Molecular cloning and expression analysis of *vasa*, *dmrt1*, *sox9* and *tryptophan hydroxylase* during sex differentiation and gonadal recrudescence in catfish

A thesis submitted to University of Hyderabad for the award of a Ph.D. degree in Animal Sciences

By

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DECLARATION

I, **Mr. K. Raghuveer** hereby declare that this thesis entitled **"Molecular cloning and expression analysis of *vasa*, *dmrt1*, *sox9* and *tryptophan hydroxylase* during sex differentiation and gonadal recrudescence in catfish"** submitted by me under the guidance and supervision of **Prof. B. Senthilkumaran** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other University or Institute for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled **"Molecular cloning and expression analysis of *vasa*, *dmrt1*, *sox9* and *tryptophan hydroxylase* during sex differentiation and gonadal recrudescence in catfish"** is a record of bonafide work done by **Mr. K. Raghuveer** a research scholar for Ph.D. programme in the Department of Animal Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or in full to this University or any other University or Institute for the award of any degree or diploma.

Signature of the Supervisor

Head, Department of Animal Sciences

Dean, School of Life Sciences

Dedicated to

my Parents

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✍ *Raghuveer K*

Contents

General Introduction

Sex Determination and Differentiation

Sexual reproduction is an important event which enables the organism to propagate and transfer genetic information from one generation to another. Successful reproduction usually depends on events that begin early in its life. In vertebrates, sex determination and differentiation are two important events in the development of gonads (testis or ovary). Sex determination is the genetic (sex chromosomes) or environmental process by which the sex (male or female) of an individual is established. Sex determination is extremely diverse and lacks conservation throughout the vertebrates. Sex differentiation refers to gonadal development once sex has been determined, i.e., a process where in an indifferent/bipotential gonads can either develop into testis or ovary, which is in turn under the control of various factors/genes and regulated by hormones (Hughes, 2001). Sex differentiation appears to be relatively conserved in several vertebrates across the phyla. Fishes are excellent models to study the events of sex determination and differentiation from evolutionary point of view as they exhibit a broad range of sexual plasticity ranging from hermaphroditism to gonochorism and from genetic to environmental sex determination (Devlin and Nagahama, 2002).

Mechanism of Sex Determination

Vertebrates have two major types of sex-determining mechanisms: genetic sex determination (GSD), where sex is determined at conception and genetic differences are expected between the sexes and the second one is environmental sex determination (ESD), where there are no consistent genetic differences between sexes and sex is determined after fertilization which in influenced by environmental factors like temperature, pH (reviewed by Baroiller *et al.*, 1999),

and social factors related to behavior of the individuals of the species (Francis and Barlow, 1993; Godwin, 2009).

Genetic sex determination

GSD is controlled by genes present on the sex chromosomes which are also influenced by dominant genes on autosomes. GSD includes monofactorial sex determination (monogenic system), multi- and polyfactorial sex determination (polygenic system). In monogenic systems, sex is determined by a gene located on a certain chromosome and genes on other chromosomes have little effect (Devlin and Nagahama, 2002). The XX/XY sex-determination system is the most familiar mode of sex-determination. In most mammals, sex determination is via XX/XY system in which males are heterogametic. A gene on the Y chromosome, *SRY* is considered as a master switch to determine testis. This gene is thought to have evolved from the transcription factor *SOX3* due to the lack of recombination (Zhang, 2004). The Y chromosome shows a reduced number of functional genes, and it has been suggested that, over time, Y chromosomes will disappear due to loss of function (Graves, 2002). In XY/XO system, females have two copies of the sex chromosome (XX) but males have only one (XO). The ZW sex-determination system is found in birds and some insects wherein the females are heterogametic. However, it is still not clear whether sex is determined by a master switch gene on the W chromosome or by a dosage effect from the Z chromosome, e.g. *Dmrt1* (Ezaz *et al.*, 2006). The situation became more cumbersome in lower vertebrates including fishes.

In most of the teleosts, morphologically differentiated sex chromosomes cannot be distinguished or difficult to identify. However, some species have heteromorphic sex chromosomes which can be identified by cytogenetic and fluorescence *in situ* hybridization studies. Some examples of this are *Aulopus japonicus* (Ota *et al.*, 2003), catfish species of the genus *Liobagrus* (Chen *et al.*,

2008) and species of sticklebacks: *Gasterosteus aculeatus* (Peichel *et al.*, 2004). In species with sex chromosomes, two systems have been reported: heterogametic males (XY) in species like medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*) and the Nile tilapia *(Oreochromis niloticus*). Heterogametic females (ZW) have been found in *Oreochromis aureus* and the poecilids (Penman and Piferrer, 2008). Till now other than *SRY* male sex determining gene in mammals has not been identified in teleosts except in medaka where a second sex determining gene *DMY/Dmrt1Yb* located on Y-chromosome has been discovered (Matsuda *et al.*, 2002; Nanda *et al.*, 2002). It has been shown that *DMY* arose from a duplication of the autosomal gene *Dmrt1* (Kondo *et al.*, 2006), a member of the family of genes with the DM domain, which is conserved in vertebrates (Raymond *et al.*, 1998). However, this gene has only been reported in two species of medaka *O. latipes* and in *O. curvinotus*, (Matsuda *et al.*, 2003), and has not been found in other species of the genus *Oryzias* (Kondo *et al.*, 2003). The functional evidence of the male sex-determining role of *DMY* in medaka has been obtained with loss-of-function and gain-of-function studies. Mutations of loss of function of the *DMY* gene cause female development of XY individuals (Matsuda 2005), where as transgenic *DMY* induced male development in genetically female medaka (Matsuda *et al.*, 2007). Nevertheless, the sex-determining region has also been identified in other teleosts like platyfish, the three-spined stickleback, pufferfish, channel catfish and rainbow trout (Penman and Piferrer, 2008).

In multifactorial systems there are three or more major sex-determining factors but the number can be characterized. In multiple sex chromosome systems: X1X1X2X2/X1X2Y, XX/X1X2Y and W1W2Z/ZZ were also observed. Chromosome fusions or fissions led to the origin of the multiple sex chromosome systems (Penman and Piferrer, 2008). For example, the platyfish has three different sex chromosomes: W, X, and Y where sex chromosome differentiation has only

been found in one population. XX, XW, and ZW females and XY and YY males are found in natural populations (Nanda *et al.*, 1992). On the other hand, in polyfactorial systems, sex is determined by the combination of several factors distributed throughout the genome.

Environmental sex determination

Environmental factors such as temperature, pH, population density and social interactions have been found to influence the proportion of the sexes in fish (Nakamura *et al.*, 1998; Baroiller *et al.*, 1999; Baroiller and D'Cotta, 2001). Temperature dependent sex-determination (TSD) is commonly noticed in fishes, amphibian and reptiles. In most thermosensitive fish species, the male proportion rises when temperature increases, and female development was observed at low temperatures (Baroiller *et al.*, 1999). TSD has been found in many species of the Cichlids (Ospina-Alvarez and Piferrer, 2008) and in the sea bass (Pavlidis *et al.*, 2000). Similarly, low temperatures can also induce ovarian differentiation resulting in more females in *Odontesthes bonariensis* (Strüssmann *et al.*, 1996). Influence of pH on the sexual proportion has been reported in several species of the cichlids and in the poecillids (Rubin, 1985; Baron *et al.*, 2002). The influence of social factors in sexual determination and differentiation has been well documented in hermaphroditic species. Sexual inversion is primarily controlled by factors such as population density and by the existent proportion of males and females in the population at a given moment (Baroiller *et al.*, 1999; Baroiller and D'Cotta 2001). Social signals used by fish may be behavioral interactions between the sexes, relative size, sexual proportion and possibly stimuli such as pheromones. This kind of sex determining system has been found in a hermaphrodite teleosts, *Thalassoma bifasciatum* and a cichlid, *Cichlasoma citrinellum* (Shapiro and Rasotto, 1993; Francis and Barlow, 1993).

Origin of gonads and gonadal development

Gonads arise from the intermediate mesoderm during embryogenesis. The initial step in the development of gonads is the formation of the genital ridge and urogenital system. The second stage of gonad formation is the development of the bipotential or indifferent gonads. The indifferent gonad arises from through a thickening and proliferation of cells on the ventromedial surface of the mesonephros. The indifferent gonad is unique as it has the capacity to differentiate into either testis or ovary. The commitment of indifferent gonads to differentiate to either testis or ovary occurs after the migration of primordial germ cells (PGCs) from extragonadal origin to the gonadal primordial (presumptive gonad). PGCs are precursors of germ cells, which eventually give rise to sperm and egg in all vertebrates. In early mammalian embryogenesis, the epiblast cells of the gastrula give rise to extraembryonic mesoderm and PGCs and signals from extraembryonic ectoderm are critical for the specification of PGCs (Zhao and Gabers, 2000). PGCs migrate to mesodermal endothelium on both sides of the hindgut and form gonadal ridges just medial to the developing mesonephros. In fishes also PGCs are formed outside the gonadal development site and later migrate to colonize the gonadal primordia/analgae along with the supporting cells which give nourishment to the proliferating PGCs (Patino and Takashima, 1995). PGCs specification in fish is accomplished by cytoplasmatic components, which are inherited maternally, known as germ plasm (Knaut *et al.*, 2000). The *vasa* gene, one of the components of germ plasm that codes for an RNA helicase, is essential for germ cell lineage specification in both invertebrates and vertebrates (Raz, 2003). The role of *vasa* in PGCs proliferation and maintenance has been extensively studied in most of the teleosts and mammals (Raz, 2003). Various molecules are known to be involved in PCGs migration. The onset of PGCs motility in the zebrafish is controlled by a RNA-binding protein '*Dead end*'. Blocking its

translation inhibits this initial step of migration of PGCs (Molyneaux and Wylie, 2004). Later, PGCs are guided towards the gonads by *SDF-1* (stromal cell derived factor 1), which acts as a chemo-attractant. Blocking the translation of *SDF-1* and of its receptor *CXCR4*, which is coupled to G proteins, hinders migration of PGCs towards the gonadal crest (Knaut *et al.*, 2003).

Most of the teleosts have paired gonads. However, in viviparous species the two gonadal primordia are fused during development to give rise to a single gonad. The undifferentiated gonad in most teleosts differentiates into one ovary or one testicle during ontogenesis, and is composed of PCGs and somatic cells, as in other vertebrates. In teleosts, the somatic cells of the genital crests are derived from the posterior part of the lateral plate mesoderm (Nakamura and Takahashi, 1973). Germ cells interact with somatic cells during gonadal sex differentiation which is essential for the differentiation of germ cells. In most teleosts species, ovarian differentiation begins with the proliferation of somatic and germ cells and differentiation of early oocytes, followed by the formation of the ovarian cavity (Nakamura *et al.*, 1998). The appearance of small number of meiotic oogonia (germ cells) is one of the histological sign of onset of ovarian differentiation in most teleosts. In addition, the number of germ cells is higher in differentiating ovaries than in gonads destined to become testis (Nakamura *et al.*, 1998). During ovarian differentiation, somatic and germ cells begin to differentiate to form follicles, comprised of oocytes and an inner layer of granulosa cells surrounded by an outer layer of theca cells (Nagahama, 1982). On the other hand, the teleost ovary is of the cystovarian type, i.e., it has an internal cavity, which forms during ovarian differentiation (Hoar, 1969). Thus, in some cases the formation of the ovarian cavity may be a criterion to identify ovarian development (Nakamura *et al.*, 1998). Testicles remain undifferentiated longer than ovaries and differentiation of the soma occurs before germ cells appear. In several species spermatogenesis begins ones the testis is fully

differentiated long time after hatching. Efferent duct formation is one of the criteria that indicate the onset of testicular differentiation. Stromal/supporting cell aggregation around the germ cells also indicates testicular differentiation in progress and later, these cells form sperm ducts and interstitial tissue (Nakamura *et al.*, 1998).

