallel Control	Thiourea-treated	Parallel Control	Thiourea-treated
Testis	94 ± 2.5	76 ± 2.0[a]	30 ± 5	6.7 ± 0.05[a]
Seminal vesicle	19.8 ± 0.4	8.3 ± 0.5[a]	42.6 ± 1.06	17.3 ± 4.37[a]

Testis and seminal vesicle histology: Sections of testis from parallel control fish (Fig 1A and B) showed a significant increase in number of spermatids/spermatozoa and a decrease in other cell types namely spermatogonia and spermatocytes when compared to initial controls (Table 3). There was a decrease in total spermatids/spermatozoa count in thiourea-treated group (Fig 1C and D) when compared to initial and parallel control groups. However, there was no significant change in the total number of spermatocytes and spermatogonia. The apparent increase in percentage distribution of spermatocytes and spermatogonia evident from Table 3 is due to a large decrease monitored in the total cell counts (spermatogonia, spermatocytes and spermatids) in thiourea-treated fish mainly due to loss of spermatids/spermatozoa heads. Seminal vesicles showed extensive fluid filled lumina with copious secretions in the control group (Fig. 1E). In the thiourea-treated group, the volume of seminal vesicular lumina greatly decreased with residual or no secretory



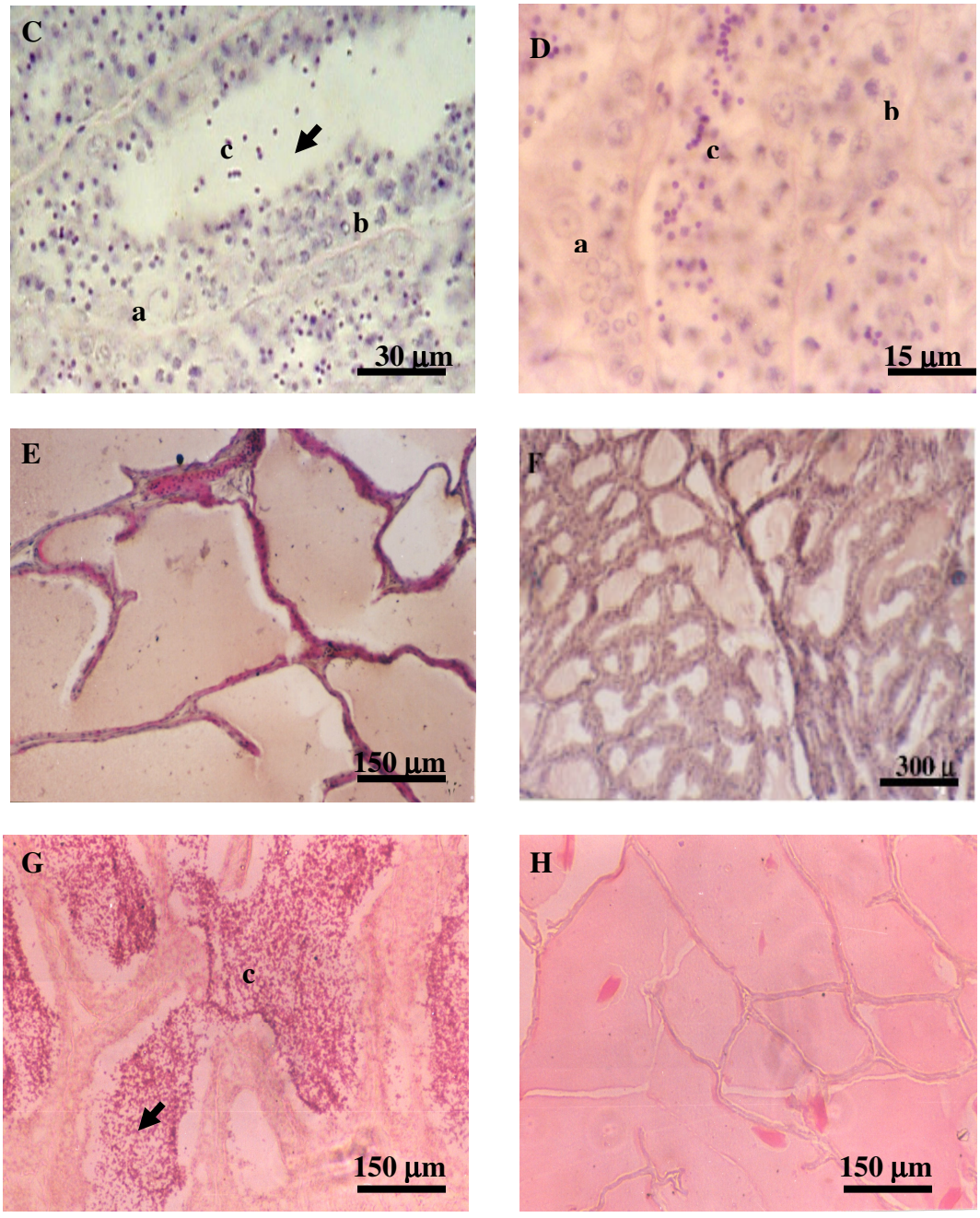


Fig 1: *Histological sections showing the effect of thiourea-induced thyroid hormone depletion on testicular tissue of C. gariepinus. (A) parallel control group, (C) thiourea-treated group, (B and D) are higher magnification images of A and B, respectively. (**a**) spermatogonia, (**b**) spermatocytes and (**c**) spermatids and spermatozoa. Long arrows indicate lumen filled with spermatids in Fig A and G while short solid arrows indicate empty lumens with very few residual sperm heads in Fig C and G. E and F - Histological*

sections showing the effect of hypothyroidism on seminal vesicular tissue of C. gariepinus in the parallel control and thiourea-treated groups. Inlay in Fig. 1F shows hypertrophy of columnar epithelial lining of the seminal vesicle of thiourea-treated fish. No such hypertrophy is evident in control fish. All images are representative of n=5.

fluid (Fig. 1F). Upon withdrawal of thiourea treatment both testis and seminal vesicle showed signs of functional recovery. There was reappearance of spermatozoa and secretory material in seminal lobules of testis and seminal vesicular lumina, respectively when fish were examined 21 days after stopping treatment with thiourea (Fig 3 G and H).

Table 3:

	Initial Control	**Parellel Control (Fig. 1A and 1B)**	**Thiourea-treated (Fig. 1C and 1D)**	**Withdrawal (Fig. 1G)**
Spermatogonia	**3.27** ± 0.95	**0.098** ± 0.01	**6.17** ± 0.64[a]	**1.05** ± 0.058
Spermatocytes (primary/secondary)	**26.42** ± 0.83	**1.34** ± 0.09	**74.07** ± 2.23[a]	**2.14** ± 0.29
Spermatids /Spermatozoa	**70.31** ± 3.67	**98.27** ± 4.51	**19.7** ± 1.32[a]	**96.65** ± 6.97

Changes in serum and tissue T and 11-KT levels: Both serum 11-KT and T levels decreased significantly following thiourea-induced thyroid hormone depletion (Fig. 2).

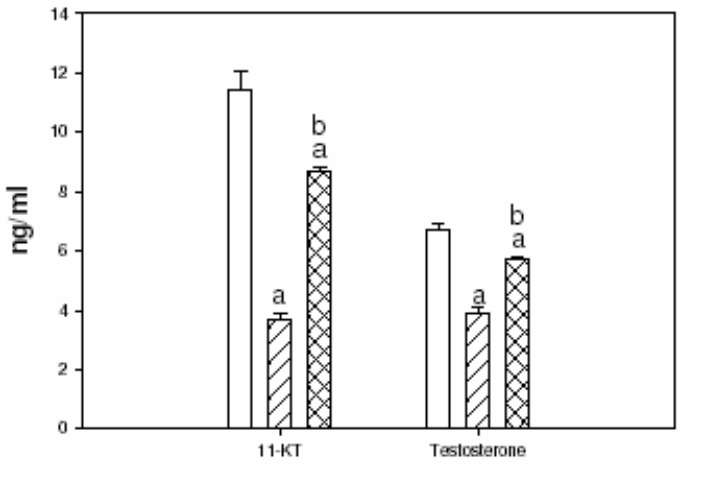


Fig 2: *Effect of thiourea-induced thyroid hormone depletion (0.03% thiourea) on serum T and 11-KT levels in C. gariepinus during preparatory phase (mean ± SEM; n = 5). P< 0.05; a, compared with the parallel control group, b, compared with the thiourea-treated group (Student's-Newman-Keuls' test).*

parallel control, thiourea-treated group, withdrawal group

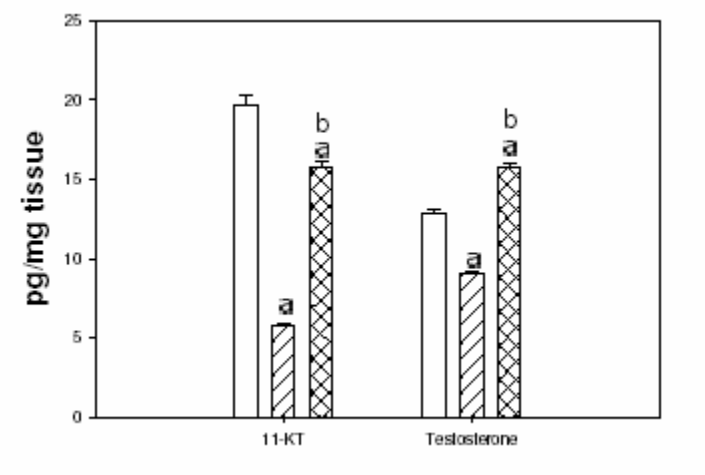


Fig 3: *Effect of thiourea-induced thyroid hormone depletion (0.03% thiourea) on testis levels of T and 11-KT in C. gariepinus, during preparatory phase (mean ± SEM; n=5). Other details are as in Fig. 2.*

However, the decrease in 11-KT was more pronounced than T. This was also true in the testicular (Fig. 3) but not in seminal vesicle tissue (Fig. 4). Both hormones in serum, testis and seminal vesicle showed a tendency of restoration, however, were yet to attain (parallel) control values, following the withdrawal of thiourea treatment (Figs 3 and 4).

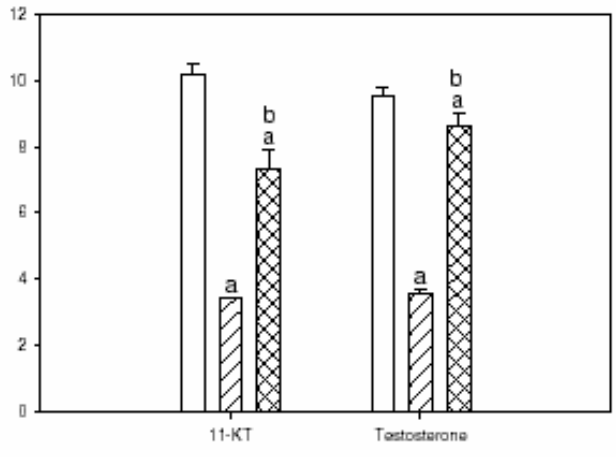


Fig 4: *Effect of thiourea-induced thyroid hormone depletion (0.03% thiourea) on seminal vesicular T and 11-KT levels in C. gariepinus during preparatory phase (mean ± SEM, n = 5). Other details are as in Fig. 2.*

ICC analysis of preoptic area-hypothalamus cfGnRH and pituitary LH, and Western blot analysis of pituitary LH: ICC analysis revealed a qualitative reduction in cfGnRH immunoreactivity (ir-) in the preoptic area-hypothalamus (POA-H) of brain (Fig. 5B) and in LH ir- pituitary (Fig 5D) following the thyroid hormone depletion of normal fish (Fig. 5A and C). Data from Western blot analysis (Fig. 6 A and B) and subsequent scanning densitometric analysis (parallel control: 13,867 ± 288; thiourea-

treated: 12,322 ± 425; n=5) showed a decrease in pituitary LH levels, which is also corroborative to the ICC data.

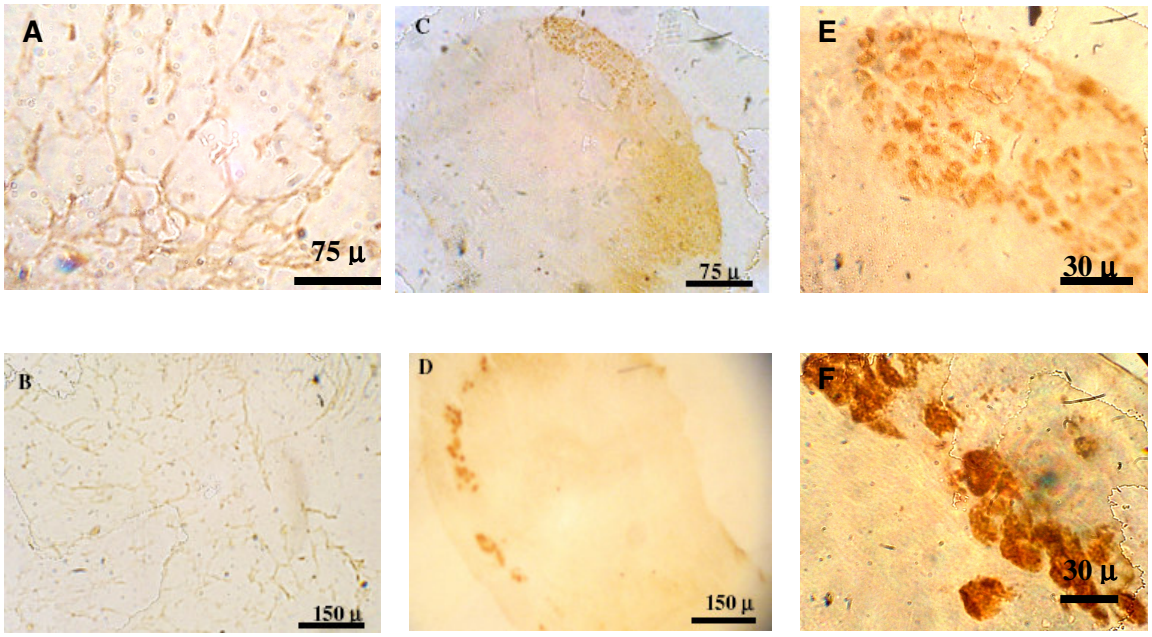


Fig 5: *Immunolocalization of cfGnRH and LH: A) cfGnRH immunoreactivity (ir-) in the preoptic area (POA) of (parallel) controls; B) cfGnRH ir- in the POA of thiourea-treated fish; C and D LH ir- in the pituitary of parallel control and thiourea-treated fish, respectively. All images are representative of n=5.*

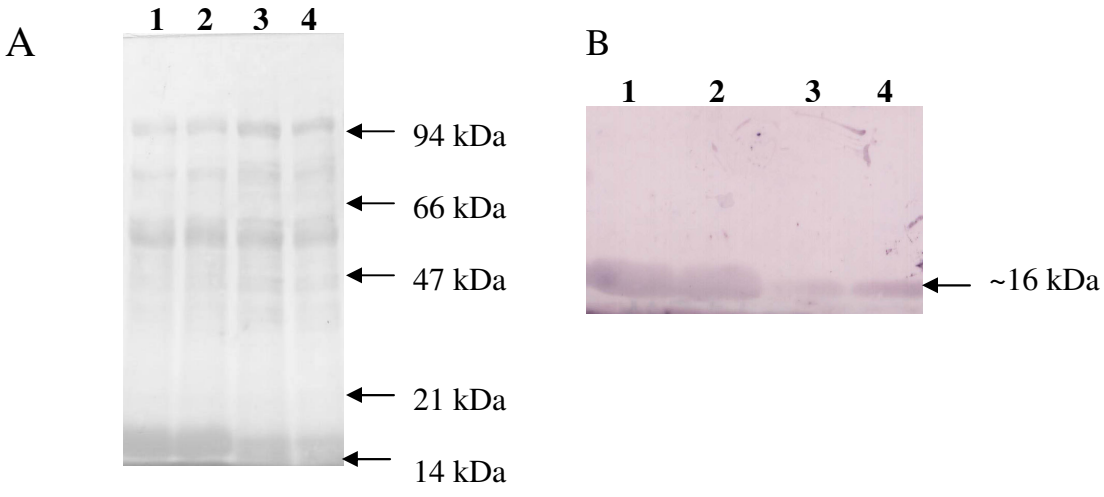


Fig 6: A. *Ponceau staining of the membrane depicting equal loading. SDS-PAGE was done on 10% gel.* ***B.*** *Western blot analysis showing qualitative decrease in LH in pituitary from thiourea-treated fish with two representative samples (Lanes 3 and 4) compared to two representative controls (Lanes 1 and 2).*

Discussion

Following the initial recognition of thiourea as an effective thyroid hormone-depleting drug (Davidson *et al.*, 1979), there have been several reports utilizing this compound to study the effects of thyroid on reproduction. The efficacy of hypothyroidism is judged based on T_3 levels, as it is the physiologically active form of thyroid hormone (Ingbar, 1985). In the present study, a major drop in serum T_3 levels after the treatment of thiourea confirms the effective induction of hypothyroidism (thyroid hormone depletion) in catfish. Data from the present study demonstrated that thyroid hormone insufficiency caused alterations in testicular cells *vis à vis* spermatogenesis, seminal vesicle lumen and interstitial cells. The markers for assessing functional capacity of seminal vesicles and testis such as protein and sialic acid (Senthilkumaran and Joy, 1993) were reduced. The reduction in serum and tissue levels of 11-KT and T after thyroid hormone depletion further confirmed the disarray in the male reproductive system. Changes in cfGnRH and LH immunolocalization in POA-H and pituitary indicated that the effect might also be orchestrated by the hypothalamo-hypophyseal axis. Partial recovery of 11-KT and T levels after withdrawal of thiourea treatment for 21 days clearly indicated that thyroid plays a role in maintaining testis functions. Our contention was further supported by with histological signs of recovery in testicular and seminal vesicular tissues. Taken together,

present study suggests that 21 days and above is required for complete recovery after short-term thyroid hormone depletion in catfish. Thus, impairing thyroid function within the physiological limit of restoration is a valid approach to understand the role of thyroid hormone in male reproduction.

Earlier studies (see Misra and Panday, 1985 and references cited therein) on teleosts including catfish reported a decrease in sperm motility and gonado-somatic index following thyroid hormone depletion without any direct experimental support on thyroid hormone or androgen status. Further most of these reports used juvenile and/or adult fish without any special mention on reproductive stage of fish. The fish used in the present study were in the preparatory phase with histological and morphological data indicating the same. The levels of various hormones including T_3 vary with the reproductive stage of the fish (Cyr *et al.*, 1988). Hence, the stage of the annual testicular cycle is important while studying the effect of thyroid hormones or any other endocrine mediator in general.

The data showed progression of control fish from early preparatory to prespawning phase during the period of acclimation and experimentation, in accordance to the transition expected in natural wild population. This situation differs from that encountered in fish routinely reared in laboratory. van Oordt *et al.* (1987) reported that fish maintained under laboratory conditions are ready for spawning throughout the year and do not show any seasonal variations. Nevertheless, natural spermiation was not observed after continued maintenance of this fish. In *C. gariepinus* like in most teleosts, spermatogenesis occurs within cysts where germ cells undergo synchronous differentiation. The control group exhibited the normal pattern of histological changes as

would occur during the preparatory phase. In the thyroid depleted fish the lobule lumens are almost empty with only a few residual spermatozoa and at first glance resemble testicular lumen of post-spawning fish. This lead to an initial suspicion that thiourea could probably causing precocious spawning. This may be possible since natural drop of serum T_4 levels just prior to spawning is reported in some annual breeders like trout (Cyr *et al.*, 1988). Thiourea by mimicking this effect could be hastening the process. However, careful examination of the fish or aquarium water did not show any signs of spermiation and further a detailed microscopic examination of the testicular sections obtained from these fish revealed the presence of cysts containing spermatogonia, spermatocytes and few spermatids/spermatozoa within the lobule towards the periphery. These cells represent the various stages of spermatogenesis and are usually characteristics of preparatory and prespawning phases rather than post-spawning phase. During post-spawning phase the lobule lumens are typically empty and the residual spermatids, spermatocytes and spermatogonia undergo apoptosis and are cleared by macrophages and Sertoli cells (Chaves-Pozo *et al.*, 2005). Only the primary germ cells remain along with the interstitial cells and Sertoli cells. Previous study in cycling catfish (Lamba *et al.*, 1983) indicated a decline and no immediate resurgence of T levels during post-spawning phase. The reappearance of spermatozoa in the lobular lumen of testis and a parallel rise in tissue and serum androgen (T and 11-KT) levels upon thiourea withdrawal further endorse that treatment with thiourea hindered spermatogenesis rather than inducing it. These observations cumulatively indicate a loss of spermatids/spermatozoa heads in thiourea-treated group during preparatory phase, a probable hindrance in normal

progression of spermatogenesis and revival of spermatogenesis following restoration of thyroid hormone levels. It was however not possible from the current experimental design to predict the mechanisms underlying the loss of spermatids/spermatozoa in thiourea-treated fish. This would form a future directive of work. Nevertheless, the observations from the present study along with the recent localization of TSH mRNA receptor on sperm (Kumar *et al.*, 2000; Vischer and Bogerd 2003) indicate a definite role for thyroid and its associated factors on spermatozoa function. In lower vertebrates, quantitative reduction in spermatozoa number and associated changes in androgens (see below) after thyroid hormone depletion were demonstrated for the first time in the present study.