Molecular basis of gonadal sex differentiation

Several genes (mostly transcription factors) implicated in the processes of gonadal sex differentiation in mammals are also similar in teleosts, which suggest conservation of the sex differentiation pathways (Fig. 1). The indifferent gonad is composed of PGCs and somatic cells of the coelomic epithelium prior to sex determination. There are genes encoding transcription factors like *Lim1*, *Emx1*, *Emx2*, *Lhx9, Wt1*, *Ad4BP/SF-1* and *Wnt4* which mediate the early events in the formation and development of indifferent/bipotential gonads. All these factors are expressed in the developing urogenital ridge of mammals (Skinner and Griswold, 2005). Both *Emx1 and Emx2* were detectable in developing urogenital system, and *Emx2* deficient mice fail to develop urogenital tissues including kidney, ureter, gonads and genital tract (Cecchi and Boncinelli, 2000). *Wt1* gene is critical for development of the urogenital system and its expression pattern is conserved in vertebrates. Null mice for *Wt1* lack kidneys and gonads (Kreidberg *et al.*, 1993). Among these factors only few of them have been identified in teleots. Two isoforms of *Wt1* has been identified in during gonadal development in zebrafish and medaka (Perner *et al.*, 2007; Klüver *et al.*, 2009). *Wnt4* is required for oocyte development during female gonadal development and has got a role in vascular formation in gonads (Bernad and Harley, 2006)

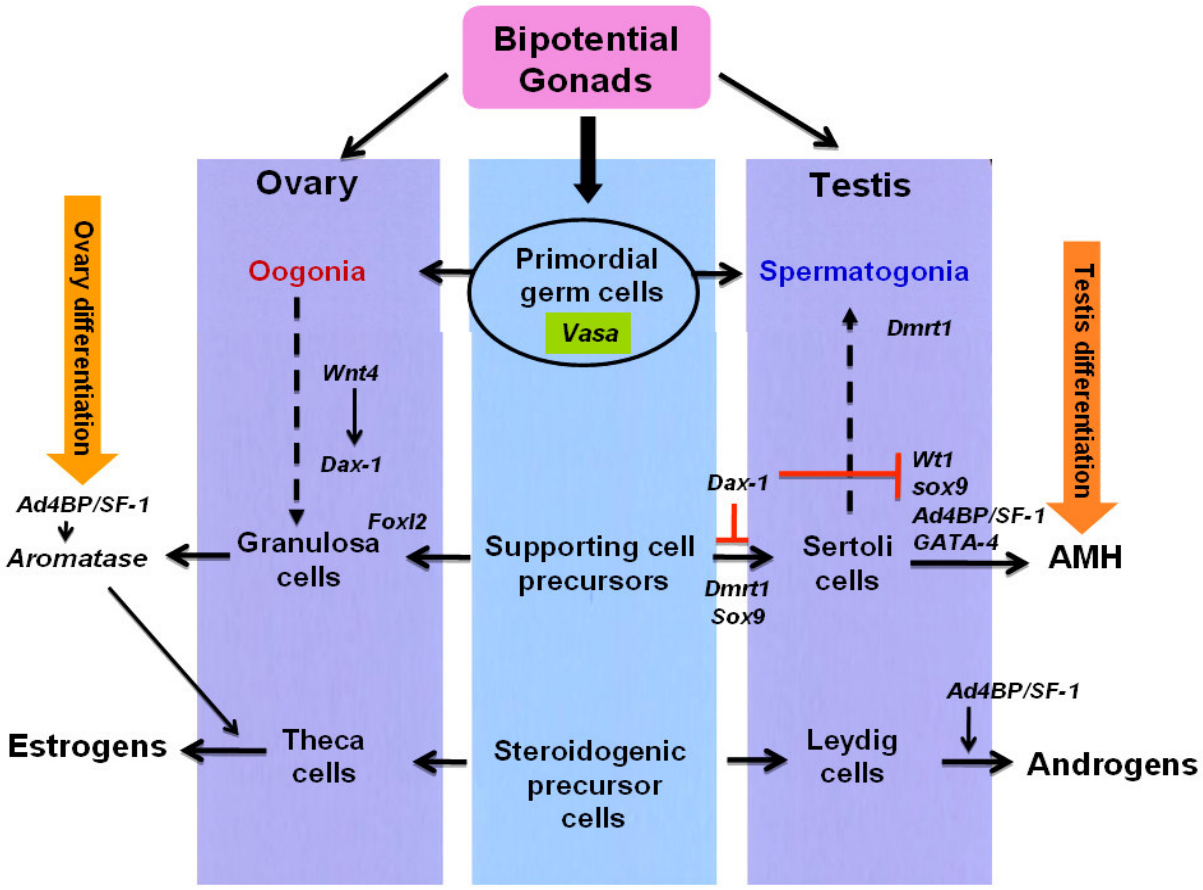

Fig. 1 Schematic representation showing interactions among various transcription factors that are involved in sex differentiation.

Transcription factors that potentially directs the differentiation of indifferent gonad:

Dmrt1 (dsx and mab-3 related transcription factor 1)

Dmrt1 belongs to gene family of putative transcription factors that has a conserved DNA binding domain known as DM domain across the phyla. *Dmrt1* has been suggested to be the first conserved gene involved in sex differentiation found from invertebrates to vertebrates. It is mostly expressed in testis of various vertebrate species implicating its role in testicular differentiation (Raymond *et al.*, 1998). Monosomy at this region in XY individuals manifests feminization and gonadal dysgenesis (Ottolenghi and Mc Elreavey, 2000). *Dmrt1*-knockout in

mice effected the differentiation of both Sertoli and germ cells (Raymond *et al.*, 2000) hampering postnatal testis development. Recent finding showed that *Dmrt1* suppresses the female pathway by repressing aromatase gene transcription and estrogen production in the gonads of tilapia (Wang *et al.*, 2010).

Sox9 (SRY-related homeo box gene)

Sox9 is the direct target of the testicular sex-determining gene, *SRY*. *SRY* interacts with *Sox9* and up regulates its transcription in developing pre-Sertoli cells during male development (Kent *et al.*, 1996). In mammals, birds, and turtle, expression of *Sox9* is up regulated in the developing male genital ridges during testicular differentiation (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996; Moreno-Mendoza *et al.*, 1999). These results suggest for a crucial role of *Sox9* in testicular development of several vertebrates. Two different forms of *Sox9* genes have been identified in teleosts and they were found to be orthologues of the tetrapod *Sox9* gene that arose during a whole genome duplication event (Klüver *et al.*, 2005).

GATA-4

GATA family of transcription factors has been shown to be important in the regulation of genes directing differentiation in multiple organs. GATA-4 is expressed in mouse Sertoli and Leydig cells throughout postnatal testicular development. *GATA-4* has been studied in detail during human gonadogenesis, implicating its role in testicular differentiation (Ketola *et al.*, 2000). *GATA-4* regulates *Dmrt1* expression in testis by binding to its promoter (Lei and Heckert, 2004) *GATA-4* enhances *AMH* expression by binding to specific promoter elements in *AMH* (Park and Jameson, 2005).

Ad4BP/SF-1 (Steroidogenic factor-1)

The *Ad4BP/SF-1* gene is a member of the nuclear receptors *Ftz-f1* of the NRA51 superfamily (Morohashi *et al.*, 1996). It plays an important role in the transcriptional regulation of steroidogenic enzymes genes like *Cyp19a1*, *Cyp17* and *3βHSD* (Parker *et al.*, 2002). In several species of teleosts such as medaka (Watanabe *et al.*, 1999), salmon (Higa *et al.*, 2000) and zebrafish (Chai and Chan, 2000) genes homologous to *Ad4BP/SF-1* have been identified. In zebrafish, *Ff1b* and *Ff1d* are functional homologues of *Ad4BP/SF-1* show higher levels of expression in the Leydig and Sertoli cells of male gonads (Chai and Chan, 2000).

Dax1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region on chromosome X, gene 1)

The gene *Dax1* belongs to family of orphan nuclear receptors, which is linked to chromosome X in humans and in mice (Zanaria *et al.*, 1994). Mutations in this gene cause X-linked adrenal hypoplasia congenita in humans (Zanaria *et al.*, 1994). In mammals, *Dax1* acts as dominant-negative regulator of steroidogenesis that inhibits the transcriptional activity *Ad4BP/SF-1* and *WT1* (Park and Jameson, 2005). In some teleosts, its pattern of expression during gonadal development has been studied, and a possible function of the gene has been suggested; however, its role during sexual determination is not clear yet. In tilapia, *Dax1* expression levels are higher in XX than in XY gonads (Ijiri *et al.*, 2008).

Foxl2 (forkhead box L2)

The gene *Foxl2* belongs to a family of transcription factors named forkhead. In mammals, *Foxl2* is female-specific and is detected during early ovarian development (Cocquet *et al.*, 2002). *Foxl2* possibly acts by regulating the transcription of *Cyp19a1* (Liu *et al.*, 2007). In teleosts the *Foxl2* shows dimorphic expression pattern, with higher expression levels in the ovary, which implicates

its role in ovarian differentiation (Wang *et al.*, 2004; Liu *et al.*, 2007). Its expression is seen in granulosa cells during ovarian development, and also expressed in pre-vitellogenic and vitellogenic follicles (Wang *et al.*, 2004). In tilapia, *Foxl2* possibly regulates ovarian aromatase expression either directly binding to the promoter of gene or interacting along with *Ad4BP/SF-1* to up regulates its expression (Wang *et al.*, 2007).

Cyp19a1 (Cytochrome P450 aromatase, ovarian form)

The gene *Cyp19a1* codes for the enzyme cytochrome P450 aromatase, a key enzyme in the conversion of androgens to estrogens (Simpson *et al.*, 1994). *Cyp19a1* is female specific gene which plays a crucial role in ovarian differentiation. It is also essential for the oocytes growth during female gonadal development and recrudescence (Nagahama, 2005).

AMH (Anti-Müllerian Hormone)

AMH is also called Müllerian inhibiting substance (MIS), a member of the TGF-β family produced by Sertoli cells, triggers Müllerian duct regression, the first step of male sex somatic differentiation (Vigier *et al.*, 1989). Onset of *AMH* expression depends on *SOX9* binding to the proximal *AMH* promoter. Subsequently, *Ad4BP/SF-1*, *GATA4* and *WT1* enhance *AMH* expression by binding to specific promoter sequences or by interacting with transactivating factors (Park and Jameson, 2005). *Dax1* impairs *GATA4* and *SF1* binding to the *AMH* promoters, resulting in lower *AMH* expression levels (Park and Jameson, 2005). The role of MIS in sexual differentiation of teleosts, which lack Mullerian ducts, is not clear. In one eel species, a substance similar to MIS labeled eSrs21 was identified in immature testicle Sertoli cells (Miura *et al.*, 2002). In zebrafish, *AMH* expression is mainly found in Sertoli cells of juvenile testis but not in ovary. However, in adult gonads *AMH* expression was seen in both testis and ovary (granulosa cells surrounding the oocytes) (Rodrı´guez-Marı´ *et al.*, 2005). Sexually dimorphic

expression of a homologue of *AMH* was observed during gonadal sex differentiation of the Japanese flounder (Yoshinaga *et al.*, 2004). In contrast, its expression was seen in both the developing male and female gonads of medaka (Klüver *et al.*, 2007).

Neuroendocrine control of gonad development

Gonadal development in both sexes is dependent on endocrine communication between the brain, pituitary and gonads, allowing developmental, physical, chemical, social and seasonal cues to be integrated with gonad maturation. A primary source of control for this pituitary-gonadal axis is mediated through the production of gonadotropins (GTH) in the pituitary gland. Two types of GTH (GTH-I and GTH-II) homologous to follicle stimulating hormone and leutenizing hormone (LH) in mammals have been identified in teleosts (Swanson *et al.*, 1991). Communication between the brain and the developing gonad is required to ensure appropriate GTH levels and rates of gonadal development and timing of maturation. Gonadotropins are well known to stimulate steroid synthesis in the teleost gonad, and sex steroids are able to feedback to the brain to alter GTH production. This process has been well elaborated for the eel testis in which prior to maturation, GTH-I is produced that promotes steroid synthesis and gonadal growth and differentiation throughout the life of fish (Miura *et al.*, 1991). During the period of sex differentiation in gonochoristic fishes like rainbow trout, GTH-I first appears in the larval pituitary gland at a time when germ cells begin to divide (Saga *et al.*, 1993). Previous studies from our laboratory showed quantitative and dimorphic differences in seabream gonadotropin-releasing hormone (GnRH) and GTH expression in POA-H and pituitary regions of XX and XY Nile tilapia during critical period of sex differentiation (Sakai *et al.*, 2005; Swapna *et al.*, 2008). As sexual maturation approaches, a switch occurs in the pituitary gland such that a new form of

gonadotropin is produced (GTH-II), which is analogous to mammalian LH. The production of LH induces receptor-mediated changes in gonadal enzymes like 11β-hydroxylase and aromatase (Kagawa *et al.*, 1982; Jiang *et al.*, 1996). The role of GnRH-GTH in the neuroenodcrine regulation of sex differentiation can be ascertained well only by studying factors that regulate hypothalamo-hypophyseal-gonadal axis.