Thyroid hormone depletion also affected seminal vesicles profoundly. The total protein and sialic acid levels dropped considerably. During the preparatory phase, the seminal vesicles secrete a viscous fluid rich in mucopolysaccharides, acidic and basic proteins, sialic acid and phospholipids (Schoonen *et al.*, 1987; Senthilkumaran and Joy, 1993). The exact function of these secretions is not known but they are believed to play a role in increasing milt volume and sperm viability (Senthilkumaran and Joy, 1993). In addition, some pheromonal properties have been implicated (Lambert and Resink, 1991; Mansour *et al.*, 2004). There was marked reduction in seminal vesicular luminal volume and secretory material in the thiourea-treated fish indicating a hindrance to normal seminal vesicle secretion and function. Further there was the columnar epithelium lining the lumina showed marked hypertrophy (Fig 1F inlay).

The percentage reduction in 11-KT is higher than T indicating reduced conversion of T to 11-KT. 11-KT is the most potent and physiologically relevant androgenic hormone with stimulatory effects on spermatogenesis (Cavaco *et al.*, 2001) and a role in LH regulation (Rebers *et al.*, 2002). Though 11-KT is a minor product during catfish testicular steroidogenesis its circulating levels are relatively high due to hepatic conversion from 11β-hydroxyandrostenedione (OHA, Cavaco *et al.*, 1997). The decrease in serum 11-KT levels might also indicate reduced conversion of OHA to 11-KT in hepatic tissue (Cavaco *et al.*, 1997).

The results obtained show a reduction in plasma androgen levels and decreased spermatogenesis on lowering T_3 levels. Another noteworthy observation obtained is that restoration of normal T_3 levels facilitates normal spermatogenesis and almost normal plasma androgen levels, establishing that T_3 depletion caused no permanent functional damage in the testicular and seminal vesicular tissue with regards to spermatogenesis and androgen production. However, the short duration of thyroid hormone depletion (21 days) is a point to debate in this context.

These effects seen on spermatogenesis by reduction of T_3 levels could be due to the lowering of plasma androgen levels which would have a detrimental effect on spermatogenesis or could be a direct consequence of T_3 depletion on the cells involved in spermatogenesis. The hypothalamo-hypophyseal axis has an important effect on the progression and coordination of different events related to spermatogenesis. Hence, the probability that T_3 depletion effected spermatogenesis via changes in the hypothalamic and pituitary secretion of GnRH and gonadotropin (GTH) was explored. Qualitative

reduction in cfGnRH and LH immunoreactivity and ir- fibers corroborate this contention that the functioning of the axis may be compromised during T_4 depletion. Thyroid hormones have also been shown to have direct influence on transcriptional regulation of GnRH peptides (Parhar *et al.*, 2000) and on the steroidogenic response of testicular tissue to GTHs.

T exerts a feed back inhibition on pituitary GTHs, hence the reduction in T levels evident in thiourea-treated fish would be expected to elevate GTH production. However, contrary to this, a decrease was observed in GTH levels indicating that T_3 depletion might act at the level of GnRH-GTH (LH) axis (present study, Parhar *et al.*, 2000). The decreased androgen levels and reduced spermatogenesis could be an effect of the decrease in GTH levels. Nevertheless, a direct effect of T_3 on spermatogenesis cannot be ruled out as functional isoforms of thyroid hormone receptors have been cloned using wide variety of tissues from few fish species (see Marchand *et al.*, 2001 and references cited therein). This is being further investigated by studies underway in our laboratory using T and LH supplementation in thiourea-treated fish.

The evidences provided above demonstrate that the effect on reproduction could be secondary to the effect on thyroid and the hypothalamo-hypophyseal axis in general could be affected in thyroid hormone irregularities. This gains significance in light of the increasing number of reports identifying many endocrine disrupters and environmental pollutants to be thyrotoxic (Kirubagaran and Joy, 1989; Schmutzler *et al.*, 2004). It also ascertains the need for caution before claiming that a compound has direct effect on

gonadal development as the effect could be mediated through disruption of thyroid or any other endocrine function.

This study, therefore, establishes the requirement of thyroid hormones for progression of testicular recrudescence and male sex steroid production. Furthermore, our study suggests a plausible role for cfGnRH, LH in this process. Additional research in this direction using specific hormone supplementation would help to identify the exact sites of thyroid hormone action in the hypothalamo-hypophyseal-gonadal axis.

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Chapter 2.1

Effect of thyroid hormone depletion on monoamines and expression patterns of catfish GnRH in air-breathing catfish, Clarias gariepinus

Abstract:

Thyroid hormone is known to have profound effect on the efficient functioning of the reproductive system. The GnRH-gondaotropin (GTH) axis is the crucial axis involved in regulation of the various aspects of reproduction like spermatogenesis, synthesis of sex steroids and regulation of courting and spawning behavior. Earlier study from our laboratory have shown that thyroid hormone depletion causes a decrease in GTH and GnRH levels in pituitary and preoptic area, respectively. GnRH secretion in pre-optic area is largely regulated by the monoaminergic system mainly dopamine (DA), epinephrine (E), norepinephrine (NE) and serotonin (5-HT). The expression pattern of catfish GnRH transcripts tends to corroborate our earlier findings. Hence, in the current study we aimed to investigate levels of monoamines in the preoptic area- hypothalamus (POA-H) so as to determine whether thyroid hormone depletion modulates the monoaminergic neurotransmission thereby affecting GnRH secretion. The levels of NE and L-Dopa decreased significantly while that of DA remained unaltered following thyroid hormone depletion. DA has an inhibitory effect on GnRH secretion. Findings from the current study suggest that the inhibitory tone is unaltered while the stimulatory tone influencing GnRH neurons in POA-H is decreased during thiourea induced thyroid hormone depletion.

Introduction:

Thyroid hormone is known to have profound effect on the efficient functioning of the reproductive system. Thyroid hormones are known for direct influence on transcriptional regulation of gonadotropin-releasing hormone (GnRH) peptides (Parhar *et al*. 2000) and

on the steroidogenic response of testicular tissue to gonadotropins. Catfish GnRH (cfGnRH) is the crucial neuromodulator involved in regulation of the various aspects of reproduction like spermatogenesis, synthesis of sex steroid and regulation of courting and spawning behavior in catfish. A decrease in LH immunoreactivity was observed in pituitary of male air-breathing catfish following thyroid hormone depletion during preparatory phase (Chapter 2, Swapna *et al.* 2006). Regulation of LH secretion from the pituitary is modulated by GnRH secretion from the preoptic area, which inturn is influenced by several neural factors acting via specific receptors present on the GnRH neurons. The chief neural factors influencing GnRH release from preoptic GnRH neurons include the neurotransmitters serotonin (5-HT), Gamma amino butyric acid (GABA), norepinephrine (NE), epinephrine (E) and dopamine (DA) in addition to other factors like neuropeptide Y (Yu *et al.*, 1991; Yu and Peter, 1992). Thus, in the present study, we aimed to investigate changes, if any in the level of various monoamines in the preoptic area- hypothalamus (POA-H) of male air breathing catfish during thiourea induced thyroid hormone depletion.

Methodology:

Experimental fish*:* Male air-breathing catfish were obtained from local ponds in and around Hyderabad. All experiments were initiated following an initial acclimation period of a fortnight under laboratory conditions. Diet consisted of minced goat liver and fish were maintained in filtered fresh water under uniform 12-hour day light and temperature conditions to prevent any changes in patterns during acclimation and experimentation.

Induction of thyroid hormone depletion*:* Hypothyroidism was induced in male African catfish by maintaining the fish in aquarium tanks containing thiourea 0.03% for 21 days following an initial two week period of acclimation to laboratory conditions (refer chapter 2). Control fish were continued to be maintained in fresh tap water without thiourea following the acclimation period.

RT-PCR Analysis of cfGnRH transcripts*:* RNA was prepared using Tri Reagent obtained from Sigma Chemicals Ltd. (Ann Arbour, MI, USA) as per the manufacturers protocol. First strand cDNA template was prepared from RNA obtained from the POA region of control and thiourea treated catfish using superscript III (Invitrogen). Semi-quantitative PCR was done with the above obtained cDNA as template using the following PCR program: 94 ^{0}C for 2 min; 94 ^{0}C for 1 min; 49 ^{0}C for 45 sec; 72 ^{0}C for 1 min; 30 cycles to 2; 72 ^{0}C for 10 min; and specific primers designed from cfGnRH mRNA sequence downloaded from Pubmed nucleotide search.

Analysis of monoamines by HPLC*:* Catecholamines from POA-H were extracted and estimated following the method described by Murai et al.(1988) in a Waters HPLC and ECD detector model No. 2465.

Statistical Analyses: All data (n=5) are untransformed and are presented as mean ± SEM.

Results:

Estimating T3 levels in serum obtained from control and thiourea treated fish assessed the effectiveness of thiourea to induce thyroid hormone depletion under the current conditions. (Treatment of thiourea significantly decreased ($P < 0.05$) serum T3 levels (0.31 ± 0.01ng/ml as against 0.69 ± 0.02ng/ml in control). The reduction was more than 50% when compared to the control group). Results obtained by semi-quantitative RT-PCR analysis of cfGnRH transcripts (110 bp sequence in open reading frame) from control and thiourea

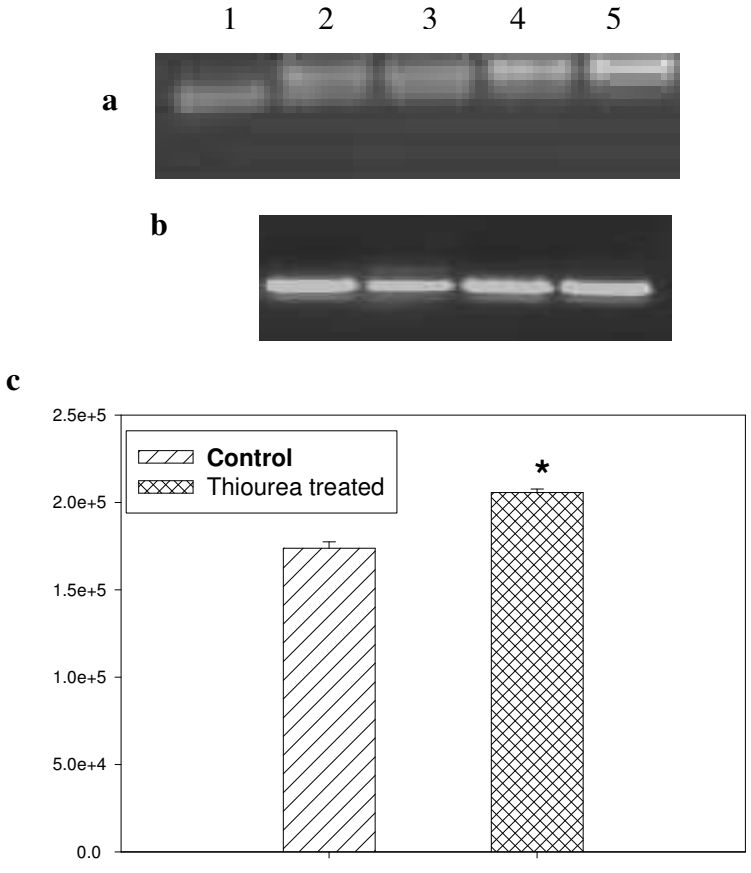

Figure 1: RT-PCR analysis of cfGnRH in POA-H of control and thiourea treated male adult catfish a) representative 1% agarose gel image showing cfGnRH amplified by RT-

PCR b) β-actin graphical. Lane 1- -ve control, 2 & 3- control male, 4 & 5- thiourea treated fish. c) graphical representation of scanning densitometry data for quantitation of cfGnRH transcripts.

treated fish indicated an increase in the level of cfGnRH transcript in POA-H of thiourea treated fish (Fig 1). The data was obtained following standardization of the optimum cycles for quantitation of cfGnRH RT-PCR as described by Kwon et al. (2001).

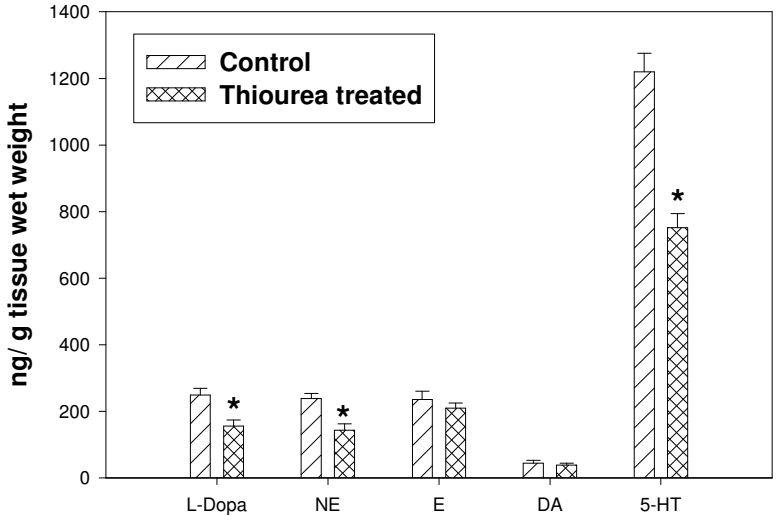

Figure 2: Monoamine levels in POA-H of thiourea treated catfish compared to stage matched controls.

HPLC analysis showed a 38%, 37% and 41% decrease of 5-HT, L-dopa and NE respectively in POA-H of thiourea treated fish compared to that of control (Fig 2). The levels of DA and E remained unaltered following thiourea treatment (Fig 2).

Discussion:

The present data provides evidence for changes in level of cfGnRH expression and 5-HT, NE in POA-H of adult male catfish treated with thiourea to deplete thyroid hormones. Parhar et al. (2000) had previously reported a specific suppression of salmon GnRH (sGnRH) transcripts in the Nile Tilapia. The Nile tilapia belongs to perciforms and has three different GnRH subtypes namely sGnRH, seabream GnRH (sbGnRH) and chicken GnRH-II (cGnRH-II). Catfish belongs to the class of siluriforms and has two major GnRH subtypes – cfGnRH and cGnRH-II. Hence, results obtained with respect to sGnRH and sbGnRH in perciforms may be correlated to cfGnRH in catfish. The present observation of an increase in cfGnRH transcripts during thyroid hormone depletion is in accordance with Parhar et al. (2000). Earlier studies from our laboratory (Chapter 2) using catfish demonstrated a decrease in GnRH and LH immunoreactivity in POA-H and pituitary respectively. In teleosts, the 5-HT and NE stimulate GnRH release from POA GnRH neurons (Senthilkumaran et al, 2001). A decrease in the 5-HT and NE levels in POA-H of thyroid hormone depleted fish compared to control fish indicates that the stimulatory tone influencing GnRH neurons is decreased. DA has an inhibitory effect on spontaneous and GnRH induced LH release (Goos et al, 1999). Levels of DA remained unaltered in thyroid hormone depleted fish compared to control fish. Depletion of thyroid hormone levels lead to changes in POA-H levels of the catecholamines L-dopa, NE and E. This is in accordance to earlier report by Chaube and Joy (2003) where they demonstrate an increase in the activity of tyrosine hydroxylase – a key enzyme in the catecholaminergic pathway following thyroidal overdose.

The neurotransmitters 5-HT and DA apart from their role in GnRH regulation are also critical in biological rhythms (Goos et al. 1999). Hence their levels are vulnerable to large shifts depending on the time of sacrifice and photoperiod. To augment circadian changes, precautions were taken to maintain the control and experimental fish in ambient photoperiod and temperature conditions and all sacrifices of were performed at midday (12 noon).

In conclusion, the study demonstrates an increase in cfGnRH transcript in POA-H of male air breathing catfish and proposes a possible role for decreased monoaminergic stimulation by 5-HT and NE signaling on POA-H GnRH neurons thyroid hormone depletion as a possible cause for this discrepancy in the level of cfGnRH transcript and the cfGnRH protein expression in POA-H during thyroid hormone depletion.

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Chapter 3

Ethynylestradiol and methyltestosterone treatment affects gonadal recrudescence by modulating hypothalamo-hypophyseal axis in adult air breathing catfish, Clarias gariepinus

Abstract:

Ethynylestradiol (EEL) and methyltestosterone (MT) were added to the aquarium tanks at concentrations of 1µg/litre holding adult male and female air-breathing catfish *Clarias gariepinus,* respectively for 21 days after daily replenishment of water. EEL treatment caused disappearance of sperm from some testicular lobules in males while MT treatment of females lead to precocious oocyte maturation. EEL caused significant water retention in all tissues including peritoneal cavity and seminal vesicles. Immunocytochemical localization of catfish GnRH (cfGnRH) and luteinizing hormone (LH) in preoptic area-hypothalamus (POA-H) and pituitary, respectively, revealed decreased immunoreactivity (ir-) following EEL treatment in males. MT treatment however caused no observable change in cfGnRH ir- and a significant increase in LH ir- in females. Semi-quantitative RT-PCR analysis indicated that cfGnRH transcripts in POA-H decreased significantly following EEL and MT treatment in males and females, respectively. Levels of POA-H inhibitory monoamine dopamine increased following EEL and MT treatment in males and females while levels of stimulatory monoamines serotonin and norepinephrine decreased. The results demonstrate a direct *in vivo* effect of sex steroid analogs on cfGnRH-LH axis via monoaminergic system.

Introduction:

Sex steroids play an important roles in development of sexual behavior and maintenance of reproductive functions (Adkins-Regan, 1987). The primary sex steroids in males and females namely testosterone (T) and estradiol-17β (E_2) are mainly synthesized in gonads

under the influence of gonadotropins (GTHs) secreted from the adenohypophysis under regulation of the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) (Peter *et al.*, 1990; Peter *et al.*, 1991; Sherwood *et al.*, 1993; Nagahama, 1994; Goos *et al.*, 1995; see Trudeau 1997). The sex steroids in turn exert negative and positive feedback at the level of pituitary and hypothalamus to affect the synthesis and release of GnRH and GTH (Goos 1987; Senthilkumaran and Joy, 1994; 1995a, b; 1996; Breton *et al.*, 1997; Trudeau 1997; Goos *et al.*, 1999; Khan *et al.,* 1999; Okuzawa *et al.* 2002). The GnRH-GTH axis plays major roles in maintaining normal reproduction and synchronization of the seasonal reproductive cycles (references as above). Like most vertebrates, teleosts also exhibit distinct cyclic reproductive patterns during which levels of GnRH, GTH and sex steroids all vary according to the reproductive phase (Lamba *et al.*, 1983; Kobayashi *et al.*, 1986; King *et al.*, 1994; Trudeau 1997; Senthilkumaran *et al.*, 1999; Joy *et al.*, 2000; Santos *et al.*, 2001; Kirubagaran *et al.*, 2005). This cyclicity is evident in both sexes of lower vertebrates like pisces and in females of higher vertebrates like mammals. In mammals, peak levels of plasma E_2 and low progesterone levels precede preovulatory luteinizing hormone (LH) surge (Looper *et al.*, 2003a; 2003b). In pisces, plasma E_2 levels drop to a nadir and increased 17α, 20β-dihydroxyprogesterone levels precede spawning (Nagahama 1997; Joy *et al.*, 1998; Nagahama, 2000; Senthilkumaran *et al.*, 2004). Any irregularity in the GnRH, GTH or sex steroid levels in a particular phase could lead to compromised reproductive functions and reduced fertility or even sterility (see Trudeau 1997).