In fishes, the factors that are involved in regulating GTH secretion include serotonin, norepinephrine, dopamine, neuropeptide-Y, and GABA. Serotonin is a stimulatory monoamine for the release of GnRH and GTHs by acting at the level of hypothalamo-hypophyseal axis. (Peter *et al.*, 1991; Goos *et al.*, 1999; Senthilkumaran *et al.*, 2001). Trytophan hydxoylase (*Tph*) a key regulator in 5-HT biosynthesis and is relatively unstable, minor changes in *Tph* abundance or activity can alter 5-HT levels in brain and serotonergic function. Previous studies showed that para-chlorophenylalanine (pCPA) treatments, a *Tph* blocker mimicked like estradiol during early sex differentiation period in the Nile tilapia which resulted in more number of females (Tsai *et al.*, 2000). Recently, we demonstrated sexual dimorphic expression of *Tph* in the brain of XX and XY Nile tilapia during early development may have a plausible role for *Tph* in brain sex differentiation (Sudhakumari *et al.*, 2010). However, to date there is no clear understanding of the role of *Tph* vis-à-vis sertonin in brain in relation to male and female sex development.

Steroidal regulation of sexual differentiation

In teleosts, sex steroids affect the development of germ cells and other cell-types during the process of gonadal sex differentiation (Devlin and Nagahama, 2002). The sex steroid estradiol-17β is considered to be responsible of inducing and maintaining ovarian development, and its levels are considerably higher in females than in males. Testicular development is mainly

regulated by the potent androgen 11-ketotestosterone (11-KT). In fish in general, testosterone is not directly involved in the mechanisms of sexual differentiation, but participates as precursor of 11-KT and estradiol-17β (Nakamura *et al.*, 1998). In early development of *Xiphophorus*, an enzyme 3β-hydroxysteroid dehydrogenase (*3βHSD*) responsible for isomerization and dehydrogenation of steroid precursors has been detected in primitive male and female gonads (Schreibman *et al.*, 1982). Immunohistochemical studies in XX and XY Nile tilpia has shown that steroidal enzymes *P*450scc, *P*450c17, *Cyp19a1* and *3βHSD* are found at high levels in female gonads at 10 days post hatch (dph), but was only seen weakly in males after testis differentiation and strong immunoreactivity (except for *Cyp19a1*) was observed in testis just before the onset of spermatogenesis (Kobayashi *et al.*, 1998). In tilapia, treatment of genetic females with a non-steroidal aromtase inhibitor (fadrozole) before and during gonadal sex differentiation causes sex reversal from females to males (Nakamura *et al.*, 1998). These findings strongly suggest that estradiol-17β is produced in the gonads around the time of ovarian differentiation and may have an important role in the differentiation. In mice, the presence of mRNAs for steroidogenic enzymes including aromatase are present in the fetal gonad before any histological evidence of sex differentiation is apparent (Greco and Payne, 1994). The manipulation of sex differentiation in fish with exogenous steroids has been extensively reviewed (Devlin and Nagahama, 2002). Androgen treatment of fish is most effective in inducing masculinization. The most common employed androgen in sex-reversal studies is 17α-methyltestosterone, while estradiol-17β and ethynylestradiol is the most regularly used estrogen to induce feminization.

Considering the knowledge gained in mammalian sex determination and differentiation studies, in depth research work in teleosts related to this area are mostly restricted to daily or fortnight

breeders like zebrafish, medaka and tilapia. In this regard, fishes (annual breeders) that undergoes seasonal pattern of gonadal attenuation and recrudescence (reproductive cycle) are good models for comparative analysis and may provide interesting highlights in understanding the expression pattern of sex-specific genes not only during gonadal development but also during recrudescence. Our laboratory is working on a teleost fish model, *Clarias gariepinus* (commonly known as North African air-breathing catfish) to study the expression pattern and understand the role of transcription factors and steroidogenic enzyme genes during sex differentiation and gonadal recrudescence. Catfish is a gonochoristic annual breeder undergoing seasonal pattern of reproductive cycle.

Present study deals with histological examination of gonadal sex differentiation and molecular analysis of genes (*vasa*, *dmrt1* and *sox9*) not only during gonadal development and also during gonadal recrudescence in adult catfish. Since, brain has got a potential role in the neuroendocrine mediated regulation of reproduction, we intend to know the occurrence of sexual dimorphism (brain sex differentiation), if any in teleosts. Hence, in this study additional efforts were made to understand the expression pattern of *tryptophan hydroxylase* in male and female brains during development. We also extended our study to understand the effect of exogenous steroid hormones on the expression of above mentioned genes in altered sex. These aspects have been studied as five major chapters discussing the results and findings.

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Chapter 1

Cloning and differential expression pattern of vasa in the developing and recrudescing gonads of catfish, Clarias gariepinus

Abstract

Vasa gene codes for a DEAD box family protein, which plays a crucial role in primordial germ cell proliferation. In this study, we report cloning of *vasa* from gonads of air-breathing catfish, *Clarias gariepinus*, a seasonally reproducing teleost fish. We studied the expression pattern of *vasa* during gametogenesis using real-time PCR. We also examined the hormonal regulation on *vasa* in gonads of catfish. RT-PCR analysis revealed that *vasa* was detectable only in the gonads. Further, real-time PCR results showed that expression of *vasa* was seen throughout the development from embryonic stage to adult. However, the expression was more in ovary than in testis during gonadal development. In adult testis, the *vasa* transcripts were significantly high during spermatogenesis and it declined during spermiation. On the other hand, during ovarian recrudescence, *vasa* transcripts were high in immature oocytes (stage I and II oocytes) when compared to mature oocytes (stage III and IV oocytes). Human chorionic gonadotropin treatment in recrudescing ovary (*in vivo*) as well as in testicular slices (*in vitro*) resulted in up regulation of *vasa* mRNA in a time-dependant manner. These results together suggest that *vasa* gene has got an important role to play in spermatogenesis and oogenesis during recrudescence in addition to development.

Introduction

Primordial germ cells (PGCs) are precursors of germ cells that migrate from the site of origin to the gonadal primordia (presumptive gonad) and eventually give rise to sperm and egg in sexually reproducing organisms (Saffman and Lasko, 1999; Wylie, 1999). In *Drosophila melanogaster*, a unique region of cytoplasm localized in the posterior region of the egg known as "germ plasm", has been well studied. Several maternally derived factors (RNA transcripts) have been identified in germ plasm that is responsible for germ cell lineage in the *Drosophila* (Illmensee and Mahowald, 1974; Eddy, 1975; Williamson and Lehmann, 1996). Later on, the germ plasm has also been identified in *Caenorhabditis elegans*, *Xenopus laevis, Danio rerio* and *Gallus gallus* (Ikenishi, 1988, 2000; Knaut *et al.*, 2000; Tsunekawa *et al.*, 2000). However, the germ plasm has not been found in higher vertebrates. In mammals, signals from extraembryonic tissues are critical for the specification of PGCs (Zhao and Gabers 2000; Ying *et al.*, 2001).

The *vasa* gene was first identified in *Drosophila* as one of the components of germ plasm essential for PGCs commitment (Schüpbach and Wieschaus, 1986; Hay *et al.*, 1988a). The *vasa* gene encodes DEAD box family protein of ATP-dependent RNA helicase (Hay *et al.*, 1988b; Liang *et al.*, 1994). Interestingly, *vasa* homologs had also been identified and found to express in germ cell lineage of several metazoans ranging from hydra to mammals (Raz, 2000; Mochizuki *et al.*, 2000). Several different isoforms of *vasa* related genes have also been identified in both invertebrates and vertebrates. Among those are homologs of *Drosophila vasa* (*glh-1, 2, 3* and *4*) identified in germ plasm of *C. elegans* essential for fertility (Gruidl *et al.*, 1996; Kuznicki *et al.*, 2000). Two isoforms of *vasa* related genes were isolated from planarians (*DjvlgA* and *DjvlgB*) and cnidarians (*Cnvas1* and *Cnvas2*) (Shibata 1999; Mochizuki *et al.*,

2000). In fishes like zebrafish and tilapia several isoforms of *vasa* have been identified (Kobayashi *et al.*, 2002; Bártfai and Orbán 2003).

Though the expression of *vasa* in adult gonads is known, little attention has been paid to understand its function in these tissues. In *Drosophila*, it has been shown that *vasa* protein is involved in oogenesis (Styhler *et al.*, 1988; Ghabrial and Schüpbach, 1999). Differential expression of *vasa* homolog in gonads of tilapia during gametogenesis suggests a possible role for *vasa* in the regulation of meiotic progression of male and female germ cells (Kobayashi *et al.*, 2000). In fishes, most of the studies related to *vasa* gene were done during embryogenesis and early gonadal development in daily or fortnight breeders like zebrafish, medaka and tilapia. The gonads of these fishes do not undergo seasonal pattern of reproductive cycle or gonadal recrudescence and mostly remain mature or have a short ovarian cycle. In this regard, catfish is an annual breeder, which undergoes seasonal pattern of gonadal attenuation and recrudescence that may provide interesting highlights to understand the role of *vasa* not only during early development but also in recrudescence. Hence in the present study, we cloned *vasa* cDNA from catfish gonads and studied its expression pattern during embryonic development, gonadal development and recrudescence (during reproductive cycle after becoming adult) in order to understand its possible involvement in gametogenesis. It is well known that gonadotropins through the action of sex steroids (estradiol-17β and 11-ketotestosterone) regulate gametogenesis in teleosts (Peter *et al.*, 1991; Nagahama, 1994). We also extended our study to understand the influence of gonadotropins (using human chorionic gonadotropin [hCG]), if any on regulation of *vasa* mRNA expression in adult gonads undergoing recrudescence.

Materials and Methods

Animals and Sampling

Catfish (*Clarias gariepinus*) is an annual breeder commonly referred to as the North African or air-breathing catfish, this species is abundantly available in ponds and lakes of Hyderabad, India. The seasonal reproductive cycle of catfish is divided into four phases (Swapna *et al.*, 2006): preparatory (February-April), prespawning (May-June), spawning (July-October) and post-spawning/regressed phases (November-January). Catfish at different age groups were reared in fresh water tanks under ambient photothermal conditions. *In vitro* fertilization was performed during breeding season using mature spermiating male and gravid female fishes injected with hCG to obtain catfish embryos. The fertilized catfish embryos were transferred to small size glass tanks containing filtered water with aeration. Generally catfish embryo takes 24 h to hatch and the hatchlings can survive for 2-3 days without any external feed by utilizing the yolk present in yolksac. Later on, the hatchlings were fed with live tubeworms until 3 months. Juvenile fishes of 3-8 months old were fed not only with live tubeworms but also with commercially available fish feed pellets. Adult catfish (1-year old) were reared in the outdoor larger tanks and fed with minced goat liver or pelleted fish food in *ad libitum*. Soon after fertilization, catfish embryos (10 each) were collected at different hours post fertilization (hpf) stages or time points: 0 hpf (single cell stage), 2 hpf (cleavage stage) 4 hpf (blastula stage), 8 hpf (gastrula stage), 12 hpf (somite/segmentation stage) and 24 hpf (larva stage) stored at –80^{o}C for total RNA extraction. Catfish fingerlings of different age groups (5, 10, 20, 30, 40, 50, 100, 200 days post hatch [dph]) were collected. Mesonephric gonadal complex (MGC) was isolated using fine sterile forceps under a stereozoom dissection microscope from 30 and 40 dph juvenile fishes. The MGC of same age groups of fishes were pooled (3 each per sample) to

get sufficient amount of total RNA. Likewise we have made 3 biological samples (n=3). Since it is very difficult to isolate MGC complex from 5, 10 and 20 dph hatchling, we used trunk region by removing most of the muscular ventral region of the fish (n=3) for total RNA preparation. Since gonadal sex differentiation is completed by 50 dph in catfish, we dissected out immature testes and ovaries from fishes at 50, 100 and 200 dph (n=3) under a stereozoom dissection microscope for the preparation of total RNA. Mature male and female gonads (n=5) of adult catfish (>1 year) were collected at different phases of seasonal reproductive cycle and stored at –80ºC for total RNA extraction.