In teleosts, the GnRH-GTH system is influenced by the central monoaminergic system in the preoptic area – hypothalamus (POA-H) and pituitary. Dopamine (DA) exerts a strong inhibitory effect on basal and GnRH induced LH release in most species including catfish (Goos, 1987; Senthilkumaran and Joy, 1996; Joy *et al.*, 1998) while norepinephrine (NE) and serotonin (5-HT) exert stimulatory effect facilitating LH release either directly or via the GnRH axis (Peter *et al.,* 1986; Somozo and Peter, 1991; Yu *et al.* 1991; Khan and Thomas, 1992; Yu and Peter, 1992; Senthilkumaran and Joy, 1996; Senthilkumaran *et al.*, 2001). In lieu with the seasonal changes in brain and pituitary GnRH (Senthilkumaran *et al.,* 1997; Senthilkumaran *et al.*, 1999; Okuzawa *et al.*, 2003) plasma GTH and sex steroid levels (Kobayashi *et al.*, 1986; Van oordt *et al.*, 1987; see Trudeau, 1997; Joy *et al.*, 2000; Kirubagaran *et al.*, 2005) with respect to reproductive phase, the POA-H and pituitary levels of monoamines (Senthilkumaran and Joy, 1993; Senthilkumaran and Joy, 1995b) also show variations indicating the importance of a regulated co-ordination and interactions of the hypothalamic-hypophyseal-gonadal and POA-H monoaminergic system for reproduction (Goos *et al.*, 1999). Taken together, sex steroids flock with monoaminergic system to modulate hypothalamic-hypophyseal axis vis-á-vis reproduction.

Sex steroids and their analogs have been extensively used in juvenile and immature fish for inducing experimental sex reversal and skewing natural mixed sex population towards controlled mono sex populations (Kwon *et al.*, 2000; Nagahama, 2000; Kobayashi *et al.*, 2003; Nagahama, 2005). E_2 and its analogs have been used for generating females while T and androgen analogs, aromatase blockers have been used for skewing population to

predominantly males (references as above). Interestingly, it has also been shown that blockade of 5-HT by para-chlorophenylalanine mimics estrogenic effect (Tsai *et al.,* 2001). During the critical period of sex reversal under exogenous steroid influence, preoptic hypothalamus GnRH levels vary indicating an impact of sex steroids on GnRH-GTH axis (Lee *et al.*, 2001). E_2 has been shown to influence the central monoaminergic system in POA-H regulating pulsatile secretion of GnRH in mammals (Smith and Jennes, 2001). Sex steroids and their analogs have been frequently administered in adult mammals to study the effects on different aspects of reproduction, GnRH-GTH axis and POA-H monoaminergic system. However, such studies in adult pisces are sparse. Further the effects demonstrated *in vitro* may vary *in vivo* based on the reproductive phase, dosage of sex steroids and fish species used (see Blazquez *et al.*, 1998). In the present study the effect of the E_2 analog ethynylestradiol (EEL) and the T analog methyltestosterone (MT) was assessed on adult male and female air breathing catfish *Clarias gariepinus* respectively during gonadal recrudescence. Such an attempt would provide insights into the effect of sex steroid analogs on gonads, POA-H GnRH and Pituitary GTH (assessed in current study using immunocytochemistry [ICC]), plasma T and E_2 levels and POA-H monoaminergic system (catfish GnRH [cfGnRH]) during gonadal recrudescence which is a crucial period of spermatogenesis and oogenesis preceding spawning. The study also caters to the aim of understanding the effect of estrogenic and androgenic compounds present as pollutants in natural water bodies on male and female reproductive systems respectively.

Materials and Methods:

Animals: Adult catfish weighing 400g brood and reared in our laboratory under natural photoperiod and ambient temperature conditions were acclimated in aquarium tanks for a fortnight. The experiment was undertaken in January 2006 when the fish were in preparatory phase i.e. undergoing gonadal recrudescence. The fish were divided into 3 groups. Group I consisted of both male and female fish maintained in filtered water as control. Group II fish comprised of all females maintained in filtered water containing 1 µg/L MT, which was replenished once a day after feeding with minced goat liver *ad libitum*. Group III fish were all males maintained in filtered water 1 µg/L EEL, which was replenished once a day after feeding with minced goat liver *ad libitum*. After the duration of 21 days, fish from both groups were weighed and sacrificed. Blood was obtained by caudal puncture prior to sacrificing. Serum was obtained by centrifugation at 1500g, lyophilized and stored briefly in -80°C freezer for hormone estimations. Testes and seminal vesicles were dissected out, weighed and processed for histological examination.

Hematoxylin-Eosin staining (H & E)*:* For histological examination, tissues were fixed in freshly prepared Bouin's fluid for 1-2h at room temperature, then processed with graded alcohol series and embedded in paraplast (Kendall, Mansfield, MA, USA). Sections of 6µ thickness were cut with a rotatory microtome (Leitz, Wetzlar, Germany) and stained with hematoxylin-eosin following a series of dehydration, rehydration steps through graded concentrations of alchohol and xylene. Microscopic examinations and

photography was done with a Nikon EFD-3 microscope fitted with a Nikon NFX-35 automatic camera (Nikon, Tokyo, Japan). Higher magnification images were obtained using a Leica microscope (Buffalo, NY, USA) fitted with a Kodak (DX 7630) digital camera.

EIA estimation for serum T and E_2: Serum and tissue T and E_2 levels were estimated using specific EIA (Cayman, Ann Arbor, MI, USA) kits as per the manufacturer's protocol. Intra- and inter-assay variations were within the limits specified in the manufacturer's protocol. Antibody specificity was very high and cross-reactivity with closely related hormones was in the range of 0.1 to 1% (see Chapter 2), which was in accordance with the manufacturer's protocol.

Imunocytochemistry for cfGnRH, LH: Immunohistochemistry (IHC) for GnRH was carried out as per the following procedure. The sections were deparaffinised and rehydrated in a gradient of decreasing concentrations of alcohol and finally in phosphate buffered saline (PBS) containing 1% tween (pH 7.6). The sections were then treated with normal goat serum (1:10 dilution). Primary antibodies against cfGnRH (Ref.:awb# 40069925892), at 1:2000 dilution were applied to the sections and incubated at 4°C for 48 h in a humid chamber. The sections were then washed with PBS thrice at 5 min interval and incubated at room temperature for 1 h with biotin labeled anti-rabbit IgG. The unbound antibody was washed away with PBS and the sections were incubated at room temperature for 30 min with streptavidin horseradish peroxidase conjugate (for

cfGnRH IHC). Alkaline phosphatase secondary antibody conjugates were used for LH IHC. The sections were then washed with phosphate buffer (PB) and then incubated with 0.05% 3,3'diamino benzidine tetra hydrochloride with 0.01% H_2O_2 in 0.05M Tris-HCl (pH 7.6) for 10 min in a dark chamber at room temperature. The slides were then rinsed with PB, dehydrated through graded alcohol series, cleared in xylene and mounted in DPX. All the chemicals and secondary antibodies used for IHC were part of the kit components supplied by Bangalore GENEI Pvt. Ltd. (Bangalore, India)

Similar IHC procedure was followed for LH using the antiserum (pmGTH-II) raised against red seabream (*Pagrus major*) LH. The antiserum was found to work efficiently at 1:2000 dilutions in our (heterologous) system as ascertained by western blot analysis (data not shown). For better clarity of staining, all the IHC analysis was done with photomicrographs of 100x magnification or higher. Lower magnification (10x) with full field view of section was not shown.

RT-PCR Analysis of cfGnRH transcripts: RNA was prepared using Tri Reagent obtained from Sigma Chemicals Ltd. (Ann Arbour, MI, USA) as per the manufacturers protocol. First strand cDNA template was prepared from RNA obtained from the POA region of control and thiourea treated catfish using superscript III (Invitrogen). Semi-quantitative RT-PCR was done as described by Kwon *et al.* (2001) with the above obtained cDNA as template using the following PCR program: 94 ^{0}C for 2 min; 94 ^{0}C for 1 min; 49 ^{0}C for 45 sec; 72 ^{0}C for 1 min; 30 cycles to 2; 72 ^{0}C for 10 min; and specific

primers designed from cfGnRH mRNA sequence downloaded from Pubmed nucleotide search (Accesion No. X78049).

Analysis of monoamines by HPLC: Monoamines from POA-H were extracted and estimated following the method described by Murai *et al*. (1998) in a Waters HPLC and ECD detector model No. 2465.

Statistical analysis: All data (n=5) are untransformed and are presented as mean ± SEM. Pairwise comparisons were done by one-way ANOVA followed by Newman-Keuls' test using Sigma Stat software.

Results:

Gonadal histology: H & E staining revealed that control fish were in the preparatory phase of reproductive cycle. Control fish showed testicular lobules completely filled with sperm typical of fish progressing from preparatory to prespawning phase (Fig 1a) while testicular section from EEL treated fish showed many empty lobules devoid of any sperm (Fig 1b). Ovarian sections from control fish showed more previtellogenic and vitellogenic follicles with less number of post vitellogenic follicles while MT treated females exhibited precocious oocyte maturation with higher number of post vitellogenic follicles (Fig 1d) compared to controls (Fig 1c) (Table 1). EEL treatment also lead to much increase in fluid content in seminal vesicles.

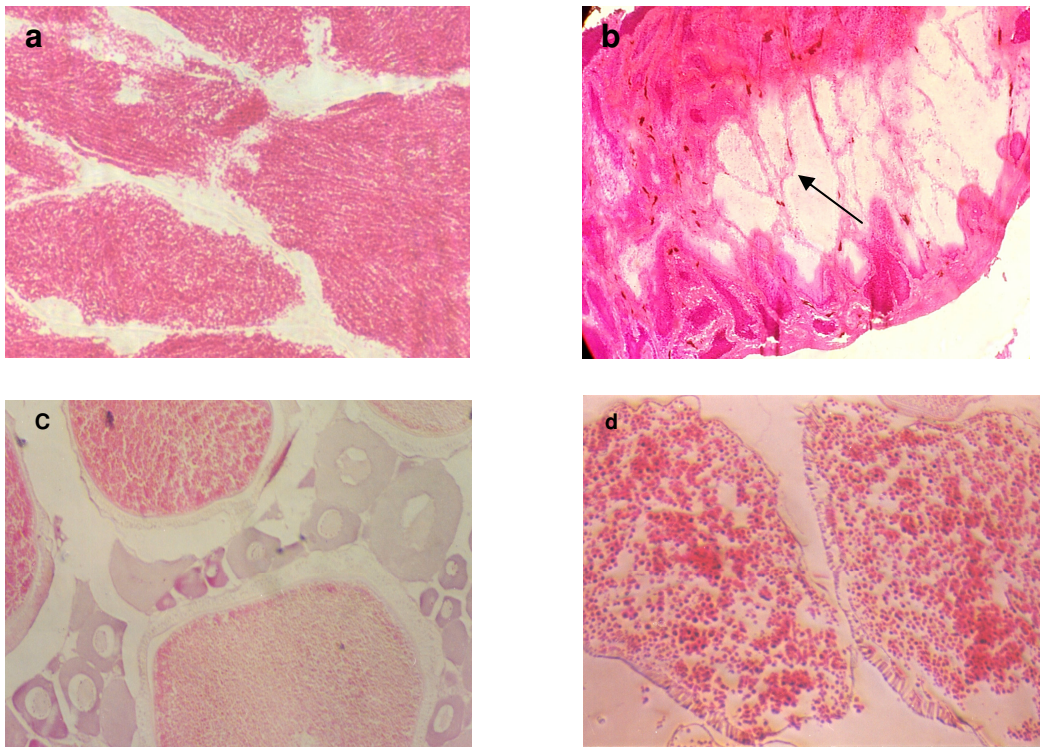


Fig 1: *H & E stained sections demonstrating changes in testicular and ovarian histology following EEL and MT treatment. a and c- control, b- testicular section from EEL treated fish, d- ovarian section from EEL treated fish. Arrow indicates empty testicular lobules.*

Table 1:

Group	Primary oocytes (%)	Non-VT oocytes (%)	VT/ Post VT oocytes (%)
Control	71.8 ± 2.97	15.7 ± 0.8	12.25± 1.08
Treated	8.6 ± 0.92	9.3 ± 1.15	81.0 ± 0.74

Fluid retention was also observed in peritoneal cavity and other tissues following EEL treatment.

Serum T and E_2 levels: Serum T and E_2 levels in males and females did not show any significant change following EEL and MT treatment compared to controls (Fig 2).

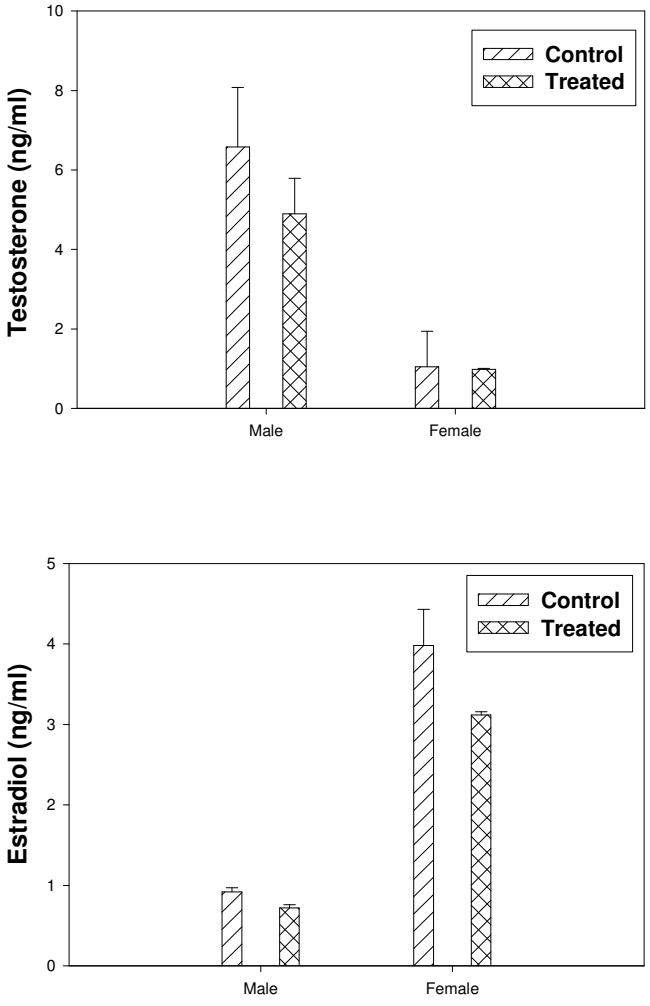


Fig2: *Serum T and E_2 levels in EEL and MT treated fish*

Immunocytochemistry for cfGnRH and LH: EEL treated fish exhibited reduced cfGnRH ir- in the POA-H region while no significant change was registered in POA-H cfGnRH ir- in MT treated fish compared to sex and stage matched controls (Fig 3). LH ir- in anterior pituitary was significantly higher in MT treated fish while a significant reduction was observed in the LH ir- in pituitary of EEL treated fish (Fig 4).

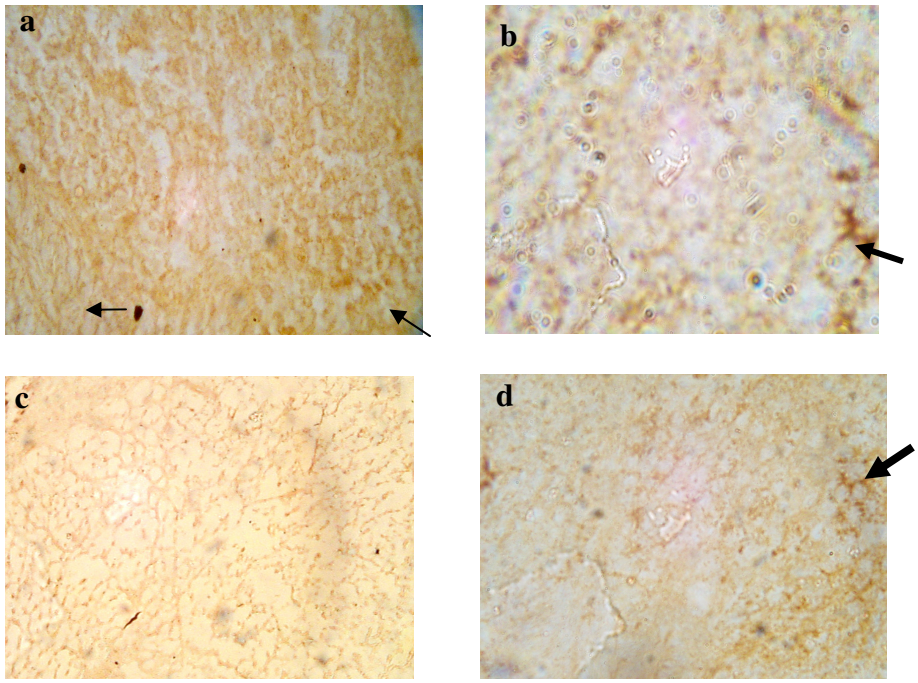


Fig 3: *cfGnRH ir- in POA-H of control male (a), female (b), EEL treated male (c) and MT treated female (d) fish.Arrows indicate positive ir-*

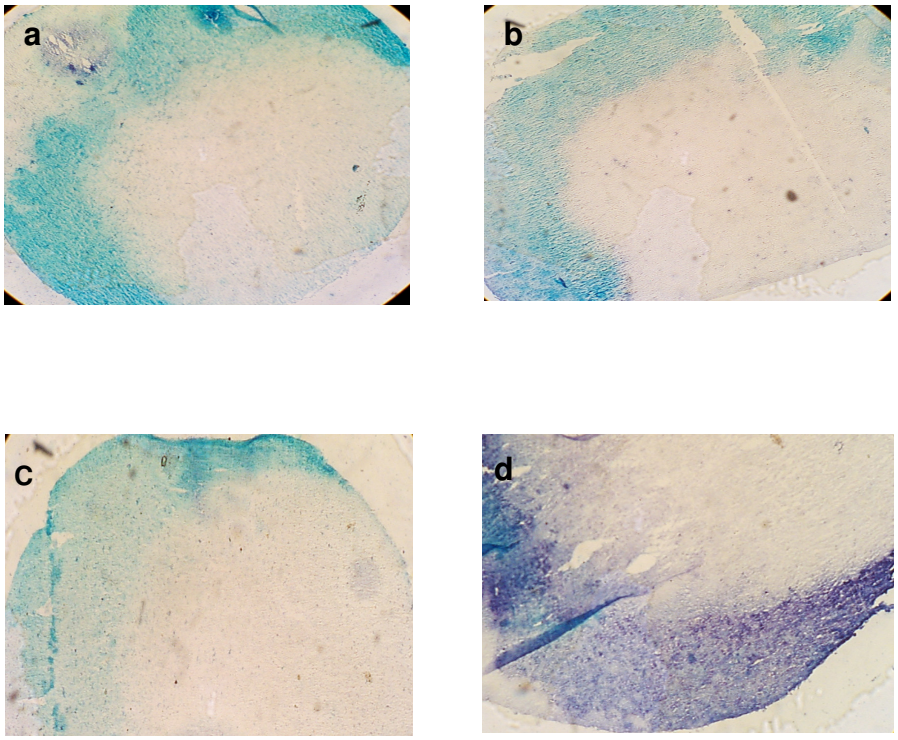


Fig 4: *LH ir- in pituitary of control male (a), female (b), EEL treated male (c) and MT treated female (d) fish.*

Monoamine levels in POA-H: Significant decrease was observed in L-DOPA, epinephrine (E), NE, 5-HT levels in POA-H of EEL and MT treated males and females compared to control male and female fish while levels of DA showed significant increase in EEL and MT treated fish (Fig 5).

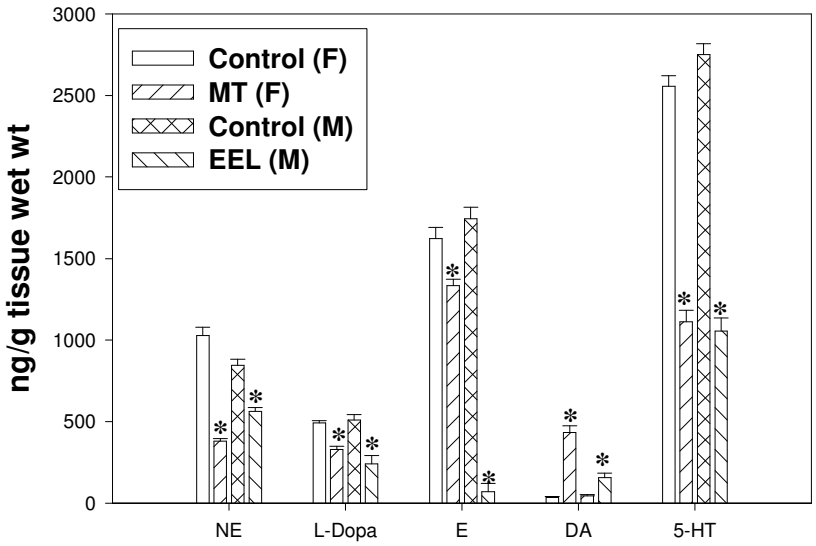
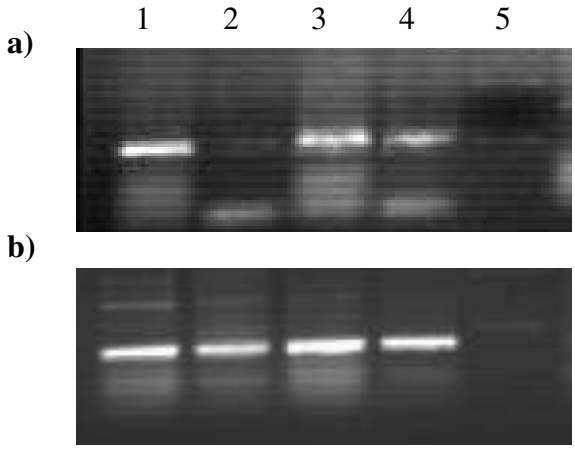


Fig 5: *Changes in POA-H monoamine levels following EEL and MT treatment.*

cfGnRH transcripts in POA-H: Semi-quantitative RT-PCR showed a significant decrease in cfGnRH transcripts in both EEL and MT treated fish. The decrease was more significant in EEL treatment compared to MT (Fig 6).



c)

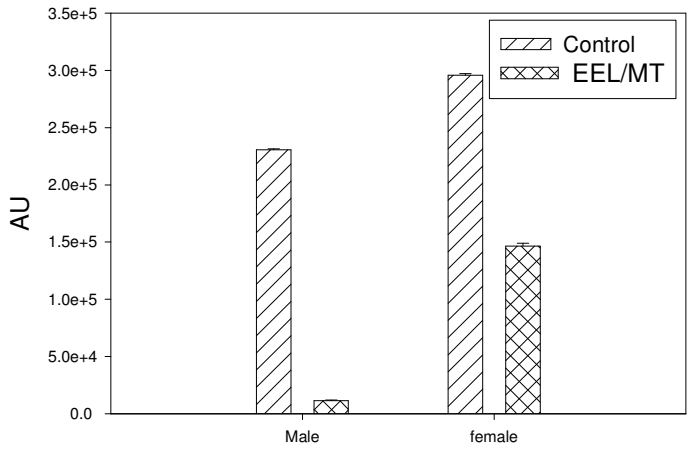


Fig 6: *Changes in cfGnRH transcripts in POA-H following EEL and MT treatment. a) 1% agarose gel showing changes in cfGnRH transcripts amplified by RT-PCR from POA-H, b) β-actin transcripts amplified by RT-PCR. Lane 1- control male, 2-EEL treated male, 3- control female, 4-MT treated, 5-marker. c)graphical representation of the scanning densitometry data obtained by analysis of image in a.*

Discussion:

Treatment with sex steroids or their analogs during juvenile phase often leads to sex reversal and skewing of mixed sex population to mono sex population irrespective of the genetic sex. (Scholz *et al.*, 2003). This effect is more evident when steroid treatment is used during the critical period of sex differentiation (see Nagahama 2000, 2005). Sex steroids and their analogs have seldom been used in adults due to an apparent belief that they would be ineffective in adults or their effects would be minimal compared to juveniles. However, such a study gains importance in light of the numerous estrogenic and androgenic compounds increasingly entering natural water bodies as industrial

effluents (Gimeno *et al.,* 1996; Sole *et al.*, 2003; Higashitani *et al.*, 2003). The study also offers interesting insights into the effect of sex steroids on GnRH-GTH axis and the POA-H monoaminergic system controlling the axis. Administration of sex steroids did not lead to sex reversal in adults unlike that observed in juveniles. This could be due to the short duration of treatment with sex steroids and also sex reversal has not been documented in *C. gariepinus* following sex differentiation, sexual maturation or even during juvenile stage. The present study demonstrates that both EEL and MT affect gonadal recrudescence. Results obtained clearly indicate that the effect may be mediated both through direct action on gonads or via the GnRH-GTH axis or both. The male reproductive axis is more grossly affected on administration of EEL compared to the female system on administration of MT.

The fish were in preparatory phase at the time of steroid analog administration and reached late preparatory phase or prespawning phase at the time of completion of treatment in males. This is evident from the testicular histology depicting lobules completely filled with spermatids/spermatozoa in control fish. EEL treatment leads to complete loss of spermatozoa/spermatids in some testicular lobules while other lobules were not significantly affected. At first glance it may appear that the fish has spermiated. But this argument does not hold weight considering the spermiating characteristics of *C. gariepinus*. In *C. gariepinus* testicular cycle progresses synchronously and during spermiation all the spermatozoa/ spermatids are released simultaneously from all the testicular lobules i.e single spermiation occurs per annual cycle. Spontaneous spawning requires LH surge (Nagahama 1997; 2000; 2005) and does not occur in nature during

recrudescence when DA levels are high to moderate (Joy *et al.*, 1998). Further spontaneous spermiation is almost absent in laboratory conditions (van Oordt *et al.*, 1987) and requires several factors (Viveiros *et al.*, 2003). The selective loss of spermatozoa/spermatids from few lobules may be due to the short duration of EEL treatment employed. If duration of treatment was extended it would probably cause loss of sperm from all lobules. MT treatment of female fish in preparatory phase lead to precocious ovarian development and higher numbers of post vitellogenic oocytes were present compared to control in MT treated fish. Tiwari *et al*. (2002) had earlier reported precocious ovarian maturation in juvenile female catfish *Hetropeneustus fossilis*. Estimation of T and E_2 levels showed no significant variations in serum level of these hormones in MT, EEL treated fish compared to controls. The EIA method used for estimation of T and E_2 are specific for these steroids and do not detect their analogs MT and EEL. Comparatively serum levels of E_2 were low in controls also probably because the fish were in pre-spawning phase at the time of steroid estimations. E_2 have been reported to decrease as the fish approach pre-spawning phase (see Senthilkumaran and Joy, 1995b; 1996). MT and EEL were not assayed in the present study as no commercial kits are available for their estimation. Specific EIA method has to be developed and validated for their estimation. This is however cumbersome and was thought to be avoidable as significant histological changes were observed in the gonads of fish treated with MT and EEL indicating that treatment was effective. Further the doses of MT and EEL were those earlier reported by (Orn *et al.*, 2003).

Reduced levels of POA-H cfGnRH transcripts and ir- are fairly evident in EEL treated fish. However, earlier studies show an increase in POA-H GnRH, following E_2 treatment (Parhar *et al*,. 2000). This may be due to the differences in reproductive stage of the fish and conditions in both experiments. Pituitary LH levels showed a decrease in EEL treated fish and an increase in MT treated fish. This observation is in accordance to earlier reports (Sohn *et al.*, 2001). Androgens like T are believed to act on the POA-H and pituitary following aromatization to E_2 (See Blazquez *et al.*, 1998). MT is a non aromatizable androgen, implying the effects of MT are due to direct effects of androgens, not mediated by estrogens. This observation is in corroboration with studies in rainbow trout indicating that T is more efficient in causing increase in GTH compared to E_2 and GnRH analog. Monoamines have been shown to be the chief regulators of GnRH and GTH release from POA-H and pituitary respectively. Hence the possibility of a direct influence of sex steroid analogs on POA-H monoamine levels was probed. Control levels of monoamines were comparable to levels earlier reported during preparatory phase in catfish (Senthilkumaran and Joy, 1993; 1995b). Both EEL and MT caused a significant decrease in E, L-DOPA, stimulatory monoamines NE and 5-HT and an increase in the inhibitory monoamine DA. L-DOPA, DA, NE and E are formed from tyrosine in a sequential manner. Decreased L-DOPA, NE, E levels and increased DA levels may indicate increased activity of the enzyme DOPA decarboxylase catalyzing conversion of L-DOPA to DA and/or decreased activity of the enzyme Dopamine-β-hydroxylase and phenylethanolamine-N-methyltransferase catalyzing conversion of DA to NE and NE to E respectively. Administration of MT caused a larger decrease in NE and increase in DA

compared to EEL treatment while changes in 5-HT were more or less similar in both MT and EEL treatment. Changes in L-DOPA and E levels in POA-H were more significant in EEL compared to MT treated fish. MT being a non aromatisable analog of T, the effects of MT on POA-H levels observed in the present study cannot be attributed to aromatization to E_2. Also the T, E_2 levels in serum are also not significantly altered between control and MT treated fish implying that the observed changes in POA-H monoamine levels cannot be attributed to changes in the levels of these natural sex steroids. Taken together these results indicate that T may be more effective in regulating NE and DA inputs compared to E_2. Recent reports in rainbow trout (Hernandez-Rauda and Aldegunde, 2002; Vetillard *et al.*, 2006) also support this view that androgens may have a direct effect on POA-H system even in absence of aromatisation to E_2.

The present study thus establishes that the effect of steroid analogs EEL and MT on gonadal histology and function in males and females respectively during preparatory and early prespawning phase. The effect may be mediated via direct action on gonads and/or via the hypothalamic- hypophyseal cfGnRH-GTH system. Sex steroid analogs can exert their effect on cfGnRH-GTH axis by altering the POA-H monoamine levels and influencing the monoaminergic input to POA-H GnRH neurons and pituitary gonadotrophs. The above observed effect of sex steroids and their analogs on the hypothalamic-hypophyseal-gonadal axis may be limited to the particular seasonal phase of reproduction and hence the results obtained in this study cannot be correlated or taken to project situation at other stages. In conclusion, the present study establishes that the male reproductive system may be more adversely affected by estrogenic compounds

compared to female reproductive system in presence of androgens during gonadal recrudescence and that androgens can exert a direct effect on the POA-H monoaminergic system without aromatization to E_2.

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Chapter 4

Cloning and analysis of the spatio-temporal pattern of sbGnRH and gonadotropins expression in the snake head murrel Channa striatus

Abstract:

Vertebrate reproduction is under the neuroendocrine control of the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) which synchronizes various reproductive events via induction of pituitary gonadotropin secretion and influences other reproduction related aspects like spawning behavior and pheromonal action in fish. Multiple forms of GnRH peptides have been reported across diverse vertebrate and invertebrate classes. Here, we report the partial Seabream GnRH (sbGnRH), follicle-stimulating hormone β (FSHβ), luteinizing hormone β (LHβ) and glycoprotein α (GTHα) cDNA sequence cloned from the brain of *Channa striatus* (snake head murrel), a fresh water perciform with immense economic and medicinal value across Asian countries. SbGnRH mRNA was found in brain, gill and ovary of mature murrel with possible implications to the effect of GnRH on pheromonal phenomena and on reinitiation of oocyte meiosis. In keeping with the earlier reported role of GnRH in initiation of oocyte meiosis, evidence is presented here from RT-PCR and immunohistochemistry demonstrating an increase in level of sbGnRH mRNA in ovary from immature to pre-vitellogenic and mature state with post-vitellogenic follicles. Similarly FSHβ, LHβ, GTHα transcrpits were identified in extrahypophyseal tissues. However, their functional implication are yet to be understood.

Introduction:

Gonadotropin-releasing hormone (GnRH), a decapeptide synthesized in nerve cells in the brain acts on the anterior pituitary, causing the synthesis and systemic release of

gonadotropins (GTHs). The GTHs in turn act on the gonads to stimulate gametogenesis and steroid synthesis. GnRH can additionally act as a neuromodulator (Muske 1993; Sherwood *et al.*, 1997; Wiechmann and Dionne 2000), pheromone (Camaron *et al.*, 1999), and regulator of reproductive behavior (Yamamato *et al.*, 1997). In fish, GnRH can stimulate the release of other pituitary hormones, including growth hormone (Marchant *et al.* 1989) and prolactin (Weber *et al.*, 1997). To date, 14 distinct GnRH peptides have been identified in vertebrates and named after the species from which they were first isolated. Although these hormones share comparable cDNA and genomic structures, their tissue distribution and regulation of gene expression are significantly dissimilar. The specific localization of GnRH in brain neurons is important for their functioning. Many forms of GnRH can activate release of GTH when injected intraperitoneally into fish. However, in the brain only GnRH in preoptic neurons is delivered to the pituitary in substantial amounts through direct innervation of the adenohypophysis. GnRH cell bodies at other locations have axons that synapse within the brain and do not terminate in the pituitary (fish) hence may not influence *in vivo* gonadotropin production.

The GTHs and thyroid stimulating hormone along with the mammalian human chorionic gonadotropic hormone (hCG) belong to a family of glycoprotein hormones and heterodimeric proteins. They posses two noncovalently bound, chemically distinct the α and β subunits. The α subunit is common among hormones within a species and is highly conserved between species, whereas the β subunit is specific to each hormone and confers biological specificity (Pierce and parsons, 1981). Teleosts were initially believed

to have only one hormone termed GTH-II homologous to the mammalian GTHs viz follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This lead to an initial assumption that a GTH gene duplication might have occurred at a point after teleost evolution, during amphibian evolution. However, subsequently two structurally and functionally distinct GTH forms from several teleosts were isolated and characterized dispelling this notion (Kawauchi *et al.*, 1987; Degani *et al.*, 2003; Hellqvist *et al.*, 2004; Hurvitz *et al.*, 2005; Kwok *et al.*, 2005; Shimizu *et al.*, 2003; Sower *et al.*, 2006; Trinh *et al.*, 1986; Yaron *et al.*, 2001; Swanson *et al.*, 2003). Fish GTH-I is believed to be homologous to mammalian FSH and GTH-II to mammalian LH but their functional similarity to mammalian GTHs has not been definitely established. Fish FSH, LH are differentially expressed and secreted during the reproductive cycle, with the level of FSH being high during oocyte growth and LH during final oocyte maturation and spawning. FSH has been shown to be important during gonadal growth, recrudescence, initial oogenesis and spermatogenesis while LH is important for sex steroid synthesis, late stages of oogenesis, spermatogenesis and spermiation/ovulation. The role of GTH-II in sex steroid synthesis, ovulation and spermiation is well established in pisces as opposed to that of GTH-I which needs further clarification.

Channa striatus, commonly referred to as snake head murrel is a local fresh water perciform in great demand due to its edible value and applicability in practices involving alternate medicine. The high demand is not appropriately substantiated by the availability hence leading to high market prices and non-affordability by general population. The limited availability of this species owes to its low reproductive efficiency. Furthur, a lack

of sufficient characterization and understanding of its reproductive and developmental events and stages severely hinders any attempts so far in artificial fertilization, seeding and rearing of murrel in fishery farms of India. *C. striatus* being a perciform would be expected to posses three distinct GnRH forms namely sbGnRH, cGnRH-II and sGnRH varying in their anatomical distribution and function. As established in most perciforms sbGnRH would be the predominant preoptic-hypothalamic (POA-H) GnRH form involved in regulation of reproduction, GTH synthesis and sexual maturation.

The present study aimed to isolate and clone the cDNA sequences corresponding to the seabream GnRH (sbGnRH), FSHβ, LHβ and GTHα from *Channa striatus* with an ultimate objective that this endeavor would help and facilitate future studies aimed at understanding the reproductive behavior of this commercially important native species. This would also be expected to lead impetus to any future attempts at artificial or *in vitro* fertilization which have so far been unsuccessful. Apart from this we also present the spatial expression pattern of sbGnRH, FSHβ, LHβ and GTHα in various tissues and the variations in the temporal expression patterns of sbGnRH in different phases of ovarian cycle. This gains importance in light of numerous reports from mammals and few from teleosts indicating that extra hypothalamic expression of GnRH peptides in gonads may have important paracrine and autocrine effects.

Methodology:

Animals and Tissue Collection: Live adult murrel were obtained from local fish markets in Hyderabad. and maintained in aquarium tanks with a daily water change at optimal

photoperiod (12L:12D) and temperature (25 °C). The fish were fed twice a day with commercial tropical fish food and minced goat liver. The fish were anesthetized in ice prior to sacrifice. Brain and other tissues were dissected out and snap frozen in liquid nitrogen. All instruments used in dissection, tissue collection and storage were subjected to prior treatment with alcohol, DEPC and sterilized by double autoclaving to prevent RNase contamination.

RNA preparation and RT PCR for obtaining partial fragments: RNA was prepared from murrel brain, pituitary and other tissues using Tri Reagent (Sigma Chemicals Ltd) as per the manufacturer's protocol. Reverse transcription was carried out with superscript III (Invitrogen). PCR for amplification of sbGnRH was carried out with following conditions: 94 °C (1 min); 49 °C (1.5 min); 72 °C (1 min); for 35 cycles using the following set of degenerate primers:

DFw : 5' CAB SRC TGC TGY CAR CAC TGG T 3'

DRv : 5' AKV ART CCT TTC AYT CKG TA 3'

PCR amplification of FSHβ, LHβ and GTHα was done using the cycling conditions 94 °C (1 min); 55 °C (1 min); 72 °C (1 min); for 29 cycles in a ABI Prism thermocycler. The following sets of degenerate primers were used for this purpose.

GTHα dFw : 5' CATAGBTGATTCCKATCCCAAC 3'

GTHα dRv: 5' CATATCTRATGAAAGTAYCAGGT 3'

FSHβ dFw:	5' GGTTYAAGTAACAACAGAGCTC 3'
FSHβ dRv:	5' GAABGTCATACAGCTRGGTATG 3'
LHβ dFw:	5' GCTCAGCAGAGCGATGGTYCTG 3'
LHβ dRv:	5' CAGGCTCTCGAAGGYGCAGTC 3'

All the cDNA fragments obtained were cloned into pGEM-T Easy vector (Promega) and sequenced bidirectionally using vector specific primers, in ABI Prism 316 sequencer.

Rapid amplification of cDNA ends (RACE): First-strand cDNA synthesis was carried out using the RNA ligase mediated-RACE system (GeneRacer kit, Invitrogen) according to the manufacturer's protocol. Approximately 5 µg of total RNA was used to synthesize RACE cDNA templates as per the manufacturer's protocol. Two sets of primary and nested gene specific primers (GSPs) for 5' and 3' RACE were synthesized based on the sequence information of partial cDNA products obtained and used for amplification of full length cDNA. All PCRs were performed in a final volume of 25 µl using the following conditions 94 °C- 45 sec, 60 °C- 1 min, 72 °C – 1 min (29 cycles) and 94 °C- 45 sec, 55 °C- 1 min (for GTH subunits) 58 °C – 30 sec (for sbGnRH), 72 °C – 1 min (35 cycles) for primary PCR and secondary PCR respectively. All second round PCR products were separated on 1% agarose gel by electrophoresis and the band of expected size was purified using MinElute Gel Extraction Kit as per manufacturer's protocol (Qiagen). The purified PCR products were cloned into pGEM-T Easy vector (Promega)and transformed into *E. coli* DH5α competent cells. The plasmid DNAs were

purified using QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. The plasmid DNA containing candidate GTH, GnRH cDNA inserts were sequenced bidirectionally using T7 and SP6 universal primers.