Cloning of partial cDNA fragment of *vasa* from catfish ovary

Total RNA was prepared from adult catfish ovary using the Sigma TRI-reagent method. Reverse transcription (RT) was carried out with oligo $d(T)_{18}$ primers and 5 µg of total RNA at 50°C using superscript-III (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. PCR amplification was done with AmpliTaq gold (Applied Biosystems, Foster city, CA) under following conditions: initial step of 94ºC (10 min), then 94ºC (1 min), 55ºC (1 min), 72ºC (1 min), for 35 cycles using the *vasaDgF* and *vasaDgR* degenerate primers (Table 1) designed based on the available *vasa* nucleotide sequences of other species from NCBI GenBank data base. PCR amplified cDNA fragments were gel purified, cloned in TOPO-TA cloning vector (Invitrogen), nucleotide sequenced and analyzed using Laser Gene software 3.05 (DNASTAR, Madison, WI). The partial cDNA fragment of *vasa* obtained from ovary was confirmed using NCBI-BLAST search.

5' and 3' Rapid amplification of cDNA ends (5' and 3' RACE)

To isolate full-length cDNAs of *vasa* from ovary, 5' and 3' RACE was performed using gene specific primers (GSP) designed based on the sequence information of partial cDNA fragment. 5' and 3' cDNA templates were made from ovary total RNA according to manufacturer's protocol using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA) according to manufacturer's protocol. For the amplification of RACE cDNA ends, touchdown primary PCR reaction was performed in a 25 µl reaction mix containing 1x PCR buffer, 100 µM dNTP mix, 1U PCR advantage *Taq* DNA polymerase (Clontech), 1 µl of the cDNA template, GSP1 primers ((Table 1) and universal primer-A mix. The Touchdown PCR cycling conditions were as follows: 94°C (30 sec) and 72°C (2 min) for 5 cycles, 94°C (30 sec) and 70°C (2 min) for 5 cycles, 94°C (30 sec), 68°C (30 sec) and 72°C (2 min) for 27 cycles. After the primary PCR, nested PCR was performed using GSP2 primers (Table 1) and nested universal primer. The nested PCR conditions were as follows: 94°C (30 sec), 65°C (30 sec) and 72°C (2 min) for 35 cycles. All the PCR amplified products were gel purified, cloned in to TOPO-TA cloning vector and sequenced. After obtaining the full-length *vasa* cDNA from ovary, RT-PCR (conditions: 94°C for 1 min, 58°C for 45 sec, 72°C for 2 min each for 35 cycles) was performed to amplify and clone the open reading frame (ORF) from catfish ovary and testis using the *vasa* ORF specific primers (Table 1).

Tissue distribution by semi-quantitative RT-PCR

The expression of *vasa* in different tissues of adult catfish was analyzed using semi-quantitative RT-PCR as described by Kwon *et al.* (2001). For this, total RNA was extracted from different tissues (brain, spleen, gill, heart, intestine, kidney, liver, testis and ovary) of

adult catfish using the Sigma TRI-reagent method and RT was carried out with 5 µg of total RNA as mentioned above. PCR amplification was done at 94°C (1 min), 60°C (30 sec), 72°C (1 min), for 30 cycles using *vasa* specific primers (Table 1). β-*actin* was PCR amplified at 94°C (1 min), 60°C (30 sec), 72°C (1 min) for 28 cycles using catfish specific β-*actin* primers (Table 1) as an internal control to test the quality of cDNA template.

Ontogenic expression pattern of *vasa* by real-time quantitative RT-PCR (qRT-PCR)

Expression of *vasa* during embyrogenesis and gonadal ontogeny after hatching was analyzed by relative qRT-PCR using SYBR Green detection method. Total RNA was extracted using Sigma TRI-reagent method from the tissue samples that were collected at different stages of development/ontogeny. The RNA quality and quantity were determined by capillary electrophoresis using the RNA 6000 Nano labchip (Agilent 2100 BioAnalyzer) according to the manufacturer's instructions. RT was carried out with 1 µg total RNA and random hexamer primers using superscript-III (Invitrogen). Real-time PCR specific primers for *vasa* and β-*actin* (Table 1) were designed using Primer Express software (Applied Biosystems) such that at least one of the primers spanned the junction of two exons, giving a single cDNA PCR product and precluding amplification of genomic DNA. qRT-PCR was then carried out in 25 µl reaction in triplicate using Power SYBRGreen PCR Mastermix (Applied Biosystems, USA) in a ABI 7500 fast real-time PCR machine (Applied Biosystems) at 95°C (15 s) 60°C (1 min) for 40 cycles according to the manufacturer's protocol. Dissociation curve analysis was performed for each sample to check single amplification. During PCR, fluorescence accumulation resulting from DNA amplification was recorded using the ABI 7500 sequence detection system software (Applied Biosystems). Cycle threshold (Ct) values were obtained from the exponential phase of

PCR amplification. Comparative Ct method was used to analyze the data. In this method *vasa* expression was normalized against β-*actin* expression, generating a ΔCt value (ΔCt = *vasa* Ct – β-*actin* Ct). Relative expression was then calculated according to the equation $2^{-\Delta\Delta Ct}$ where initial stage/phase was taken as calibrator.

Primer Name	Nucleotide Sequence (5′– 3′)	Usage
VasaDgF	GCTGG(CA)(CA)G(AG)GA(TC)CT(GCA)ATGGC	Degenerate RT-PCR
VasaDgR	GGCTCAAA(CAG)CCCAT(GA)TCCA(GA)CAT	Degenerate RT-PCR
Vasa 5'GSP1	GAATAGGCAGCAGGAAGGCAGCCGTT	5'RACE
Vasa 5'GSP2	TGTCATTATTGCACTGGGCGGGTTGCT	5'RACE
Vasa 3'GSP1	TGTGGGACCCCTGGAAGATTGCTTGACA	3'RACE
Vasa 3'GSP2	TCTGGTGCTGGATGAAGCTGACCGAATG	3'RACE
Vasa ORF-F	ATGGAGAACTGGGAAGATGATC	ORF cloning
Vasa ORF-R	CTACTCCCATTCATCCTCATC	ORF cloning
Vasa Sp-F	AGCTCCCACTAGGGAGCTCAT	Tissue distribution
Vasa Sp-R	TCTCAAACCCCATATCCAACA	Tissue distribution
VasaRTF	GGTCTGAGTAAAGTTCGTTATCTG	qRT-PCR
VasaRTR	GCCAACCTTTGAATATCCTCTG	qRT-PCR
β-actinF	ACCGGAGTCCATCACAATACCAGT	qRT-PCR
β-actinR	GAGCTGCGTGTTGCCCCTGAG	qRT-PCR

Table1. List of primers used for cloning and expression analysis of vasa

Expression of *vasa* in mature gonads at different stages of gamatogenesis by qRT-PCR

Total RNA was extracted from the adult catfish testis and ovary samples, which were collected during four different phases (preparatory, prespawning, spawning and post-spawning phases)

of seasonal reproductive cycle using the Sigma TRI-reagent method. RT was carried out with 1 µg total RNA and random hexamer primers using superscript-III (Invitrogen). qRT-PCR was performed to analyze the expression pattern of *vasa* gene in gonads during gamatogenesis.

Expression of *vasa* in different stages of oocytes by qRT-PCR

Expression of *vasa* was analyzed in different maturational stages of oocytes by qRT-PCR. For this, oocytes from prespawning female catfish were separated under stereozoom microscope in F.O. solution (NaCl 113 mM, KCl 1 mM, $CaCl_2$ 2 mM, Hepes 5 mM, pH 7.5) in four different stages: stage I, perinucleolar/primary oocytes (< 50 µm); stage II, pre-vitellogenic oocytes (50-100 µm); stage III, vitellogenic oocytes (150-300 µm); and stage IV post-vitellogenic oocytes (300-800 µm). After separation, total RNA was immediately prepared and qRT-PCR was performed to analyze the expression of *vasa* gene.

hCG-induced oocyte maturation, *in vivo* in mature female catfish

We performed hCG-induction in adult female catfish during prespawning phase by injecting 1000 IU of hCG intraperitonially in the abdomen as described earlier (Sreenivasulu and Senthilkumaran 2009). Oocytes were collected from the hCG induced female catfish by gently stripping from ovipore at different time intervals (0, 4, 8, 12 and 24 h). Controls were injected with physiological saline. Total RNA was prepared and RT was carried out. The expression pattern of *vasa* in hCG-induced oocytes at different time points was analyzed by qRT-PCR.

In vitro culture of testicular slices in L-15 medium

Testes from catfish in late preparatory phase (April) were dissected under sterile conditions and kept in ice-cold Leibovitz (L15) culture medium (Sigma). Testicular slices of 20 µm thickness were prepared using McILwain tissue chopper (Vibrotome). Testicular slices were individually transferred to tissue culture plates containing 2 ml of L-15 medium supplemented with 10 mM Hepes–10% FBS and antibiotic (penicillin, 100 IU/ml, streptomycin, 0.1 mg/ml). Testicular slices were cultured for 24 hours at 20-22° C in the presence 100 IU/ml of hCG. At each time interval of 0, 4, 8, 12 and 24 h slices were collected, washed with ice-cold phosphate buffer (pH 7.4) and total RNA was prepared using sigma TRI-reagent. The expression of *vasa* was quantified for each time interval using qRT-PCR.

Statistical analysis

All real-time PCR data were analyzed by the "Comparative Ct method" (ΔΔCt method) and normalized to β-*actin* cDNA as a reference. We did not find any significant change in the β-*actin* expression during development or upon treatments. All the results were expressed as mean ± SEM. Data were analysed by One-Way ANOVA followed by Student-Newman-Keuls post hoc test or Duncan's multiple range test using SigmaStat 3.1 software. A probability of $P < 0.05$ was considered statistically significant.

Results

cDNA cloning of *vasa* from catfish gonads

A partial cDNA fragment (480 bp) of *vasa* was cloned from catfish ovary by degenerate RT-PCR (Fig. 1A). Later, full-length cDNA of *vasa* from ovary was isolated using 5' and 3' RACE strategy (Fig. 1B and C). Then we also cloned the ORF region of *vasa* from both ovary and testis. We obtained 2.5 Kb full-length cDNA of *vasa* which encodes a putative protein of 682 amino acids which contain eight conserved signature domains characteristic of the DEAD box protein family, including ATP-binding motifs (Fig. 2, boxed regions). The N-terminal region it has got multiple arginine-glycine (RG) repeats and arginine-glycine-glycine (RGG) repeats. The nucleotide sequence of *vasa* of catfish had been submitted to the GenBank and the accession number is GU562470. The amino acid sequence comparison of catfish *vasa* with other species revealed considerable homology with rainbow trout (71%), human (56%), frog (51%) and fruit fly (43%). The phylogenetic tree analysis of *vasa* proteins from different species (Fig. 3) showed the existence of three main clades. The first clade represents invertebrate *vasa* homologs, while the second one is teleosts *vasa* homologs and the third clade includes mammals, frog and chicken *vasa* homologs.

Tissue distribution pattern of *vasa* in adult catfish

Tissue distribution pattern of *vasa* by semi-quantitative RT-PCR revealed that the expression was restricted to gonads i.e. testis and ovary (Fig. 4).

Expression pattern of *vasa* at different stages of embryonic development and gonadal ontogeny

We have monitored the expression pattern of *vasa* during embryonic development at different time points (0 hpf to 24 hpf) and also during gonadal ontogeny from 5 dph to 200 dph by real-time PCR. We observed stable expression of *vasa* during the course of embryonic development from 0 hpf to 12 hpf which was then followed by a decrease in transcript levels at 24 hpf (just hatched embryo). Later, the expression of *vasa* began to increase around 10-20 dph and it reached the peak at 50 and 100 dph. The expression was less in males compared to females (Fig. 5).

Differential expression of *vasa* in adult testis and ovary at different stages of seasonal reproductive cycle

The real-time PCR results showed that the expression of *vasa* in testis was significantly high during preparatory and prespawning phases when compared to spawning and post-spawning/resting phases of testicular cycle (Fig. 6A). This result showed that the transcript was abundantly expressed during active spermatogenesis stages (preparatory and prespawning phases). Later on the expression declined drastically during spermiation stage. On the other hand, the expression of *vasa* in ovary was abundant in the preparatory phase i.e. during oogenesis when compared to other phases. The expression in mature ovary was significantly low in spawning (maturation stage) and post-spawning phases (Fig. 6B).

Expression of *vasa* in different stages of oocyte growth

We isolated four different stages of oocytes from catfish ovary during prespawning period: Stage I (perinucleor/primary oocytes), stage II (pre-vitellogenic oocytes), stage III (vitellogenic

oocytes) and stage IV (post-vitellogenic oocytes). The *vasa* mRNA levels were quantified in these four stages of oocytes by qRT-PCR. The real-time PCR results showed that the *vasa* mRNA were abundantly expressed in oocytes at stage I and II while the levels decreased in stage III and IV (Fig. 7).

Effect of hCG treatment on *vasa* expression in ovary, *in vivo* and in testicular slices, *in vitro*

We analyzed the effect of hCG on the expression of *vasa* in both adult male and female gonads by qRT-PCR. In females, during prespawning phase, hCG induction *in vivo* showed a gradual increase in *vasa* expression from 0 h to 24 h time interval (Fig. 8A). The expression of *vasa* was significantly increased at 6 h and peaked at 12 h after hCG treatment in testicular slices (Fig. 8B)

Figures

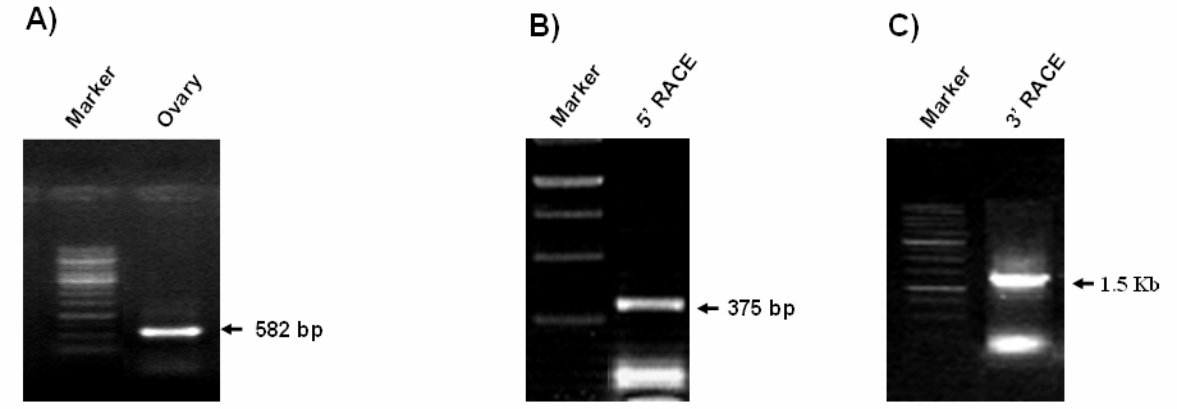

Fig. 1. 1% Agarose gels showing RT-PCR products. A) Partial cDNA fragment of vasa from catfish ovary. B) and C) 5' and 3' RACE products using gene specific primers to obtain full-length cDNA of vasa.

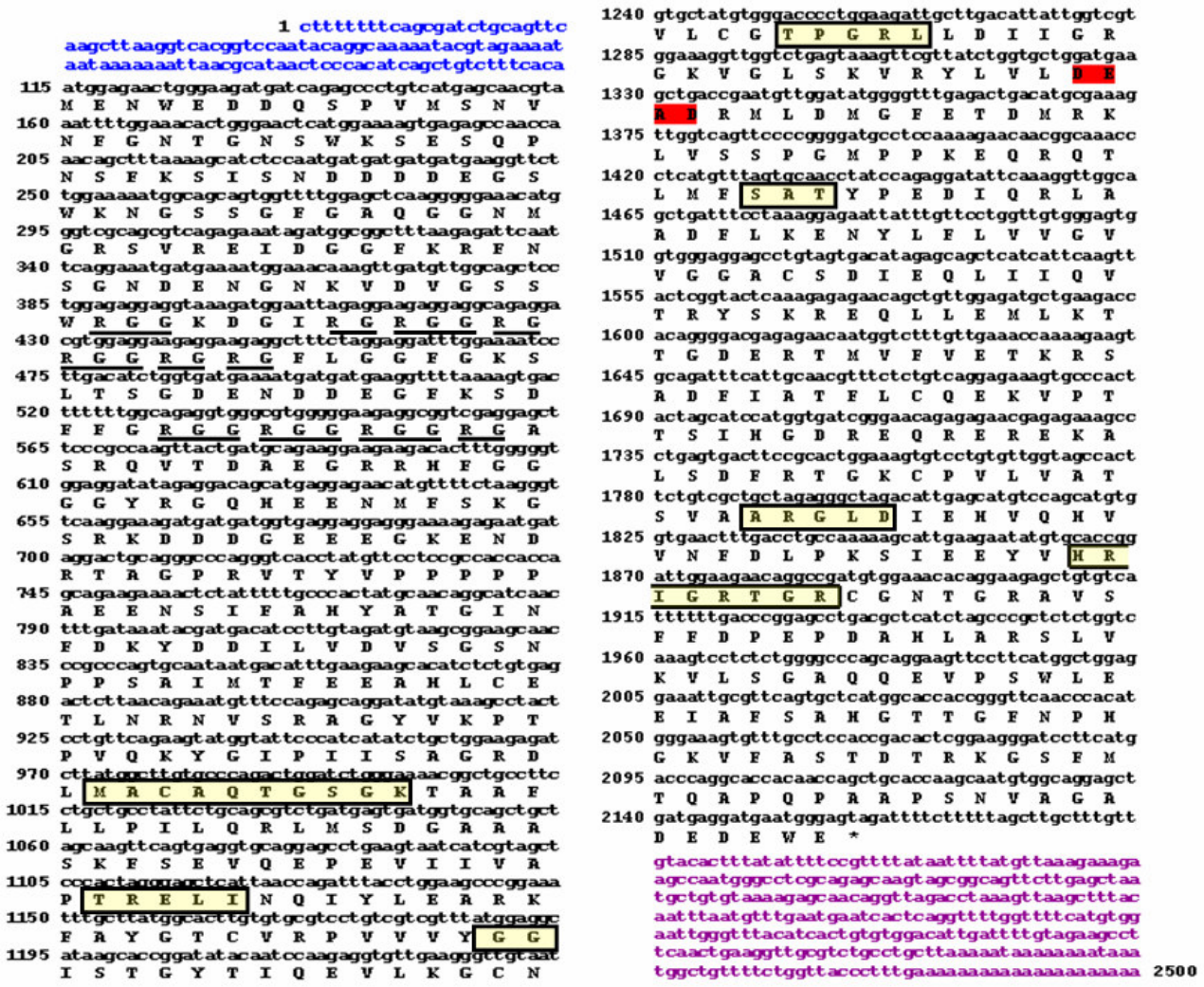

*Fig. 2. Nucleotide and deduced amino acid sequence of catfish full-length vasa cDNA sequence. 5' UTR was shown in blue letters and 3 UTR in red letters. The DEAD box is indicated in red color and other conserved domains were shown in yellow color boxes. The arginine-glycine (RG) repeats and arginine-glycine-glycine (RGG) repeats in the N-terminal region were underlined. * indicates stop codon.*

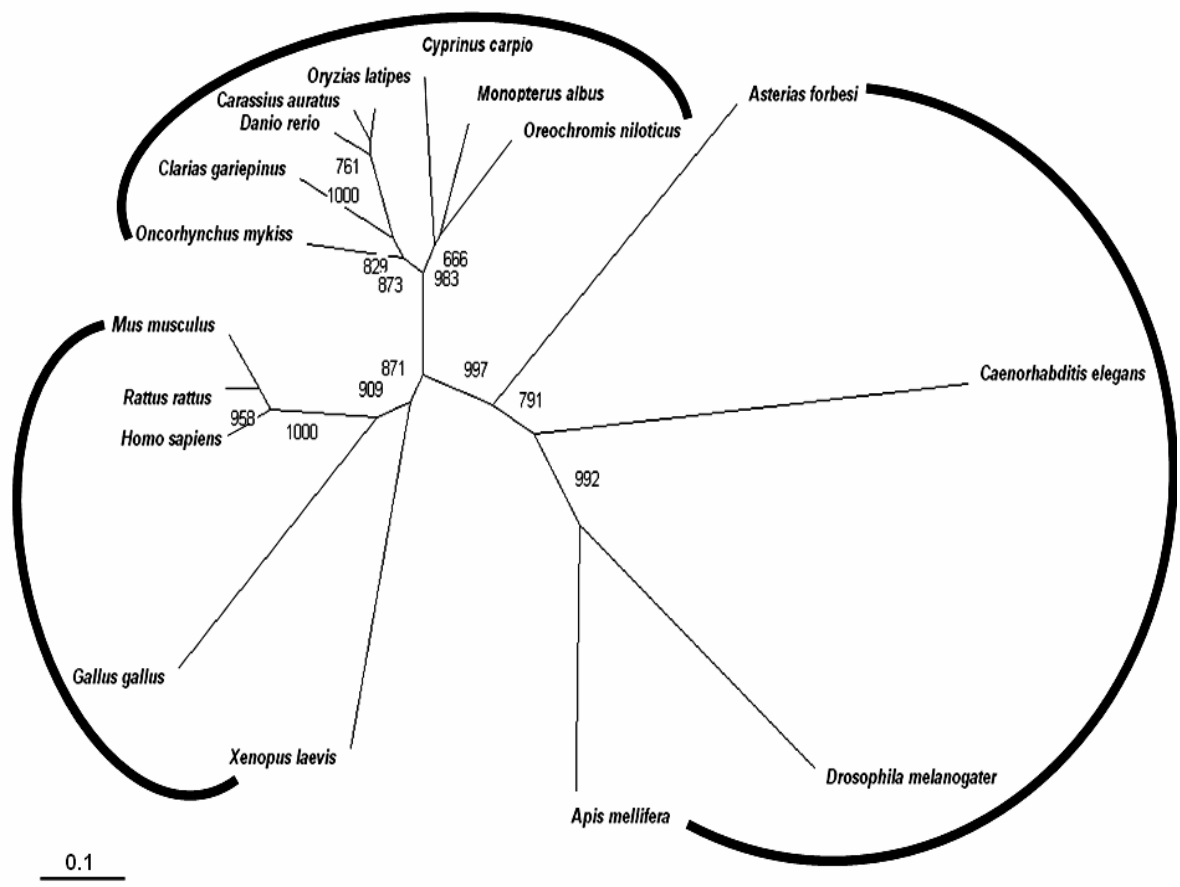

Fig. 3. Phylogenetic tree showing the evolutionary status of catfish vasa. Phylogenetic analysis was constructed with ClustalX using the Neighbor-Joining (NJ) method and a bootstrap analysis with 1000 replicates was used to assess the strength of nodes in the tree (http://www.ddbj.nig.ac.jp). Phylogenetic analysis was generated using the TreeView software package version 1.4. GenBank accession numbers of the sequences used in phylogenetic tree were as follows: Drosophila melanogaster: AAF53438; Caenorhabditis elegans: NP_001021793; Asterias forbesi: ACM80365; Apis mellifera: ABC41341; Homo sapiens: AAF72705; Mus musculus: BAA03584; Rattus rattus: AAB33364; Gallus gallus: BAB12337; Xenopus laevis: AAC03114; Danio rerio: AAI29276; Oryzias latipes: BAB61047; Oncorhynchus mykiss: BAA88059; Monopterus albus: ABA54551; Oreochromis niloticus: BAB19807; Carassius auratus: AAX22126; Cyprinus carpio: AAL87139; Clarias gariepinus: GU562470 .