GSP1	:	5'GGTGATTCTTCTTCACAGCCCAA 3'
GSP2	:	5'GCCCAGGAGGGAAGCGGGAACTG 3'
GSP3	:	5' CTGTATGGTTTCTCACCTTAATGCC 3'
GSP4	:	5' CACCTCTCAGAGCCACGAAGACA 3'
GSP5	:	5' CACCAAGGGTGGGTGATCTTTA 3'
GSP6	:	5' CAGAGGATGCAGCTGGTTGTCA 3'
GSP7	:	5' CAGGCTGCAGGCTCTCGAAGGTGC 3'
GSP8	:	5' CTCATCAACCAGACGGTGTTTCT 3'

Analysis of spatial expression of sbGnRH in murrel by RT-PCR: Total RNA (5 µg) was isolated from various tissues of adult murrel. Then cDNA was synthesized and RT-PCR was employed for analysis of sbGnRH expression in various tissues of adult fish. The PCR reaction for sbGnRH amplification consisted of 94 °C (2 min) followed by 35 cycles at 94 °C (1 min); 58 °C (30 sec); 72 °C (1 min) followed by final extension 72 °C (10 min). Specific primers were employed for this purpose. cDNA encoding GTH subunits was amplified using the PCR reaction 94 °C (2 min) followed by 35 cycles at 94 °C (1 min); 55 °C (1 min); 72 °C (1 min) followed by final extension 72 °C (10 min).

Analysis of expression in ovary*:* RNA was prepared from immature and mature ovary and semi-quantitative RT-PCR analysis of sbGnRH transcripts was done using gene specific primers as described earlier by Kwon *et al.* (2001).

GnRH Immunocytochemistry: Ovary tissues were fixed in freshly prepared Bouin's fluid for 1-2h at room temperature, then processed with graded alcohol series and embedded in paraplast (Kendall, Mansfield, MA, USA). Sections of 6µ thickness were cut with a rotatory microtome (Leitz, Wetzlar, Germany) and Immunohistochemistry (ICC) for GnRH was carried out as per the procedure described by Shimizu *et al.* (2003). Characterization and immuno-crossreactivity were described earlier by Senthilkumaran et al. (1999). Specific antisera against sbGnRH was used. Sections were visualized in a Leica laser activated confocal microscope following immunostaining with fluorescent FITC labeled anti rabbit secondary antibodies.

***In vitro* bioassay for GVBD:** Fill grown folliculated oocytes with centally located germinal vesicles (GV) were chosen and distributed in 30 follicles per well. The incubation medium consisted of 3.74 g NaCl, 0.32 g KCl, 0.16 g caCl2, 0.1 g NaH_2PO_4, 0. 16 g $MgSO_4$, 0.8g glucose and 0.008 g phenol red in 1 litre of sterilized triple distilled water (Upadhaya and Haider, 1986). Effects of different concentrations of GnRH analogs were assayed by adding 5 µg/ml; 25 µg/ml and 50 µg/ml to the incubation medium containing the oocytes. 100 IU/ ml hCG was used as control. Following

incubation for 36 h at 25 ± 1 °C ten oocytes were taken from each well and placed in clearing solution to determine the rate of GVBD.

Statistical analysis: All assays were carried out in triplicates and data analysed by One way ANOVA using Student Newman-Keuls' test in Sigma stat software.

Results:

Molecular characterization of GnRH cDNAs: RT-PCR with degenerate primers for sbGnRH yielded a 225 bp sbGnRH sequence. In 5' RACE for sbGnRH GSPN1 and 5' RACE nested primer yielded 274 bp sequence while in 3' RACE. GSPN2 and 3' RACE nested primer yielded 264 bp sequence. 5' and 3' RACE with GSPN3 and GSPN4 primers for GTHα yielded a 333 bp fragment and 236 bp fragment. 5' and 3' RACE with GSPN5 and GSPN6 primers for FSHβ yielded a 480 bp fragment and 436 bp fragment while that with GSPN7 and GSPN8 yielded a 453 bp and 420 bp cDNA sequence encoding LHβ. The overlapping of 5' and 3' RACE sequences provided 433 (sbGnRH, including poly A tail), 538 (LHβ including poly A tail), 445 (GTHα, excluding polyA tail) bp and 535 (FSHβ) bp long cDNAs (including poly A tail) encoding 99 amino acids (aa), 149 aa, 118 aa and 121 aa respectively (Fig 1,2,3 and 4). All the sequences have a characteristic polyadenylation signal. Nucleotide sequence of *C. striatus* FSHβ showed 91% identity with *Channa maculata*, 88% with *Trichogaster trichopterus*, 86% with *Micropogonias undulates* and *Morone saxatalis* while GTHα from *C. striatus* exhibited 99% identity to *Oreochromis niloticus*, 98% to *Oreochromis mossambicus* and 86% to

Pagrus major. C. striatus LHβ showed 91% nucleotide identity with *Channa maculate*, 89% with *Oreochromis niloticus* and 88% with *Morone saxatalis* while the sbGnRH cDNA from *C. striatus* has 83% identify with *Rachycentron canadum sbGnRH*, 82% with *Verasper moseri,* 81% with *Monopterus albus pejerrey form GnRH*.

5’ tg gac act gac atg gac tga agg agt aga aaa ttc act gac gac tgc aga caa gga

taa atc aga cac tcc agc ctt ata cac aga aga **atg** gct atg aaa atc ttg gtg ctg

M A M K I L V L

ctg ctt atg ggg gcg ttg gtg aac tgc tgc cag cac tgg tcg tac gga ctt agc cca

L L M G A L V N C C Q H W S Y G L S P

gga ggg aag cgg gaa ctg aac agt att tca gac aca ctg gac aac ata gtt gaa

G G K R E L N S I S D T L D N I V E

ggg ttt tca cat gtg gac atg ccc tgg agt gtt ttg ggc tgt gaa gaa gaa tca cct

G F S H V D M P W S V L G C E E E S P

ttt gcc aga ctt tac aaa ata aaa gga ttc ctt gaa agt gtc aca gac agg caa aat

F A R L Y K I K G F L E S V T D R Q N

gga cgc cga aca tat aag aaa tat gtt gat gat att gat att gtt **tga** ttc aac **aat**

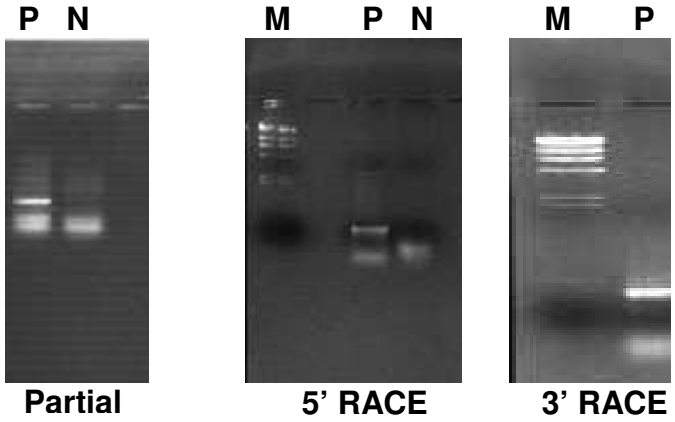
G R R T Y K K Y V D D I D I V *

aaa ttg tta cat gat gag cat aaa aaaaaaaaaaaaa 3’

Fig 1: *Nucleotide and deduced amino acid sequence of pre-pro sbGnRH from C. striatus. Numbers on the left indicate the nucleotide positions. The open reading frame and polyadenylation signal are indicated by different color coding as indicated. The aminoacids 19- 29 i.e. NH2-QHWSYGLSPG- COOH constitute the sbGnRH decapeptide preceded by the signal peptide. The remaining aminoacids encode the GnRH associated*

peptide (GAP) whose fuction is not yet known. Triplets in bold indicate the start and stop codons.

a)



b)

5'g aaa aga agc ctg cag atg ttt ctg gac taa cag aag aaa ttt gca act gag gtt caa gta

aca aca gag ctc tac agg tgt ctg tgc tgc acc cga cag agg **atg** cag ctg gtt gtc atg

M Q L V V M

gca gca gtg tta gca ctg gca ggg gcg ggg cag ggc tgc agc ttc ggc tgt cat cca acc

A A V L A L A G A G Q G C S F G C H P T

aac atc agc atc ccc gta gac agc tgt ggc atc acc gag tac atc ttc acc acc ata tgt

N I S I P V D S C G I T E Y I F T T I C

gca gga cag tgc tac cac gag gat ccc atc tac atc ggc cac cat gac tgg act gaa cag

A G Q C Y H E D P I Y I G H H D W T E Q

aag atc tgt aac ggg gac tgg tct tat gag gtt aaa cac att cat gga tgt ccg ttg gct gtc

K I C N G D W S Y E V K H I H G C P L A V

acc tac ccc gtg gcc aga aaa tgc gaa tgt act gca tgc aat gca gga aac acg tac

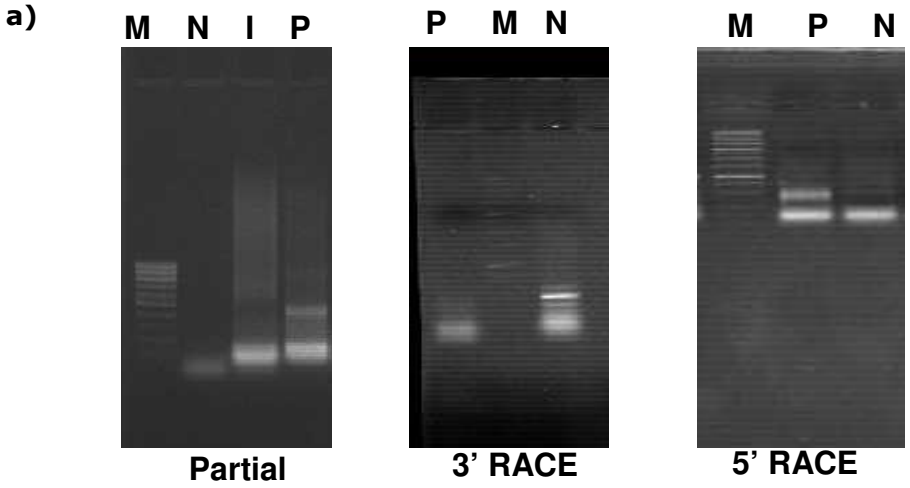
T Y P V A R K C E C T A C N A G N T Y

tgt ggc cgc ttt cct gga gac ata ccc agc tgt atg aca ttc **taa** aga tca ccc acc ctt

C G R F P G D I P S C M T F *

ggt gtc ctt ttt ctg atg ttt cgg **aat aaa** cag ata tca ctt caa aaaaaaaaa 3'

Fig 2: *a) 1% agarose gel images showing Etbr stained PCR amplified products using degerate primers (for amplification of partial FSHβ cDNA fragments) and GSPs for 3' 5' RACE to obtain full length FSHβ cDNA sequence. Lane M-marker, P-pituitary, N- -ve control. b)Nucleotide and deduced amino acid sequence of FSHβ from C. striatus. Numbers on the left indicate the nucleotide positions. The open reading frame and polyadenylation signal are indicated by different color coding as indicated. Triplets in bold indicate the start and stop codons.*



b)

5' ag aaa aaa aac ccg ctg ctg acg gca gac agg tga gtc agg

atg acg gcg gtg cag ctc agc aga gcg atg gtc ctg ctg ctg cag tgt ttg ctt cta gga

M T A V Q L S R A M V L L L Q C L L L G

gcc tca tcg tcc atg tgg cct ctg gct cca gca gag gcc ttc cag ctg ccg ccc tgt cac

A S S S M W P L A P A E A F Q L P P C H

ctc atc aac cag acg gtg ttt ctg gag aag gag ggc tgc ccc aag tgt cac cca gtg gaa

L I N Q T V F L E K E G C P K C H P V E

aca acc atc tgc agc ggc cac tgc atc acc aag gac cct gtc atc aag atc ctg ttc agc

T T I C S G H C I T K D P V I K I L F S

aat gtg tac cag cat gtg tgc acg tac agg gac ttg tac tac aag acg ttt gag ttt ccc

N V Y Q H V C T Y R D L Y Y K T F E F P

gac tgt cct cct ggt gtg gac ccc atc gtc acc tac ccc gtg gct ttg agc tgc cac tgc

D C P P G V D P I V T Y P V A L S C H C

ggc cgc tgt gtg atg gac aca tcc gac tgc acc ttc gag agc ctg cag cct gac ttc tgc

G R C V M D T S D C T F E S L Q P D F C

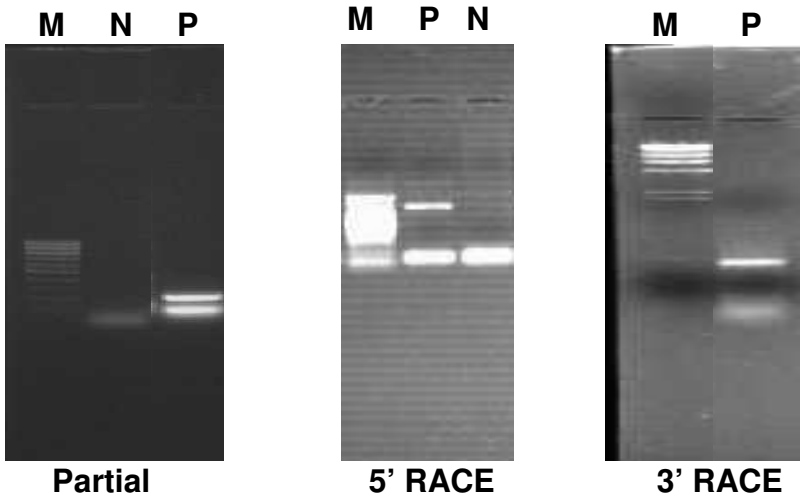
atg aat gac ata cct tac tac tac **tag** gcg tca aga aaa gaa aaa cat taa gac att gac

M N D I P Y Y Y *

agt gtc tcc aat taa cta tga ta**a ata aa**g att aga aag att aaa aaaaaaaaaaaaaa 3’

Fig 3: *a) 1% agarose gel images showing Etbr stained PCR amplified products using degerate primers (for amplification of partial LHβ cDNA fragments) and GSPs for 3’ 5’ RACE to obtain full length LHβ cDNA sequence. Lane M-marker, P-pituitary, N- -ve control, I-pituitary from fish in resting phase. b) Nucleotide and deduced amino acid sequence of LHβ from C. striatus. Numbers on the left indicate the nucleotide positions. Color coding is as Fig 1 and 2. Triplets in bold indicate the start and stop codons.*

a)



b) 5' ta act gct gca acc acg atg ggc tca ctg aaa tca cct gga ctg tcc ctt ctg ctg

ctg tct ttc ctt ctt tac ata gct gat tcc tat ccc aac att gat tta tca aac **atg** ggc

M G

tgt gaa gaa tgc acg ttg aga aag aac aat ctt ttc tca agg gat cgt ccg gtt tac

C E E C T L R K N N L F S R D R P V Y

cag tgc atg ggc tgc tgc ttc tcc aga gca tac cca aca cct ctc aga gcc acg

Q C M G C C F S R A Y P T P L R A T

aag aca atg aat atc cca aaa aac atc acc tca gag gcg acg tgc tgc gtc gca

K T M N I P K N I T S E A T C C V A

agg cac agc tac gag atc gag ata gct ggc att aag gtg aga aac cat aca gac

R H S Y E I E I A G I K V R N H T D

tgc cat tgc agc acc tgt tac ttt cat aag ata **tga** cag atg ggg act gga gac cgt

C H C S T C Y F H K I *

tct gca tcc ttc ggc ttg gct aca cgt cgg gct tca ttt aat atg cac gag ct 3’

Fig 4: *a) 1% agarose gel images showing Etbr stained PCR amplified products using degerate primers (for amplification of partial GTHα cDNA fragments) and GSPs for 3’ 5’ RACE to obtain full length GTHα cDNA sequence. Lane M-marker, P-pituitary, N- -ve control. b) Nucleotide and deduced amino acid sequence of GTHα from C. striatus. Numbers on the left indicate the nucleotide positions. Color coding is as Fig 1 and 2. Triplets in bold indicate the start and stop codons.*

Sequence analysis and phylogenetic tree:

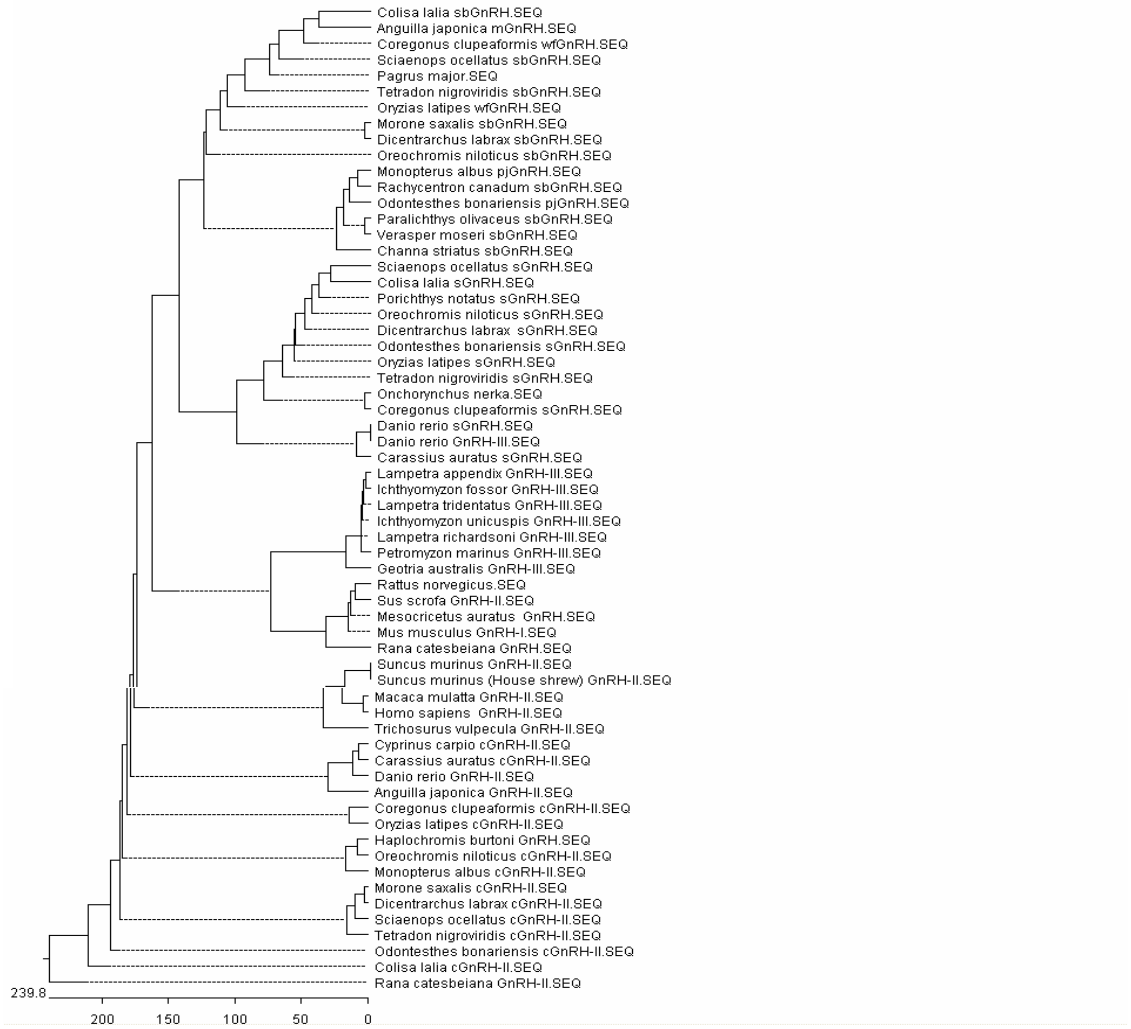


Fig 5: *Phylogenetic tree showing evolutionary status of C. striatus sbGnRH in relation to GnRH forms isolated from other species (Pubmed accession No. AB066360, AY677175,*