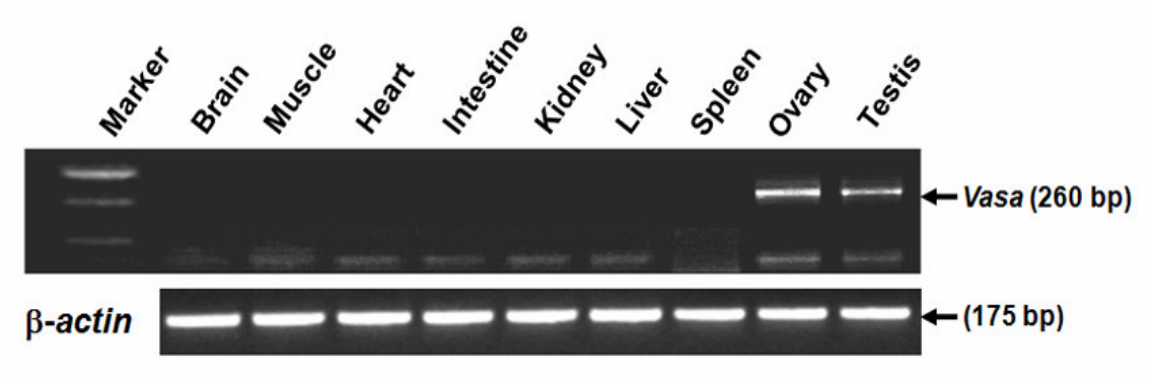

Fig. 4. RT-PCR analysis of vasa expression in different tissues of adult catfish. Upper gel panel showing vasa expression in different tissues and lower gel panel showing expression of β-actin as an internal control.

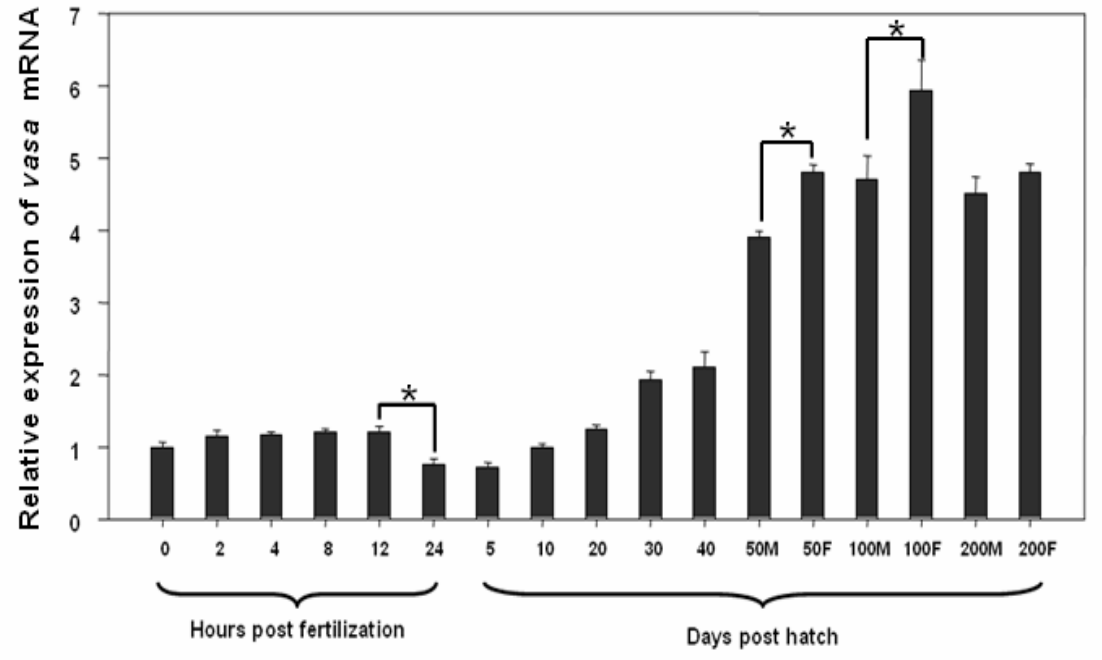

Fig. 5. Relative mRNA expression levels of vasa in different stages of embryonic and gonadal development using real-time PCR. * indicates P < 0.05, one-way ANOVA followed by Duncan's multiple range test.

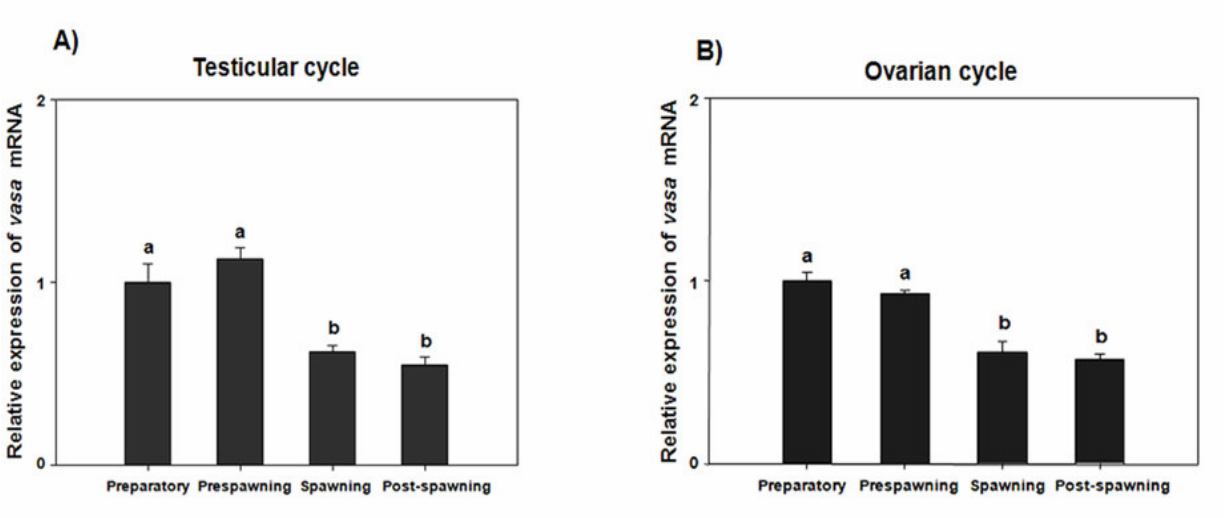

Fig. 6. A) Relative mRNA levels of vasa during different phases of testicular cycle in catfish using real-time PCR. B) Relative mRNA levels of vasa during different phases of ovarian cycle in catfish using real-time PCR. Common letters indicate means that are not significantly different. Means with different letters differ significantly (P < 0.05, one-way ANOVA followed by Student-Newman-Keuls post hoc test).

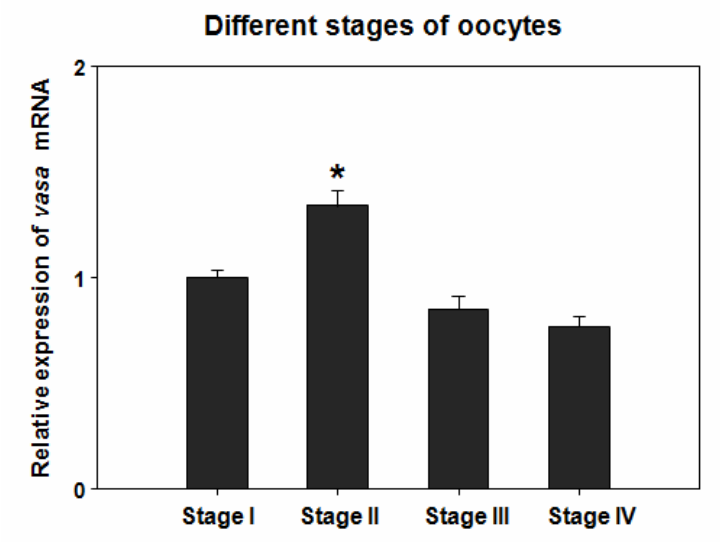

Fig. 7. Relative mRNA levels of vasa in different stages of oocytes. * indicates significantly high levels of vasa mRNA in stage II oocytes, when compared with other stages of oocytes (P < 0.05, one-way ANOVA followed by Student-Newman-Keuls post hoc test).

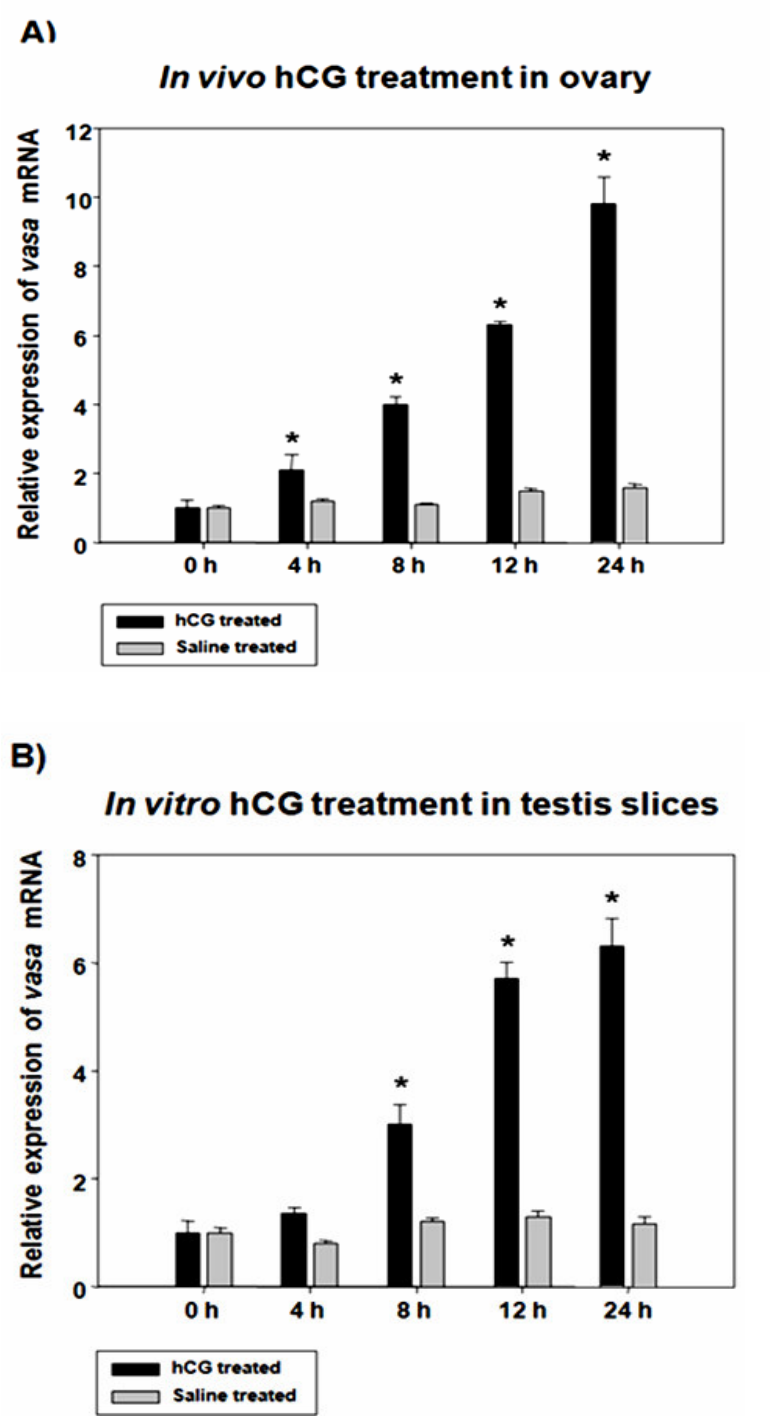

Fig. 8. A) Relative mRNA levels of vasa mRNA in the oocytes collected at different time points from females induced with hCG, in vivo. B) Relative mRNA levels of vasa in the testicular slices treated with hCG, in vitro at different time intervals. * indicates P < 0.05, one-way ANOVA followed by Duncan's multiple range test.

Discussion

In the present study, we isolated the full-length *vasa* cDNA from ovarian tissue using 5' and 3' RACE strategies. We also cloned ORF of *vasa* from catfish testis using RT-PCR whose sequence was identical with that of the ORF obtained from ovary. The deduced amino acid sequence of catfish *vasa* contained eight conserved regions characteristic of DEAD box family protein including the ATP-binding motifs (Hay, *et al.*, 1888b; Liang *et al.*, 1994). The *vasa* family of DEAD box helicases are conserved across different phyla and thought to have originated from *PL10* family of helicases before the cnidarian evolution (Mochizuki *et al.*, 2000). Phylogenetic analysis using neighbor-joining method revealed that the cloned catfish *vasa* was closely related to its teleosts counterparts. Tissue distribution analysis in adult catfish showed that the expression of *vasa* was only detectable in gonads (testis and ovary). Similar kind of expression pattern for *vasa* was evident in other vertebrates as well (Castrillon *et al.*, 2000; Kobayashi *et al.*, 2000, 2002; Yoshizaki *et al.*, 2000; Xu *et al.*, 2005; Ye *et al.*, 2007; Nagasawa *et al.*, 2009). In the catfish, qRT-PCR analysis revealed no significant change in *vasa* mRNA levels throughout the course of embryonic development from 0 hpf to 12 hpf. However, the transcript levels dropped at 24 hpf as soon as the embryo hatched. Northern blot analysis in zebrafish revealed stable *vasa* mRNA expression till 6 hpf embryo followed by gradual decrease till 24 hpf (Wolke *et al.*, 2002). As the gonadal development progresses, the mRNA levels gradually increase from 10 dph and reach significantly high around 50 and 100 dph, due to the increase in the number of germ cells in testis and more number of primary oocytes in ovary. We observed differential expression of *vasa* mRNA in adult male and female gonads during seasonal reproductive cycle by qRT-PCR. The *vasa* mRNA levels were significantly high during spermatogenesis and its expression decreased drastically around the

phase of spermiation. This kind of expression pattern in testis during recrudescence can be explained based on the localization studies of *vasa* reported in tilapia testis where in the *vasa* signal was high in spermatogonia (germ cells), moderately present in primary spermatocytes while no signal was detected in secondary spermatocytes, spermatid and spermatozoa (Kobayashi *et al.*, 2000). However, in human and mouse, *vasa* expression was noticed in germ cells at stages from the spermatogonium to spermatids (Castrillon *et al.*, 2000; Toyooka *et al.*, 2000). In the case of mature female catfish, the expression was high in the preparatory phase i.e. during early oogenesis when compared to other phases of ovarian cycle. These results together suggest that *vasa* might have an important role to play in gametogenesis. The qRT-PCR results showed that *vasa* transcript was abundantly expressed in stage I and II (immature) oocytes when compared to stage III and IV (mature) oocytes. Similar kind of expression pattern was also observed in tilapia and gibel carp by *in situ* hybridization and in humans by immunostaining where in the signal was high in immature oocytes while in mature oocytes the signal became weak, perhaps due to dispersion of transcripts throughout enlarged mature oocyte that accumulate large amount of vitellogenin (Castrillon *et al.*, 2000; Kobayashi *et al.*, 2000; Xu *et al.*, 2005). In mouse ovaries, strong immunoreactivity was observed in primary oocytes and the expression decreased as maturation proceeded, and finally reached to undetectable levels in mature oocytes (Toyooka *et al.*, 2000). On the contrary, Cardinali *et al.* (2004) observed abundant expression of *vasa* in oocytes of mature seabream when compared to immature oocytes by semi-quantitative RT-PCR. In this study, for the first time we report the regulation of *vasa* mRNA by gonadotropin (hCG) induction in oocytes (*in vivo*) and testicular slices (*in vitro*) during late preparatory/prespawning phase. In both the cases hCG treatment resulted in the up regulation of *vasa* mRNA in a time-dependant manner. This further supports

our findings during seasonal cycle wherein the expression of *vasa* was found to be high during gametogenesis.

In conclusion, present study clearly demonstrated differential expression of *vasa* mRNA in male and female gonads during gametogenesis which might be under the regulation of gonadotropins.

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Chapter 2

Identification of multiple dmrt1s in catfish: Localization, dimorphic expression pattern and changes during testicular cycle

Abstract

The double sex and mab-3 related (DM) transcription factor 1 (*dmrt1*) plays an important role in testicular differentiation. Here, we report cloning of multiple *dmrt1*s, a full-length and two alternative spliced forms from adult catfish (*Clarias gariepinus*) testis, which encodes predicted proteins of 287 (*dmrt1*a), 253 (*dmrt1*b) and 233 (*dmrt1*c) amino acid residues, respectively. Interestingly, *dmrt1*c lacks majority of DM domain. Multiple *dmrt1*s (*dmrt1*a and *dmrt1*c) were obtained from *C. batrachus* as well. Tissue distribution (transcript and protein) of catfish *dmrt1* revealed exclusive expression in testis. Semi-quantitative RT-PCR revealed the presence of multiple *dmrt1*s with high levels of *dmrt1*a in adult testis but not in ovary. Potential role for *dmrt1* in testicular differentiation and development was evident from its stage-dependent elevated expression in developing testis. Real-time RT-PCR analysis during testicular cycle showed higher levels of *dmrt1* transcripts in preparatory and pre-spawning when compared to spawning and post-spawning phases. Immunocytochemical and immunofluorescence localization revealed the presence of catfish Dmrt1 protein in spermatogonia and spermatocytes, which indicates plausible role in spermatogenesis. These results together suggest that multiple *dmrt1*s are testis-specific markers in catfish.

Introduction

Several transcription factors like *SRY*, *DMY*, *Sox9, Ad4BP/SF-1*, *Dmrt1*, *Dax1* and *Wt1* have been identified to play an important role in sex determination and differentiation of vertebrates including teleosts (Swain *et al.*, 1999; Hughes, 2001; Matsuda *et al.*, 2002; Wang *et al.*, 2002; Kobayashi *et al.*, 2004). Molecular similarity in sexual development across different phyla found so far is among *Drosophila doublesex*, *Caenorhabditis mab-3* and vertebrate *dmrt1* (Raymond *et al.*, 1998). *Dmrt1* (*dsx* and *mab-3* related transcription factor 1) belongs to gene family of putative transcription factors that share a highly conserved novel zinc finger DNA binding domain (DM domain) across different phyla (Raymond *et al.*, 1998). It has been cloned from several vertebrate species including mammals, birds, reptiles, amphibians and fishes (Nanda *et al.*, 1999; Grandi *et al.*, 2000; Guan *et al.*, 2000; Kettlewell *et al.*, 2000; Osawa *et al.*, 2005; Nagahama 2005) and has been implicated in testicular differentiation. Further, *dmrt1* has been localized in 9p23.4 and monosomy at this region in XY individuals manifests feminization and gonadal dysgenesis (Ottolenghi and Mc Elreavey, 2000). In mouse, *dmrt1* expresses in both male and female embryonic genital ridges, but as the differentiation proceeds it is gradually lost from the ovary and expressed only in the Sertoli and germ cells of testis. High expression of *dmrt1* was observed in testis at 13.5 days post coitum and its expression is maintained throughout the adult after birth (Raymond *et al.*, 1999; Grandi *et al.*, 2000). Localization of *dmrt1* in the Z chromosome of birds designated it as a male master testis-determining gene (Nanda *et al.*, 1999). *Dmrt1* knockout mice showed its dispensability in females yet required in males for postnatal testicular development by effecting the differentiation of both Sertoli and germ cells (Raymond *et al.*, 2000). These expression patterns indicate that *dmrt1* is likely to have a conserved role in the early stages of testis development. Studies from lower vertebrates are mostly done in teleosts to specify the conserved role of *dmrt1*. *DMY*, a Y-linked male sex determination gene, similar to

Sry discovered in medaka has been shown to be a duplicate of autosomal gene *dmrt1* (Matsuda *et al.*, 2002; Zhang, 2004). Nevertheless, most of these reports are from daily or fortnight breeders like zebrafish, medaka and tilapia (Guan *et al.*, 2000; Kobayashi *et al.*, 2004; Guo *et al.*, 2005). Research reports on this line using annual breeders with a focus on seasonal reproductive cycle are limited (Marchand *et al.*, 2000; Huang *et al.*, 2005). In this regard, fish that undergoes seasonal pattern of gonadal attenuation and recrudescence rather than continuously mature individuals may provide interesting highlights to understand the role of *dmrt1* not only during development but also during recrudescence. The fate of *dmrt1* transcripts after testicular differentiation is not clear at present. Such an attempt may provide more insights to understand its role in adult, if any. Though tracking of *dmrt1* may not provide a direct role to this issue but may contribute to understand whether timing of *dmrt1* expression coincides with the beginning of testicular recrudescence vis-à-vis spermatogenesis. Catfish, *Clarias gariepinus* is an annual breeder, which takes one-year time to attain maturity. It's domesticated in south India and interestingly it exhibits seasonal pattern of reproductive cycle. In the present study, we aimed to clone *dmrt1* from catfish testis and also explored the possibility of multiple spliced forms. In addition to this, we also aimed to study the spatio-temporal expression pattern of *dmrt1*(s) during early stages of gonadal development in juveniles as well as at different phases of testicular recrudescence in adult catfish. We also intend to confirm the presence of *dmrt1*(s) from a closely related catfish species *C. batrachus* to augment our findings. Further, our study was extended to localize *dmrt1* in juvenile and adult testis.

Materials and Methods

Animals and Sampling

Breeding and rearing of catfish (*C. gariepinus*) at different age groups was already described in chapter 1. Adult male gonads at different phases of reproductive cycle were collected and stored at –80^{o}C for total RNA extraction. Juvenile catfish fries at different age groups (50, 100 and 150 days post hatch [dph]) were dissected and gonads (testis and ovary) were removed using a fine forceps under stereo zoom microscope (Leica, Germany). Gonads of similar age group and same sex were pooled (5 fishes per each sample) and stored at –80^{o}C for total RNA extraction. Adult *C. batrachus* were obtained from north India. These were maintained and fed with minced goat liver *ad libitum* during acclimation in the laboratory for a fortnight before sacrifice. Testis tissue was collected and stored at –80^{o}C for total RNA extraction.

Cloning of partial cDNA fragment of *dmrt1* from catfish testis

A set of degenerate primers (Table 1) were designed by aligning the available *dmrt1* nucleotide sequences of other species from NCBI GenBank data base. A partial cDNA fragment was amplified from testis cDNA template by RT-PCR and cloned as per the methodology described in chapter 1.

Cloning of full-length cDNA and alternatively spliced forms of *dmrt1*

5’ and 3’ cDNA templates were made from testis total RNA according to manufacturer’s protocol using the SMART RACE cDNA amplification kit (Clontech, USA). For the amplification of 5' and 3’ cDNA ends, touchdown PCR reactions were performed using 5’ and 3’ GSP1 and GSP2 primers (Table 1) and as well as anchor primers supplied in the kit as described in chapter 1. All the amplified cDNA fragments were gel-purified, cloned in TOPO TA cloning

vector (Invitrogen) and nucleotide sequenced. Later on we aimed to amplify the open reading frame (ORF) of *dmrt1* using *dmrt1orfF* and *dmrt1orfR* primers (Table 1) designed at ORF flanking region of full-length cDNA obtained by RACE. The PCR conditions used for ORF amplification were as follows: 94 °C (30 sec), 60°C (30 sec) and 72 °C (1 min) for 35 cycles. We obtained multiple PCR products which were cloned in TOPO cloning vector and nucleotide sequenced.

Tissue distribution pattern of *dmrt1* by RT-PCR

RT-PCR was carried out as described in chapter 1 to study expression pattern of *dmrt1* in different tissues of adult catfish using *dmrt1* specific primers (Table 1).

Semi-quantitative RT-PCR for expression analysis of *dmrt1*

Semi-quantitative RT-PCR as described by Kwon *et al.* (2001) was carried out to study the expression patterns of multiple *dmrt1*s in adult and developing gonads. Total RNA was prepared from adult gonads and developing gonads collected from juvenile catfish at 50, 100 and 150 dph using the Sigma TRI-reagent method. Reverse transcription was carried out using superscript-III reverse transcriptase (Invitrogen) with oligo $d(T)_{18}$ primers and 5 µg of total RNA at 50°C. PCR amplification was done using using thermal cycler (Applied Biosystems) under following conditions: 94°C (1 min), 60°C (30sec), 72°C (1min), for 30 cycles using specific primers *dmrt1aF, dmrt1aR, dmrt1bF, dmrt1bR, dmrt1cF* and *dmrt1cR* designed for amplification of respective *dmrt1* forms (Table 1). Catfish β-*actin* was PCR amplified at 94°C (1 min), 60°C (30sec), 72°C (1min) for 28 cycles using specific β-*actinF* and β-*actinR* primers (Table 1) as an internal control.