AB113209, AB113208, AB113207, AF224281, AF224280, AF224279, AF056314, AF056313, AY677172, AY677171, AY677170, AY786183, AB212814, AB212813, AB212811, AY744689, AY744688, AY744687, L27435, AY245104, AY245103, AY245102, AB041336, AB041335, AB026990, AB026989, AY189961, AY189960, AY147400, U40567, U30301, AY657019, AY657018, AJ304429, AF193516, AF107315, AF097356, NM001083111, NM000825, U41669, D31869, D86582, NM008145, NM012767, AY542893, AF186096, AY307178, AY307177, AY307176, AY307172, AY307175, AY307174, AY052628, U91938, AB101667, AB101666, AB101665)

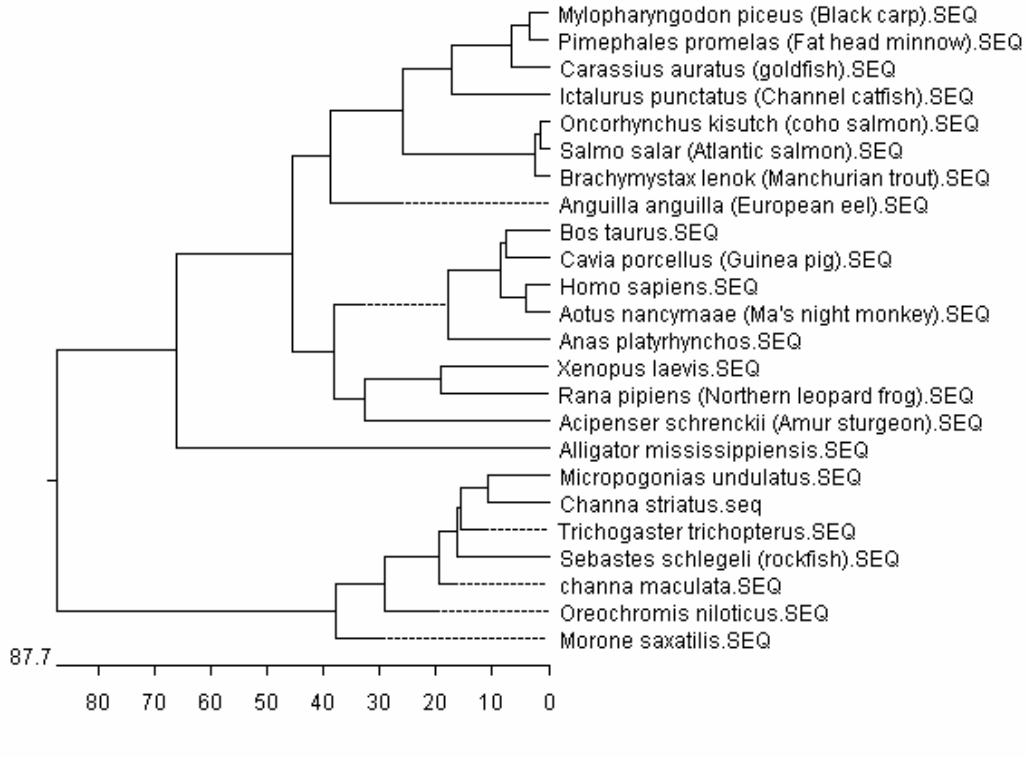


Fig 6: *Phylogenetic tree showing evolutionary status of C. striatus FSHβ in relation to FSHβ isolated from other species.(Pubmed accession No.s AF319961, AY447038, D88023, AF146152, AF146152, NM008045, DQ242616, NM174060, DQ838580, DQ232890, DQ200807, AY424303, AY609079, AY082514, AY250761, AF324541, AF146152, AY169722, AF112191, AF289174, DQ054790, AY515500)*

Sequence comparison was performed using Clustal analysis in Lasergene software and a rooted phylogenetic tree was constructed by the addition of available GnRH, LHβ, FSHβ and GTHα sequences from pubmed. Phylogenetic analysis of *C. striatus* sbGnRH showed that sbGnRH diverged into two distinct clades that giving rise to *C. stiatus* sbGnRH and *Oreochromos niloticus* sbGnRH very early during teleost evolution, from which subsequently other sbGnRHs developed (Fig 5). *C. striatus* FSHβ shows high similarity with that of other perciforms which together form a separate clade quite distinct from that of salmonids and mammals (Fig 6).

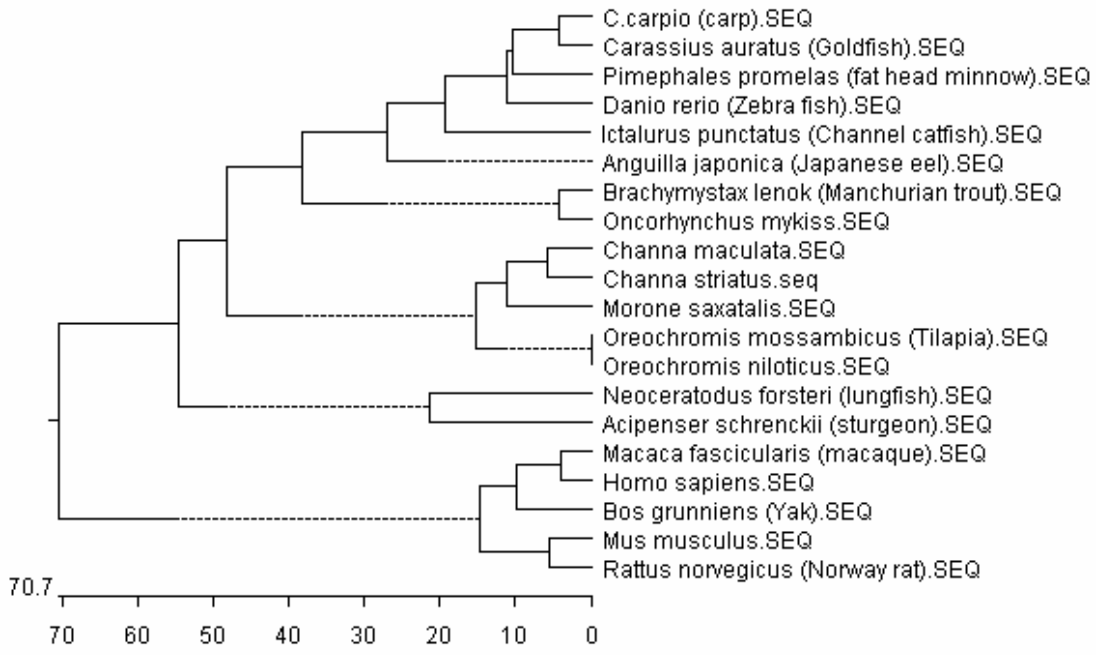


Fig 7*: Phylogenetic tree showing evolutionary status of C. striatus LHβ in relation to LHβ isolated from other species (Pubmed accession No. AY447037, L35096, NM000894, AY424304, , AY541609, AY294016, NM_001033975, AY575921, AY515501, AF112192, AY082379, AY026360, DQ242617, D88024, M37380, AB050836, AY026360, NM_000825)*

C. Striatus LHβ showed high similarity to LHβ from other perciforms. LHβ from other advanced and primitive teleosts species formed a separate clade from that of perciforms (Fig 7). GTHα from *C. striatus* shows high similarity with tilapia which form a separate clade distinct from that of other perciforms like red seabream.

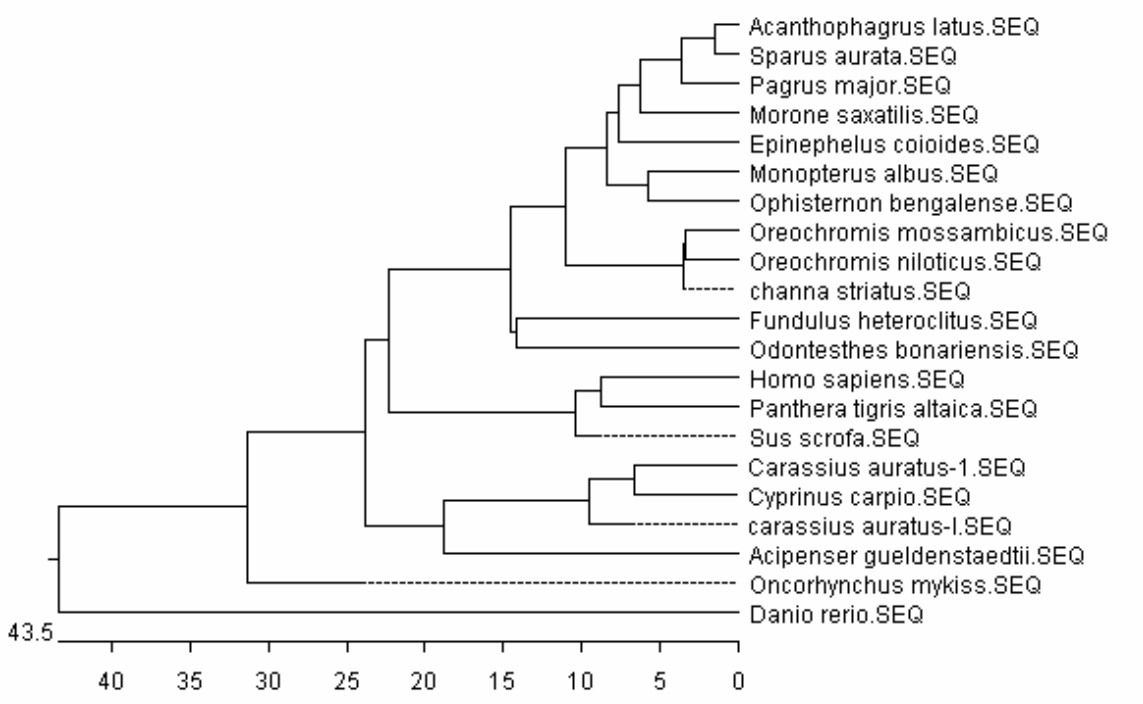


Fig 8: *Phylogenetic tree showing evolutionary status of C. striatus GTHα in relation to GTHα isolated from other species (Pubmed accession No. AF408393, AB050834, U12923, NM_214446, AY129309, AF502395, M94038, AF300425, L35071. AF303087, AF303087, AY294017, NM_000735, AY800266, AY800267, NM_205687, X56497, AB050834, AY519658, DQ382280)*

Spatial expression of sbGnRH, FSHβ, LHβ and GTHα in adult *C. striatus*: Using specific primers 183 bp sequence of sbGnRH was amplified in RT-PCR. sbGnRH transcripts were identified in gill and ovary apart from the classical POA-H hypothalamic (brain) site of expression (Fig 9). The amplified products in ovary and gill were confirmed to be sbGnRH transcripts by sequence analysis.

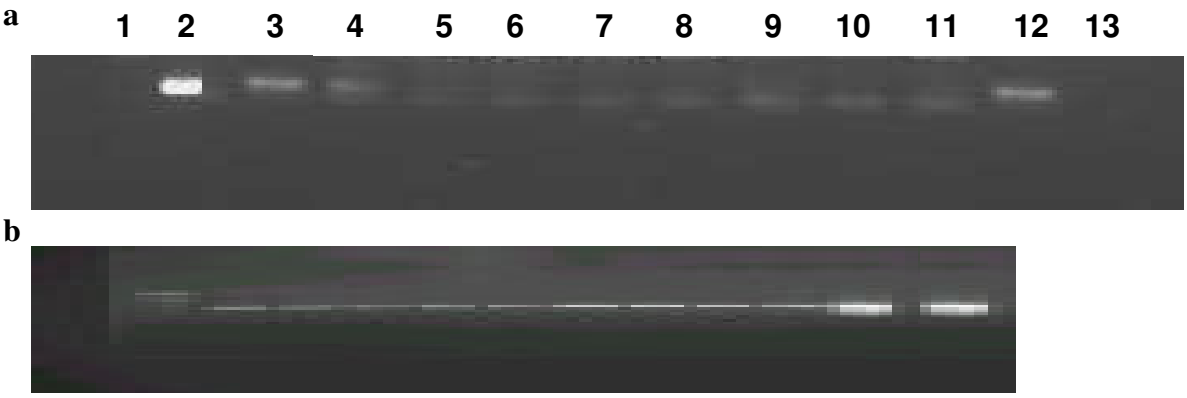


Fig 9: *a) sbGnRH distribution in various tissues; Lane 1- Marker, Lane 2- +ve control, Lane 3- Brain, Lane 4- Gill, Lane 5-Heart, Lane 6- Muscle, Lane 7- Liver, Lane 8- Spleen, Lane 9- Anterior kidney, Lane 10- Posterior kidney, lane 11- Intestine, lane 12- Ovary, Lane 13- -ve control. b) β- actin amplification in various tissues. +ve and –Ve control are not indicated in b. Lane order of remaining samples same as in a.*

FSHβ transcripts were identified in ovary, brain and liver in addition to pituitary (Fig 10 a, b). A slightly longer transcript was amplified in brain while LHβ transcripts were present in ovary and pituitary only (Fig 11a, b). Identity of all the amplified products was confirmed by sequence analysis. However, Western blot analysis revealed FSHβ and LHβ peptides only in pituitary extracts (Fig 10 c and 11 c). RT-PCR analysis showed GTHα transcripts only in pituitary while PCR southern depicted low level of GTHα transcripts in brain, ovary and kidney apart from pituitary (Fig 12).

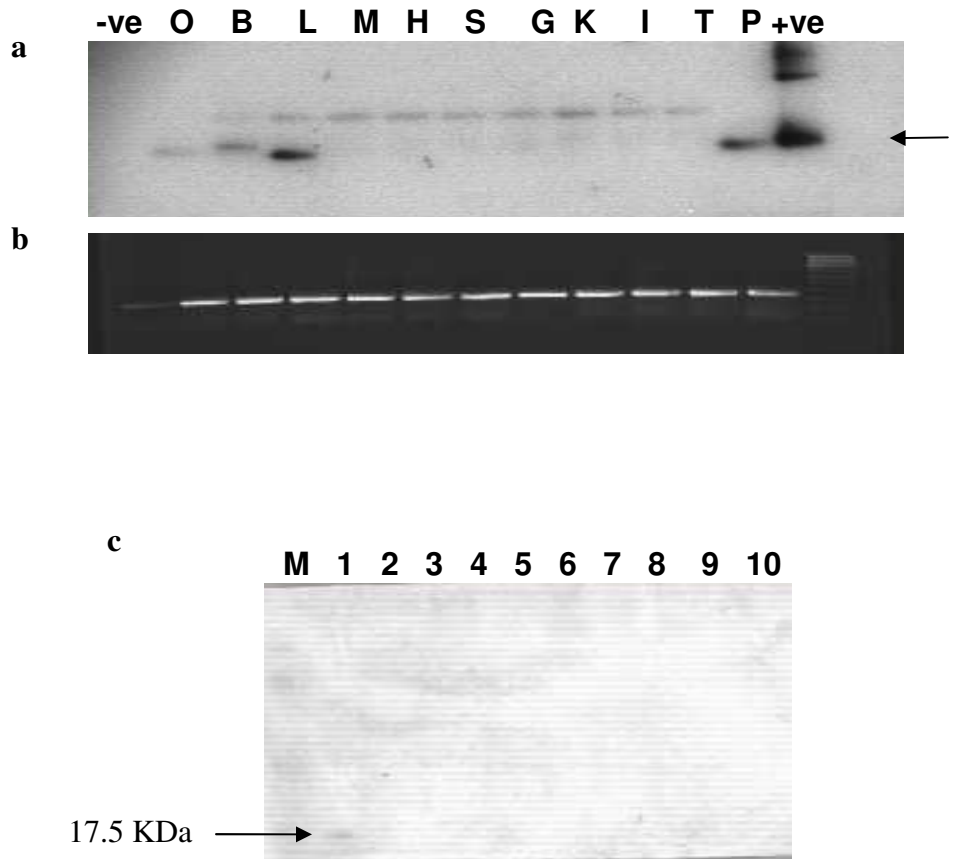


Fig 10: *a) PCR Southern for identification of FSHβ transcripts in various tissues. b) β-actin demonstrating cDNA quality in various tissues. c) Western blot analysis for identification of FSHβ peptides in different tissues. Lanes in a are as follows: B- Brain, L- liver, M- Muscle, S- Spleen, G- Gill, K- Kidney, H- Heart, I- Intestine, T- Testis, O- Ovary, P- Pituitary. Lane order in b is similar as in a except that the positive control is not depicted. Lane order in c: 1- Pituitary, 2-Brain, 3- Gill, 4- Heart, 6- Liver, 7- Spleen, 8- Kidney, 9- Testis, 10- Ovary.*

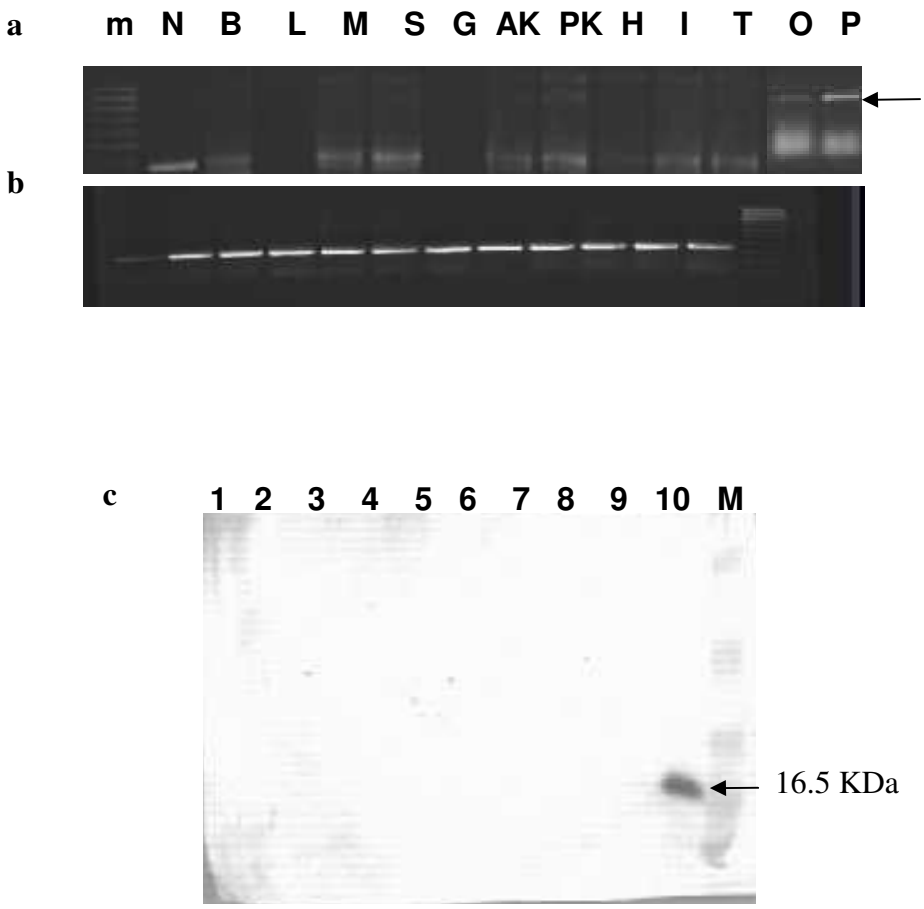


Fig 11: *a) 1% agarose gel depicting LHβ transcripts amplified by RT-PCR from various tissues. b) β-Actin amplified by RT-PCR from different tissues. c) Western blot analysis showing distribution of LHβ peptides. Lanes in a) are as follows: m –marker, N- negative control, B- Brain, L- liver, M- Muscle, S- Spleen, G- Gill, AK- Anterior Kidney, PK- Posterior kidney, H- Heart, I- Intestine, T- Testis, O- Ovary, P- Pituitary. Lanes in C are as follows: 1- Brain, 2- Gill, 3- Heart, 5- Liver, 6- Spleen, 7- Kidney, 8- Testis, 9- Ovary, 10- Pituitary, M- Molecular weight marker*

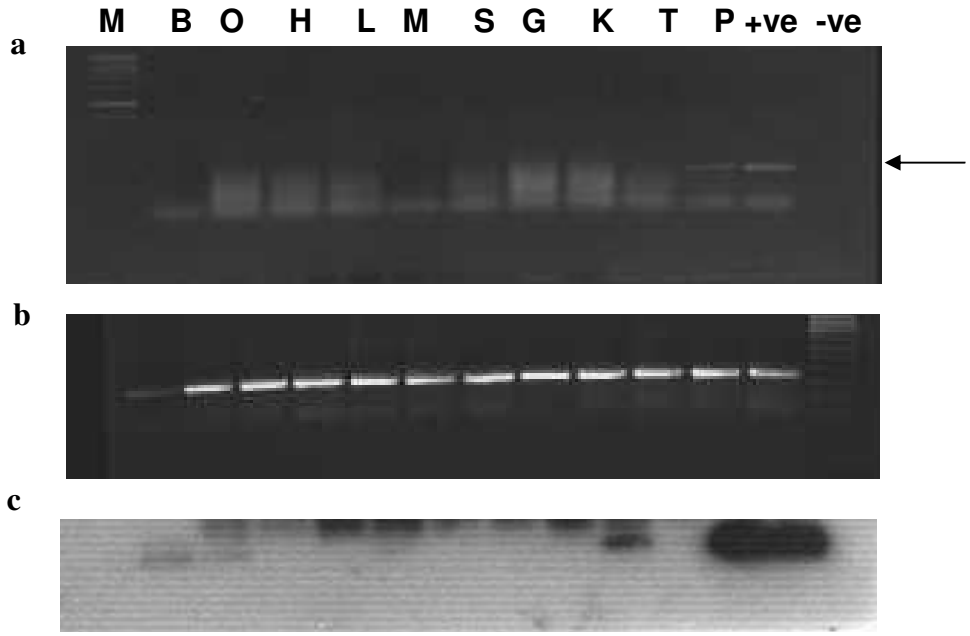


Fig 12: *a) 1% agarose gel depicting GTHα transcripts amplified by RT-PCR from various tissues. b) β-Actin amplified by RT-PCR from different tissues. c) PCR Southern distribution of GTH α peptides.Lanes m indicates marker, B- Brain, L- liver, M-Muscle, S- Spleen, G- Gill, K- Kidney, H- Heart, I- Intestine, T- Testis, O- Ovary, P-Pituitary.*

Temporal distribution of sbGnRH transcripts in ovary: Expression of sbGnRH transcripts increased in ovary along with progression of oocyte development and vitellogenesis. Semi-quantitative RT-PCR revealed increase in ovarian sbGnRH transcripts from ovaries with primary follicles to post vitellogenic follicles (Fig 13). sbGnRH protein levels as assessed by immunocytochemistry also exhibited a similar trend (Fig 14).

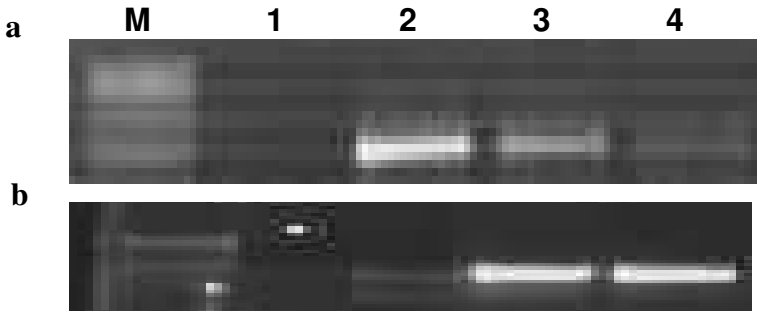


Fig 13: *Semi-quantitative RT-PCR analysis of a) sbGnRH in ovary b) β- actin Lane M indicates marker, 1- -ve control, 2- +ve control, 3- ovary with post vitellogenic follicles, 4- ovary with post vitellogenic follicles.*

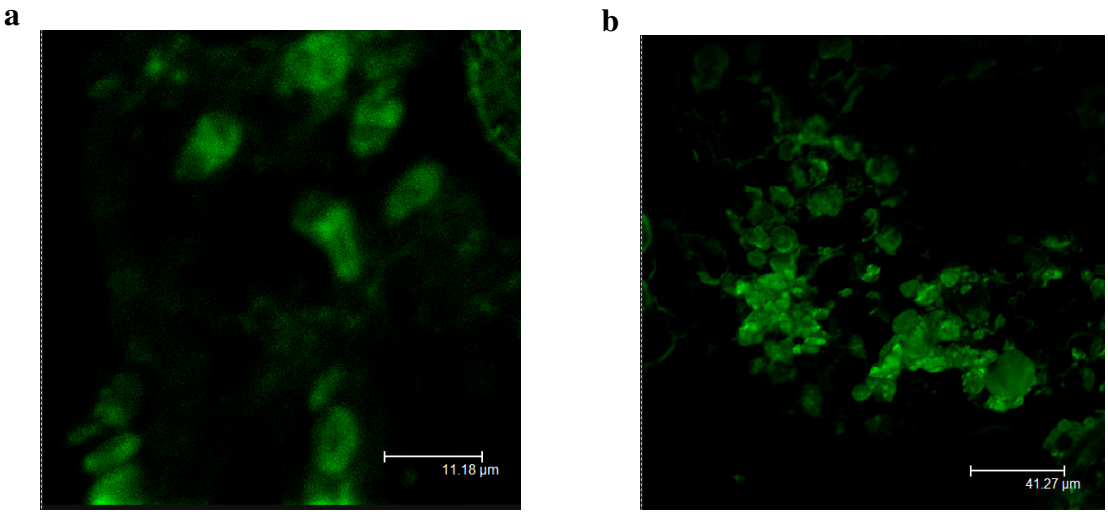


Fig 14: *Immunocytochemical localisation of sbGnRH in ovary a) ovary with previtellogenic follicles b) ovary with postvitellogenic follicles*

Effect of GnRH analog on GVBD: GnRH analog (GnRH-A) at 5mg/ml, 25 mg/ml and 100 mg/ml concentration was able to induce 45% and 57% GVBD respectively in vitro after incubation for 36 h as compared to 96% in hCG and 1.6% in negative control (Table 2).

Table 2:

Sample	**Control**	**hCG**	**GnRH-A (5µg/ml)**	**GnRH-A (25µg/ml)**	**GnRH-A (100µg/ml)**
% GVBD	16% ± 1.5%	96% ± 2.3	45% ± 1.4	57% ± 3.8	57% ± 2.6

Discussion:

Complete cDNA sequence and therein deduced amino acid sequences of sbGnRH, FSHβ, LHβ and GTHα from *C. striatus* are presented herewith. Also presented are the phylogenetic position of sbGnRH and GTH subunits of *C. striatus* in relation to sequences from other teleosts and few representative mammalian species. The POA-H form of GnRH transcript in *C. striatus* codes for the sbGnRH decapeptide as in other perciforms. In phylogenetic analysis it shows high similarity with perjerrey form GnRH and sbGnRH isolated from few other teleosts but not with that of sbGnRH from *Morone saxatalis*, *Oreochromis niloticus* and other related species (Fig 5). This may be due to the fact that GnRH is a decapeptide hence is not generally considered for phylogenetic

analysis by itself. Instead Phylogenetic analysis is carried out taking into consideration the entire cDNA sequence encoding the pre-pro GnRH. This includes the signal peptide, GnRH decapeptide and GAP sequence which is highly variable even between related species. Similarly it may be observed that the sequence percentage identity in BLAST and phylogenetic tree reveal distinct similarity patterns. This may be due to the different convergence, divergence criteria used in matching sequence similarities.

Analysis of spatial distribution, demonstrated expression of sbGnRH in brain, gill and ovary of *C. striatus* and an increase in expression in ovary from immature resting stage to mature stage with post-vitellogenic follicles. Evidence from earlier reports support the expression of GnRH subtypes in brain and some non-neuronal tissues including gonads. Apart from the hypophyseal regulation of GTH secretion, GnRH has also been shown to regulate important processes in ovarian cycle through its autocrine and paracrine action. The expression of sbGnRH in gill is not fully understood but may be ascribed to the role of GnRH in pheromonal action (Sherwood *et al.*, 1997). GnRH has been shown to cause apoptosis in testis of goldfish, through induction of death receptor pathway involving activation of fas, fasL-like proteins, caspase -3 and caspase-8 (Andreu-Vieyra *et al.*, 2004). GnRH subtypes have also been shown to have a stimulatory effect on reinitiation of oocyte meiosis and germinal vesicle breakdown (Pati and Habibi 2002). Following this line of thought is the current observation (though preliminary) that GnRH analogs are able to induce reinitiation of oocyte meiosis. This may explain the observed increase in sbGnRH transcripts and immunoreactivity in mature ovary with vitellogenic oocytes

compared to the resting phase ovary. GnRH subtypes have also been shown to induce expression of novel LHβ and FSHβ transcripts in ovary (Wong and Zohar, 2004).

The LHβ transcripts were identified in ovary and FSHβ transcripts in extra pituitary tissues like brain and liver. Similarly GTHα transcripts were identified in kidney and brain. This is corroborated by the recent demonstration of LHβ transcripts in fish ovaries by Wong and Zohar, 2004. However, their physiological significance in extra pituitary tissues is not well understood. GTHα transcripts were not observed in ovary hence he physiological relevance of beta transcripts in ovary in absence of the alpha transcripts is questionable as it is generally believed that the GTHs are physiologically active following dimerization of α and β subunit. Furthur, Western blot analysis showed that FSH and LHβ were present only in pituitary extracts raising speculation that the LHβ, FSHβ and GTHα transcripts though present in extrapituitary tissues may not be transcribed in these tissue locations else their half life and quantity is very low making them undetectable on Western blots.

Thus, in the present study we describe expression of sbGnRH transcripts in gill and ovary, in addition to the well-established site of expression in brain (POA-H). Presence of FSHβ, LHβ and GTHα in extrapituitary and gonadal tissues has been reported for the first time in course of this study though their functional implications have to be assessed. We also report an increase in transcripts in ovary with the progression of oogenesis with plausible implications for understanding its role in reinitiating of oocyte meiosis.

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Chapter 5

Cloning, in silico and functional analysis of sbGnRH upstream promoter motifs from Channa striatus

Abstract: The central role of gonadotropin-releasing hormone (GnRH) as the chief regulator of vertebrate reproduction has long been established, but the molecular mechanisms underlying its synthesis, pulse frequencies and regulation still remain largely unexplained. Analysis of GnRH promoter and upstream regulatory elements would lend important insights into these mechanisms. In the present study a 1038 bp promoter sequence upstream of the *Channa striatus* sbGnRH gene has been isolated. *In silico* analysis of this sequence revealed putative binding sites for transcription factors Oct-1, SF-1, FOXL 1, thyrotrophic embryonic factor and GATA binding domains. Functional analysis with promoter luciferase reporter constructs indicated that the -1038 to -660 region was important for promoter activity. Studies also revealed that testosterone, estradiol-17β and T_4 had repressive effect on the basic promoter activity.

Introduction:

Gonadotropin-releasing hormone (GnRH) - a decapeptide of hypothalamic origin acts as the central regulator for reproduction in all mammals (Peter *et al.*, 1991). First isolated from porcine hypothalamus (Matsuo *et al.*, 1971), this decapeptide was later identified in several vertebrates where it stimulates synthesis and secretion of gonadotropins (GTHs) from adenohypophysis. To date, 14 distinct GnRH forms have been characterized from vertebrates and multiple GnRH forms have been identified in species varying in their anatomical distribution, ontogenic origin and physiological function (Parhar 2002; Iwakoshi *et al.*, 2004). GnRH producing neurons are chiefly distributed in the olfactory bulb/ telencephalon, midbrain tegmentum and pre-optic area hypothalamus. Teleosts lack

a hypothalamo-hypophyseal portal system and hence contribute a useful model for studying the physiological implications and function of multiple GnRH system in a species (Bla´zquez *et al.*, 1998). Moreover, multiple GnRH in a single species was first identified in teleosts (Powell *et al.*, 1994). *In situ* and immunocytochemical studies have shown that the POA-H GnRH neurons innervate the pituitary and are the most relevant GnRH population involved in control of GTH release. In C*hanna striatus*, a natively available perciform species, the predominant POA-H neurons are sbGnRH neurons.

Reproduction in vertebrates is a highly synchronized phenomenon influenced by different environmental parameters like photoperiod, temperature, seasonal cycles and social cues. GnRH levels and pulse frequencies vary according to all these social, environmental and social cues (Peter *et al.*, 1991; Senthilkumaran and Joy, 1995; Senthilkumaran *et al.*, 1999). The mechanism of endocrine regulation of GTH secretion in response to GnRH is widely understood as are the effects of monoamines and sex steroids on GnRH and GTH release (Peter *et al.*, 1986; Yu *et al*., 1991; Yu and Peter, 1992; Goos *et al.*, 1999; Senthilkumaran *et al.*, 2001; Okuzawa *et al*., 2002). However, the monoaminergic effect and positive, negative feedback by sex steroids on GnRH neurons does not completely explain the variations in GnRH pulse frequencies and levels during sexual maturation, differentiation and in response to social, physiological cues. Further, the molecular mechanism controlling the differential expression of different GnRH genes in different brain loci has received considerable attention in mammals, but remains unresolved. Understanding the regulatory mechanisms of GnRH genes will expand our knowledge on GnRH neuron physiology and putative transcription factors involved in its expression.

Such studies are however sparse and limited in teleosts (Kitahashi *et al.*, 2005). The few studies in teleosts addressing these issues are limited to *in silico* analysis of the gene sequence upstream of the transcription start site to identify putative transcription factor binding sites (Kitahashi *et al.*, 2005).

sbGnRH being the most relevant form of GnRH for reproduction in *Channa striatus*, we aimed to isolate the upstream (promoter) sequence of the gene encoding sbGnRH. The obtained promoter sequence was subjected to *in silico* analysis for identification of the potential regulatory motifs involved in sbGnRH gene expression. Further, functional analysis of the isolated promoter sequence was also carried out to identify the location of transcriptionally important upstream elements. The effect of the thyroid hormone (T_4) and sex steroids analogs methyltestosterone (MT) and ethynylestradiol (EEL) on promoter activity was also assessed.

Materials and Methods:

Isolation of genomic DNA: Genomic DNA was prepared from brain of adult *Channa striatus* using the genomic DNA preparation kit supplied by Bangalore Genei Pvt. Ltd. (India) following the manufacturer's protocol.

Isolation of sequence upstream of transcription start site by Genome Walking: Genome walking was carried out using the Universal genome walking kit from BD Biosciences (Clonetech, USA). 25 µg of genomic DNA was used to construct restriction digestion based genome walking DNA templates as per the manufacturer's protocol.

Primary and secondary genome walking was carried out using gene specific primers (GSP) designed from the sbGnRH cDNA sequence obtained from *Channa striatus* and genome walking primary and nested primers supplied in the kit. Primary walk PCR conditions were as follows: 94 C – 30 sec; 94 C – 45 sec, 72 C – 2 min (5 cycles); 94 C - 45 sec, 69 C- 1 min, 72 C – 2 min (25 cycles); 72 C – 10 min. Secondary walk PCR conditions: 94 C – 30 sec; 94 C -45 sec, 65 C- 1 min, 72 C – 2 min (35 cycles); 72 C – 10 min. All secondary walk PCR products were separated on 1% agarose gel by electrophoresis and the band of expected size was purified using MinElute Gel Extraction Kit as per manufacturer's protocol (Qiagen). The purified PCR products were cloned into pGEM-T Easy vector (Promega) and transformed into *E. coli* DH5α competent cells. The plasmid DNAs were purified using QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. The plasmid DNA containing candidate sbGnRH promoter inserts were sequenced bidirectionally using T7 and SP6 universal primers.

GSP fw primer	:	5'CTGTTCAGTTCCCGCTTCCCTC 3'
GSP nested fw primer	:	5' GCTGCCAGCACTGGTCGTACGG 3'

***In silico* analysis:** Sequence identity was confirmed by BLAST. Potential transcription factor binding sites, primarily nuclear receptor response elements, were identified using MatInspector software along the 1038 bp upstream of the transcription start site.

Construction of sbGnRH promoter-luciferase reporter constructs and transfection into CHO cells: The 1038 bp sbGnRH promoter region was PCR amplified from the Promega T-easy plasmid containing the cloned sbGnRH promoter sequence using specific forward and reverse primers with Hind-III and Xho-I restriction sites respectively (Table 2). The amplified PCR products were cloned into Promega T easy vector following which the plasmids with inserts were double digested with Hind-III and Xho-I. The digested products were then ligated into PGL2 basic luciferase vector, similarly cut with Hind-III and Xho-I. Serial PCR based sbGnRH promoter deletion constructs were made in PGL2 basic vector by designing primers at different locations in the promoter sequence. The obtained sbGnRH promoter- PGL2 luciferase reporter constructs along with the PRLTK plasmid were then transfected into CHO cells grown in DMEM medium (Gibco BRL) using the Promega TFX reagent and lipofectamin.

Table 2:

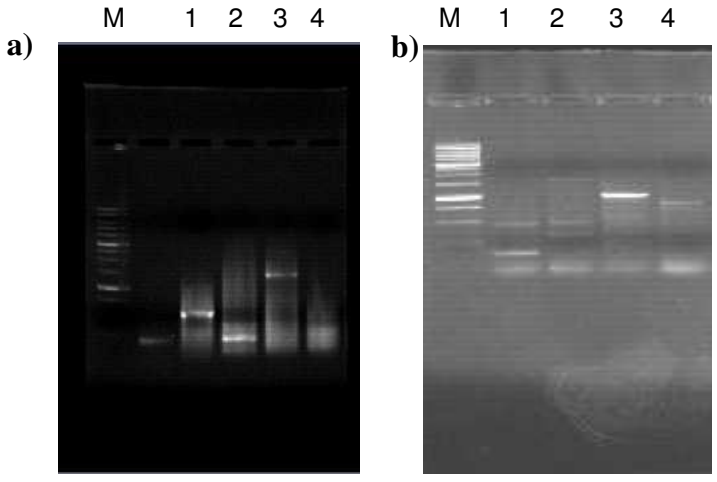
GSP used for PCR based promoter deletions:		
GSPF1 (for Del1)	:	5'GCTAGCGGCGTCGCGGCTCCGGCCGCCATG 3'
GSPF2 (for Del 2)	:	5'GCTAGCCATTCTCACAGGCTGGGTACCTAC 3'
GSPF3 (for Del 3)	:	5'GCTAGCCATGCAAAACGACAAAGTCCAGT 3'
GSPF4 (For Del 4)	:	5'GCTAGCCAATTATGTGATATATATCAATAC 3'
GSPR (common rev primer)	:	5'CTCGAGCGAATTCACTAGTGATTACTATA 3'

Luciferase assay: Promoter activity was assayed using the dual luciferase assay kit supplied by Promega.

Effect of MT, EEL and T_4 on promoter activity: Effect of T_4, E_2 and T on promoter activity was assessed by addition of 2 ng/ml, 2 ng/ml, 0.05 ng/ml of MT, EEL and T_4 respectively to the cell culture medium 6 hr after transfection.

Results:

***In silico* analysis:** Computational analysis of the obtained 1038 bp sbGnRH promoter sequence (Fig 1) revealed putative binding sites for the transcription factors -octomer binding factors (OCT-1, OCT-2), steroidogenic factor 1 (SF-1), thyrotropic embryonic factor, SOX 5, GATA binding factors, CREB/TAX complex, Fork head proteins FOXL-1 (Table 1).



c)

5'GGCGTCGCGGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTCCAATTG

ATATATGGCACCATAACAGATTTTATAAATTATCACTTTTATATTATAATTTA

AAACACTAATTTATTTAAAGCTAAGACAGACTAAAAGAAAAAATACTGACTA

AAATGTTTCTTCATTATAGTTTATTTATAACAGTCATTAATGGAAATATATTTC

CACAGTCGAGTGCAATAATCATACAGGGAGACCTACACCACTCCCCACCTTA

AATACAAACCATATATTGCCCCATATTGCATGTAGTGAGAAGTATTAAAATA

AATAACTTTAGCTCTTACATTGATTTTCACACTCAAGGTGAGACACGTGATTG

CCTTCATCATTCATTCTCACAGGCTGGGTACCTACTTTTGAACTGAGTGTGTA

TAGTATTGCATCGGAGCAGCTTTGAGCTACAGCTAGAGAAACTTCACCAGTC

AAAGACACACTTCACACATGCAAAACGACAAAGTCCAGTAAGAAGTTAGAC

AAGATCCTTGACATAACAGCTCACACAACCTATGCTAACTGTTTTTTTGTTTG

TTTTTTCTTGAGAGGGGAAGATTTGGAAGCACTGGATTCTTTTACATAAGATT

ACAAGCACCAAACATACTTCGTGAAAAGGACAATACAACATACAAGTTAACA

TAAACTTGTGCAAATAACTTCAAAGCACAATTATGTGATATATATCAATACAT

GAAGCATATATCCAAGCAAACCCAGTTTAACATGAACAATGGCTTTCTGCGC

ATCAGTTTGATTTTTTTTTCATGATGGGAATCTGATGATGAATGACAGACATA

AACATTTCATAAGCAGTGCCACAAACAAATACATGCTGTTGTGGATCACTTA

CACACCAGATTAATATAAGAGGTAGATTCACAGAGAGAAAAACAAGGGGAA

GCGTTTTAAGAACACAGTAAGTAGGAAAGGAGAAACCACACAGACCAGCCC

GGGCCGTCGACCACGCGTGCCCTATAGTAATCACTAGTGAATTCG **3'**

Fig 1: 1% agarose gel showing PCR amplified products after primary (a) and secondary walk (b). Lane M- marker, Lane 1- amplification from DraI library, 2- EcoRV, 3- StuIV, 4- PvuII. c) 1.038 kbp sbGnRH promoter sequence upstream from transcription start site.

Table 1: Putative transcription factor binding sites identified in sbGnRH gene upstream sequence by MatInspector software

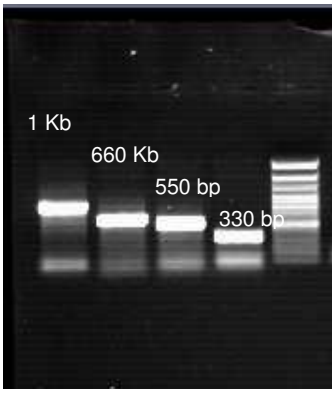
Factor	**Position (+ strand)**	**Sequence**
GATA binding factor 3	66-78	aacAGATtttata
GATA binding factor 2	77 – 89	aagtGATAattta
OCT 1	91-105	tatattatAATTtaa
Thyrotropic embryonic factor	110-126	ctaatttatTTAAagct
OCT 1	193-207	ttAATGgaaatatat
Nuclear factor of activated T cells 5		aatGGAAatatatttccac
Serum response factor	263-275	caaaccaTATAttgcccca
HOX cluster of homeobox proteins	306 - 322	attaaaataAATAactt
FOXL1	307-323	ttaaaaTAAAtaacttt
SF-1	344-356	cactCAAGgtgag
GDNF inducible zinc	411-423	TGAGtgtgtatag

finger protein		
Heak shock factor 2	445-469	agctacagctagAGAAacttcacca
OCT-1	487-501	cacATGCaaaacgac
cAMP binding element protein 1	526-546	gatcctTGACataacagctca
PAR-type chicken vitellogenin promoter-binding protein	527-543	atccttgacATAAcagc
Fork head homologous X binds DNA with a dual sequence specificity (FHXA and FHXB)	530-546	cttgacATAAcagctca
OCT-1	552-566	cctatgctAACTgtt
Thyrotropic embronic factor	707-723	cacaattatGTGAtata
Interferon regulatory factor (IRF-3)	648-668	tcgtgaaaagGACAatacaac
SOX-5	654-670, 765-781	aaaggaCAATacaacat
OCT-1	666-680	aaCATAcaagttaac

Photoreceptor-specific nuclear receptor subfamily 2, group E, member 3 (Nr2e3)	695-712	ataactTCAAagcacaattat
FOXA2	670-686	tacaagttAACAtaaac
TAX/CREB complex	728-748	aatacaTGAAgcatatatcca

Functional analysis of sbGnRH promoter activity using promoter deletion constructs: Considerable luciferase activity was obtained with the 1.038 promoter constructs which was significantly reduced by deletion of the -1038 to -660 bp sequence (Fig 2).

a)



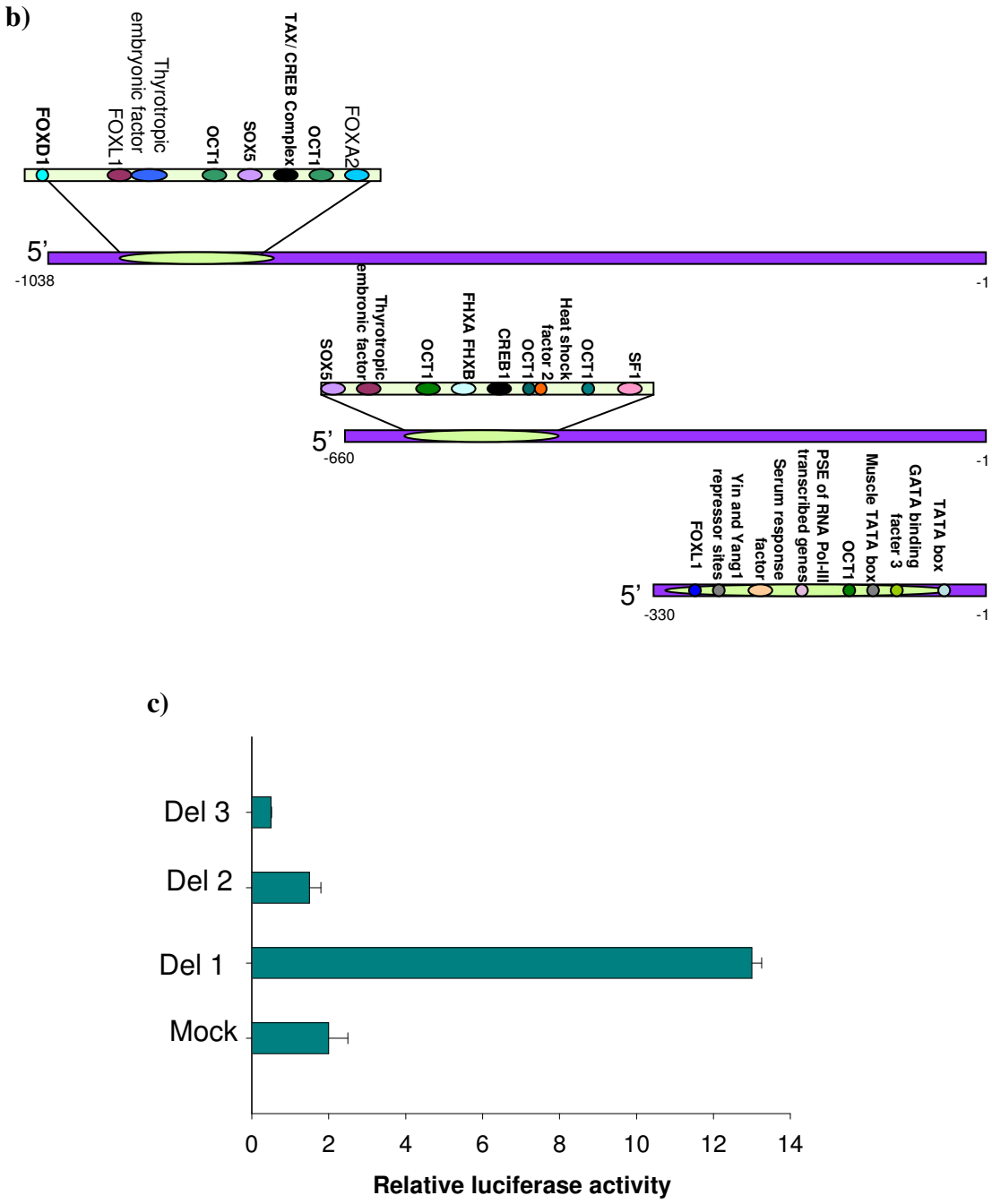


Fig 2: a) 1% agarose gel showing generation of different PCR based deletions. b) Schematic representation of putative regulatory motifs in variation deletion constructs of sbGnRH promoter Channa striatus. C) Relative liciferase activity of different sbGnRH promoter-luciferase reporter constructs.

Effect of MT, EEL, T_4: T, E_2, T_4 added to the cell culture medium lead to decrease in basic promoter activity using the complete 1038 bp sequence (Fig 3).

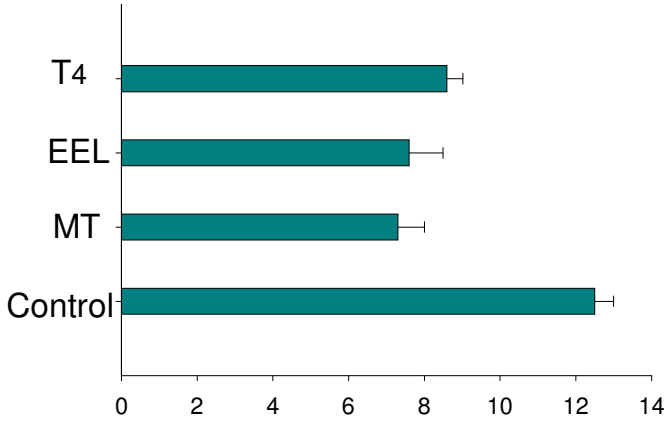


Fig 3: Relative luciferase activity of the Del 1 construct in presence of T, E_2 and T_4

Discussion:

The present study demonstrates presence of putative sites for binding of Oct-1, 2, SOX-5, FOXL-1, SF-1, and thyrotrophic embryonic factor in the promoter of sbGnRH. SOX proteins are involved in sexual and neural differentiation (Wegner, 1999) therefore, it is possible that sbGnRH is involved in sexual differentiation (Parhar, 1997). This may also contribute to sex related differences in sbGnRH expression during early sex differentiation (see chapter 1). Further, the presence of binding sites for SOX sbGnRH promoter is interesting because progesterone is known to regulate SOX (Wegner, 1999). Parhar *et al.* (1998) had earlier demonstrated an absence of influence of progesterone on any of the three GnRH systems in the male tilapia. This may probably be due to the dose,

reproductive stage of animals or the lack of estrogen priming, which is essential for sbGnRH synthesis by progesterone (Kim *et al.*, 1989).

Oct-1 is believed to be responsible for regulation of GnRH pulses and cell specific expression of sbGnRH transcripts (Clark and Mellon, 1995). Furthur, Oct-1 interacts with GR and is considered to mediate glucocorticoid repression of sbGnRH gene (Chandran and DeFranco, 1999). The presence of binding sites for myogenic factors and muscle TATA box in the promoter of sbGnRH gene provides indirect evidence for the speculative role of sbGnRH and GnRH receptors in skeletal muscle physiology (Okubo *et al.*, 1999; Kakar and Jennes, 1995; Jodo *et al.*, 2003). Presence of binding sites embryonic thyrotrophic factors indicates that sbGnRH expression may be influenced by thyroid related factors during early development. Putative photoreceptor-specific nuclear receptor binding sites have also been identified in the 1038 bp sbGnRH sequence. This may indicate a possible mechanism for direct influence of photoperiod changes on GnRH expression.

Functional reporter assays for analysis of promoter activity showed that the -1038 bp to -660 bp region was required for optimal promoter activity. GnRH promoters may require specific transcription factors for optimal function as evident from the *in silico* analysis. This is further exemplified by the cell specific expression of sbGnRH in few brain loci. Hence, it would be appropriate to use cells capable of GnRH expression for transfection and assaying GnRH promoter activity. sbGnRH expression has been earlier observed in ovarian tissue (Habibi and Pati, 1993, Chapter 4) prompting the usage of CHO cells for assaying promoter activity in the present study as opposed to commonly used COS and

HEK 293 cells. In agreement with this line of thought, non neuronal cell lines capable of expressing GnRH (eg. spleen cell lines, Farahmand *et al.*, 2003) have been used in addition to neural cell lines (Muraoka *et al.*, 2003) for analysis of neuropeptide promoter activity. Preliminary studies also demonstrated that T, E_2 and T_4 have a repressive effect on sbGnRH promoter activity at the dosages used. This is however yet to be further ascertained by using varying concentrations of these compounds. Finally it may be cautioned that the functional analysis of the sbGnRH promoter presented here is at a very preliminary stage and further efforts are underway in this direction with attempts to isolate native POA-H neuronal cell lines and carry out promoter analysis in these cells.

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Consolidated summary

The present thesis is an effort to understand the role of gonadotropin-releasing hormone and gonadotropins (GnRH and GTH) in early sex differentiation; the effect of thyroid hormones and sex steroid analogs on GnRH-GTH axis and GnRH promoter. These aspects have been studied as five major chapters and the results and important findings are summarized below. Chapter 1 and 2 have been divided into two sub-chapters 1, 1.1 and 2, 2.1 respectively in the thesis but the results are herewith consolidated and summarized as Chapter 1 and Chapter 2 in this section for better understanding and clarity.

Chapter 1: Variations in GnRH ontogeny between sexes and its impending role during sex differentiation were studied. 0 dah (days after hatching) to 50 dah tilapia XX, XY fingerlings were collected, wax embedded, serially sectioned and incubated with anti-sbGnRH, Anti-LHβ and Anti-FSHβ (generated in a heterologous system, Red sea bream, *Pagrus major*) sbGnRH is the predominant POA-H GnRH form in tilapia, a perciform. Significant variations were observed in sbGnRH immunoreactivity (ir-) during critical period of gonadal sex differentiation with possible implications to sex differentiation. sbGnRH ir- apperas in POA of earlier in XY fish compared to XX and is detectable by 7 dah and in pituitary by 10dah. sbGnRH ir- appears in POA and pituitary of XX fish by 15 dah . GnRH ir- reduces in XY fish from 11 dah onwards and is almost undetectable by 30 dah. During this period the sbGnRH ir- in XX fish steadily increases reaching a peak by 30 dah. LHβ ir- appeared in pituitary of XY fish by 20dah. No LH ir- was observed at 6dah and 14dah. LHβ ir- was less in pituitary of 20dah XX fish compared to age matched

XY fish. FSHβ ir- was observed in XX fish by 29 dah and in XY fish by 20dah. LHβ ir- was more diffusely distributed in pituitary while FSH ir- was localized in distal regions. LHβ ir- was less intense in XX when compared to XY fish. The study revealed subtle differences in POA, pituitary sbGnRH ir-, LH ir- and FSH ir- during early development of male and female fish. These findings may have implications in early sex differentiation.

Chapter 2: Thiourea-induced thyroid hormone depletion was used as a strategy to understand the influence of thyroid hormones on testicular recrudescence of the air-breathing catfish, *Clarias gariepinus*. Treatment with 0.03% thiourea via immersion for 21days induced hypothyroidism (thyroid hormone depletion) as evidenced by significantly reduced serum T_3 levels. Thiourea-treated males had narrowed seminiferous lobules with fewer spermatozoa in testis, very little or no secretory fluid in seminal vesicle, reduced protein and sialic acid levels in seminal vesicles when compared to controls. The histological changes were accompanied by reduction in serum and tissue levels of testosterone (T) and 11-ketotestosterone (11-KT), a potent male specific androgen in fish. Qualitative changes in the localization of catfish gonadotropin-releasing hormone (cfGnRH) and luteinizing hormone (LH, heterologous system) revealed a reduction in the distribution of immunoreactive neuronal cells and fibers in thyroid depleted fish. Thyroid hormone depletion also caused an increase in stimulatory monoamines norepinephrine and serotonin while the levels of inhibitory monoamine dopamine remained unaltered in POA-H. Interestingly, thiourea-withdrawal group

showed physiological and histological signs of recovery after 21days such as reappearance of spermatozoa and partial restoration of 11-KT and T levels. These data demonstrate that thyroid hormones play a significant role in testicular function of catfish. The mechanism of action includes modulating sex steroids either directly or through the hypothalamo (GnRH)-hypophyseal (LH) axis.

Chapter 3: Exogenously administered steroid analogs were used to study the effect of sex steroids on POA-H GnRH and monoamines. Male and female fishes treated with 1µg/lt of ethynlestradiol (EEL) and methyltestosterone (MT) respectively exhibited considerable gonadal changes as observed from histological studies. Male fish treated with EEL showed loss of sperm in selected testicular lobules while female fish treated with MT showed precocious oocyte maturation. EEL treatment caused decrease in POA-H GnRH ir- and transcripts while no significant change was observed in POA-H GnRH ir- and expression in MT treated fish. Similar results were obtained with pituitary LH levels. POA-H levels of serotonin and norepinephrine (stimulatory for GnRH secretion) decreased by more than 2 fold in fish treated with EEL and MT while levels of the inhibitory monoamine dopamine increased to a significant extent. Data obtained demonstrates a direct effect for sex steroids on POA-H monoamine levels and GnRH expression.

Chapter 4: *Channa striatus* is a local fresh water species in great demand due to its edible value and applicability in practices involving alternate medicine. The high demand

is not appropriately substantiated by the availability hence leading to high market prices and non affordability by general population. The limited availability of this species owes to its low reproductive efficiency and lack of sufficient characterization and understanding of its reproductive and developmental events and stages, thus hindering any attempts so far in artificial fertilization, seeding and rearing in southern part of India. GnRH is the chief endocrine regulator of various reproductive events in fish, itself subject to complex regulatory processes, less understood and subject to different internal and external cues like nutritional status, environmental factors, photoperiod and social factors. Unlike as in higher vertebrates like mammals where there exist only one or two forms of GnRH, fish display multiple forms of GnRH, the evolutionary significance of which still remains unclear. Thus in an initial attempt to follow and characterize the reproductive events of murrel we propose to clone sbGnRH – the predominant POA-H form and LHβ, FSHβ and GTHα from *Channa striatus*- a perciform fish species. sbGnRH transcripts from murrel comprise of 433nt sequence encoding a preprohormone of 297 amino acids. GTHα cDNA from murrel consists of 445nt encoding a 354 amino acid sequence. FSHβ cDNA consists of 535nt encoding 363 amino acids, while the LHβ transcript comprises of 581nt and encodes 447 amino acids. Analysis of spatial distribution of GTH, sbGnRH transcripts showed that sbGnRH transcripts were present in gill and ovary, in addition to the classically known POA-H (brain) region. Immunocytochemistry revealed presence of sbGnRH ir- in ovary. sbGnRH ir- and transcripts in ovary increased from pre-vitellogenic to post vitellogenic stage indicating a possible role in reinitiation of oocyte meiosis. GnRH analog could induce ~60 GVBD in

in vitro oocyte cultures compared to hCG indicating that GnRH could induce oocyte maturation. FSHβ transcripts were observed in testis, ovary and liver in addition to pituitary. LHβ transcripts were observed in ovary. This was confirmed by PCR Southern, cloning and sequencing. RT-PCR revealed GTHα transcripts only in pituitary but minute amounts were also detected in testis and ovary using PCR-Southern. While implications of the presence of GTH transcripts in extra-pituitary tissues are yet to be understood, the study clearly establishes their presence.

Chapter 5: Regulation of GnRH secretion and pulse frequencies is barely understood. Important insights could be obtained regarding different transcription factors and gene elements involved in GnRH expression by analyzing GnRH promoter motifs. 1.038nt sequence upstream of sbGnRH transcription start site was isolated by genome walking and cloned into PGL2 basic luciferase reporter vector. *In silico* analysis of the 1.038nt upstream sequence using genomatix Matinspecter software tool showed putative binding sites for the transcription factors CREB/TAX, FOXD2, FOXL2, octomer binding proteins OCT-1 and OCT-2, SF-1 and SOX-5. Deletion analysis of the region showed that the -550 to -1038 region was important for basic promoter activity when transfected into CHO cells. Preliminary assays with sub-physiological doses of T_4, EEL and MT showed that they repressed basic promoter activity.

In conclusion the present study clearly demonstrates the differential ontogeny of POA-H GnRH in XX, XY fish, repressive effect of thyroid hormone depletion and EEL on POA-

H GnRH ir- and pituitary LH ir-, effect of thyroid hormones, EEL and MT on POA-H monoamine levels. The study also establishes the expression of LHβ, sbGnRH transcripts in ovary in a stage dependent manner.

Publications

From thesis work:

Swapna I, Rajasekhar M, Supriya A, Raghuveer K, Sreenivasulu G, Rasheeda M K, Majumdar K C, Kagawa H, Tanaka H, Dutta-Gupta A, Senthilkumaran B. 2005. Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. Comp Biochem Physiol 144A: 1-10.

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