Real-time Quantitative RT-PCR (qRT-PCR)

Pilot experiments for multiple *dmrt1*s expression during testicular cycle were done by semi-quantitative RT-PCR using ORF-specific primers. The expression of multiple *dmrt1*s during four different phases (preparatory, pre-spawning, spawning and post-spawning phases) of testicular cycle was analyzed by qRT-PCR using real-time specific primers (Table 1) as described in chapter 1. The *dmrt1* expression was normalized against β-*actin* expression, generating a ΔCt value (ΔCt = *dmrt1* Ct – β-*actin* Ct). Relative expression was then expressed according to the equation $2^{-\Delta CT}$.

Primer Name	Nucleotide sequence (5′– 3′)	Usage
dmrt1DgF	TGCCG(ACG)(CA)G(AG)TGC(CA)G(AG)AA(CT)CACG	Degenerate RT-PCR
dmrt1DgR	TAGTAGGA(GC)TGCAT(AG)CGGTAC	Degenerate RT-PCR
dmrt1orfF	GAAGCACGAGGCCGCGCAGAG	ORF cloning
dmrt1orfR	GTTGGTATACTCTCTGAGACTT	ORF cloning
dmrt1 5'GSP1	GCTATCTCCACTGGGCATCTGCTGGT	5'RACE
dmrt1 5'GSP2	AATCATTTCCTGGCTCATCCTTCACC	5'RACE
dmrt1 3'GSP1	ATGCCGAAGTGCTCCCGGTGCAGG	3'RACE
dmrt1 3'GSP2	5'GTCCCGCCAGTTACAGAAGCGCTTG	3'RACE
β-actinF	ACCGGAGTCCATCACAATACCAGT	qRT-PCR
β-actinR	GAGCTGCGTGTTGCCCCTGAG	qRT-PCR
dmrt1abRTF	ATGGCCGCTCAGGTGGCTCTGCGG	qRT-PCR
dmrt1abRTR	GCGGCTCCCAGAGGCAGCAGGAGA	qRT-PCR
dmrt1cRTF	CCAGGGCCAGGTGGCTCTGCG	qRT-PCR
dmrt1cRTR	GCGGCTCCCAGAGGCAGCAGGAGA	qRT-PCR
dmrt1aF	ATGCCGAAGTGCTCCCGGTGC	Tissue distribution
dmrt1aR	AGCGGCTCCCAGAGGCAGC	Tissue distribution
dmrt1bF	AAGGATGAGCACCAGGGGACA	Tissue distribution
dmrt1bR	TTACTTAGCAGCTCCCTCTAT	Tissue distribution
dmrt1cF	CCAGGGCCAGGTGGCTCTGCG	Tissue distribution
dmrt1cR	TTACTTAGCAGCTCCCTCTAT	Tissue distribution

***Table1. List of primers used for cloning and expression analysis of multiple dmrt1*s**

Production of polyclonal antibody against Dmrt1 protein

Polyclonal anti-catfish Dmrt1 antibody was raised in rabbit using catfish partial Dmrt1 recombinant protein of the conserved DM-domain as antigen. The partial Dmrt1 protein was expressed in *E. coli* DE3 LacI host using 0.5M IPTG by cloning the partial *dmrt1* cDNA fragment of 340 bp into bacterial pET BLUE2 vector system (Novagen, USA). The expressed fusion protein with the histidine tag (His-tag) was then purified using Ni-NTA Agarose column (Qiagen) according to the manufacture's protocol. This purified recombinant protein was used to raise polyclonal antibody in rabbit after confirming by immunoblot using the monoclonal His-tag antibody. For raising polyclonal antibody against the purified protein, rabbit was first injected with Freund's complete adjuvant (Bangalore Genei Pvt Ltd) at the vola. Later, after 1 week 500 µg of the purified protein emulsified in Freund's complete adjuvant was injected into the swollen lymphoid node. Then two booster injections were given with the same antigen amount but using Freund's incomplete adjuvant (Bangalore Genei Pvt Ltd, India) for each week. After the last booster dose the rabbit was bled 2 weeks later and serum sample was stored at −80 °C until use. All the rabbits used in the present study by following the guidelines of Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals and also after taking prior permission.

Western blot analysis

Western blot analysis was carried out to verify the specificity of the polyclonal antibody raised in rabbit. For this, different tissues (testis, ovary, gut, spleen, liver, muscle, heart, brain) of catfish were homogenized in 250 µl of homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM DTT, 0.1% TritonX-100 and 0.1mM PMSF using a Sigma hand homogenizer. 50 µg of each sample with a pre-stained marker was electrophoresed through a

15% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Pall-Life sciences, USA). Membrane was blocked in 5% skim milk powder in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hr at room temperature. After blocking, membrane was washed three times for 15 min each in TBST solution and incubated with purified anti-catfish Dmrt1 antibody at 4°C for overnight in 0.5% skim milk powder/TBST solution. Blot was washed and incubated with secondary antibody Alkaline phosphatase conjugated goat anti-rabbit IgG (Bangalore Genei) for 1 hr. After washing, blot was developed using BCIP-NBT (Bangalore Genei).

Immunocytochemical and Immunofluorescence localization of Dmrt1

Adult and juvenile gonads were fixed in 4% paraformaldehyde (PFA), processed and embedded in paraplast (Sigma). Sections were cut at 3µm thickness using microtome (Leica, Germany). Testis sections from adult male catfish were deparafinnised in xylene, rehydrated in successively lower graded concentrations of ethanol and then treated with 0.1% H_2O_2 for 10 minutes to prevent endogenous peroxidase reaction. The sections were washed in 0.1M phosphate buffered saline with 0.1% Tween 20 (PBST) and then blocked with 10% normal goat serum in 0.1M PBS for 30 minutes at room temperature. Sections were then incubated overnight at 4^{o}C in 1:1000 dilution of Dmrt1 antiserum or antiserum pre-absorbed with excess of peptide used for immunization (as negative control). Later sections were washed in PBS with 0.1% Tween 20 (PBST) for 10 min and then incubated with 1:500 diluted biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at room temperature for 1 h. The sections were later incubated with ABC reagent (Avidin-Biotinylated horse-radish peroxidase complex) provided in VECTASTAIN Elite ABC kit (Vector Laboratories) for 30 min at room temperature. The sections were washed in PBS and color was developed using commercially supplied 3' 3'-diaminobenzidine as chromogen and H_2O_2 as substrate for horse-radish peroxidase (Vector

Laboratories). The sections were washed, dehydrated in graded ethanol, cleared in xylene and mounted using DPX mountant. Photomicrographs were taken using Olympus CX41 bright field microscope (Olympus, Japan). For immunofluorescence studies, both adult testis and ovary sections were processed in the same way as mentioned above up to primary antibody incubation step except for 0.1% H_2O_2 treatment. The sections were then in PBST and incubated with FITC fluorescent-labeled secondary antibody at room temperature for 1 hr. After that the sections were washed and mounted using 90% glycerol. Photomicrographs were taken immediately using Olympus fluorescence microscope (Olympus, Japan). Hematoxylin and propidium iodide (PI) was used as a counter stain for better clarity. All reagents and secondary antibodies to perform immunocytochemistry were obtained from Bangalore Genei Pvt Ltd.

Results

cDNA cloning of multiple alternative transcripts of catfish *dmrt1*

A partial cDNA fragment (421 bp) of *dmrt1* was cloned from catfish testis by degenerate RT-PCR (Fig. 1A). To isolate full-length cDNA of *dmrt1* from catfish testis 5' and 3' RACE was performed. After aligning the 5' and 3' cDNA end regions of *dmrt1* sequence that overlap in the DM-domain region, we obtained 1.1 Kb full-length cDNA sequence of *dmrt1* (here after referred as *dmrt1*a) which encodes a putative protein of 287 amino acids (Fig. 2). The amino acid sequence comparison of catfish *dmrt1*a with other vertebrate *dmrt1* proteins revealed considerable homology with other teleosts like zebrafish (61%), rainbow trout (59%), the eel (57%), and the Nile tilapia (54%). The phylogenetic analysis of vertebrate *dmrt1* proteins (Fig. 3) showed the existence of two main clades. The first clade represents mammalian *dmrt1* sequences while the second one is for teleost *dmrt1* sequences. Interestingly, we obtained multiple *dmrt1* transcripts (Fig. 4), when we attempted to amplify the ORF region of *dmrt1* using specific primers designed at ORF flanking region of full-length *dmrt1*a cDNA. Sequence information of these multiple bands revealed that these products represented *dmrt1* transcripts generated by multiple alternative splicing. Two alternative spliced forms *dmrt1*b and *dmrt1*c were obtained in catfish along with *dmrt1*a, which encodes different size predicted proteins with 253, 233 and 287 amino acids, respectively (Fig. 5). The nucleotide sequence data of multiple *dmrt1*s have been submitted to the GenBank and the accession numbers are *dmrt1*a (FJ596554), *dmrt1*b (FJ596555) and *dmrt1*c (FJ596556). The entire alternative splicing events of the *dmrt1* in catfish occurred within the ORF towards the 5' end region. Interestingly, the *dmrt1c* isoform is lacking most of the DM-domain region at 5' end. Cloning of *dmrt1*a and *dmrt1*c ORFs from a

closely related species, *C. batrachus* confirmed our findings in *C. gariepinus*. The GenBank accession numbers of *dmrt1*s of *C. batrachus* are FJ596557 and FJ596558.

Tissue distribution and expression pattern of *dmrt1* in adult and developing gonads

Tissue distribution pattern of *dmrt1* revealed exclusive expression in testis (Fig. 6A). Semi-quantitative RT-PCR analysis showed that *dmrt1* spliced forms a, b and c were detectable only in adult testis (Fig. 6B). Further the expression of *dmrt1*a was higher than those of *dmrt1*b and *dmrt1*c. Stage-dependent increase in *dmrt1*a expression was observed in testis at different age groups of catfish (50, 100, and 150 dph). Expression of *dmrt1*b and *dmrt1*c were also evident in developing male gonads. In contrast, *dmrt1*a could not be detected in developing female gonads (Fig. 6C).

Expression pattern of multiple *dmrt1*s in different phases of testicular cycle

Real-time qRT-PCR analysis showed that *dmrt1*a and *dmrt1*b expression was high during pre-spawning when compared to preparatory, spawning and post-spawning phases. Similar kind of expression pattern was true for *dmrt1*c that lacks majority of DM-domain (Fig. 7). This indicates that *dmrt1* transcripts are abundantly expressed during active spermatogenesis i.e. in preparatory and pre-spawning phases where in spermatogonia and spermatocytes are more in number. In addition, *dmrt1*a transcript is abundantly expressed in testis when compared to *dmrt1*b and *dmrt1*c transcripts. Studies from semi-quantitative RT-PCR pilot experiments revealed similar pattern of expression for multiple *dmrt1*s (densitometric quantification data not shown). Representative gel image was shown in the inlet of Figure 7.

Detection of Dmrt1 protein in testis using Western blot

SDS-PAGE gel picture showing over-expression of 21 kDa recombinant Dmrt1 protein using bacterial pET BLUE2 vector system and later then was purified using Ni-NTA column (Fig. 8A and B). The purified protein was confirmed by immunoblot using monoclonal His-tag antibody (Fig. 8C). The polyclonal catfish Dmrt1 antibody raised in rabbit revealed an expected band of approximately 31 kDa by western blotting in testis but not in other tissues. This band corresponds to the *dmrt1*a, which encodes a 31 kDa putative protein (Fig. 9). However, we could not detect other two variants of *dmrt1*a by western blot which may be due to their low expression levels as mentioned in the real-time PCR results.

Immunocytochemical and immunoflouresence detection of Dmrt1 in testis

Our immunocytochemical data revealed that Dmrt1 expression was observed in the surrounding regions of the testicular lumens filled with spermatozoa (Fig. 10B). Absence of immunoreactivity using pre-absorbed antiserum confirms that the primary antibody of Dmrt1 is specific for catfish (Fig. 10A). At higher magnification, Dmrt1 immunoreactivity was detected in primary spermatogonia (SG1), secondary spermatogonia (SG2) and spermatocytes (SC), while spermatids/sperm (SP) did not show any immunoreactivity in both mature testis (Fig. 10C and 10D) and juvenile testis at 200 dph (Fig. 10E). We also carried out immunofluoresence studies, wherein Dmrt1 specific immunofluorescence signal (green) was detected in spermatogenic cells surrounding the lumens of adult testis except SP (Fig. 10F and 10G). But no signal was detected in the pre-vitellogenic oocytes (PO) of ovary (Fig. 10H). Red fluorescence is due to PI indicating SP.

Figures

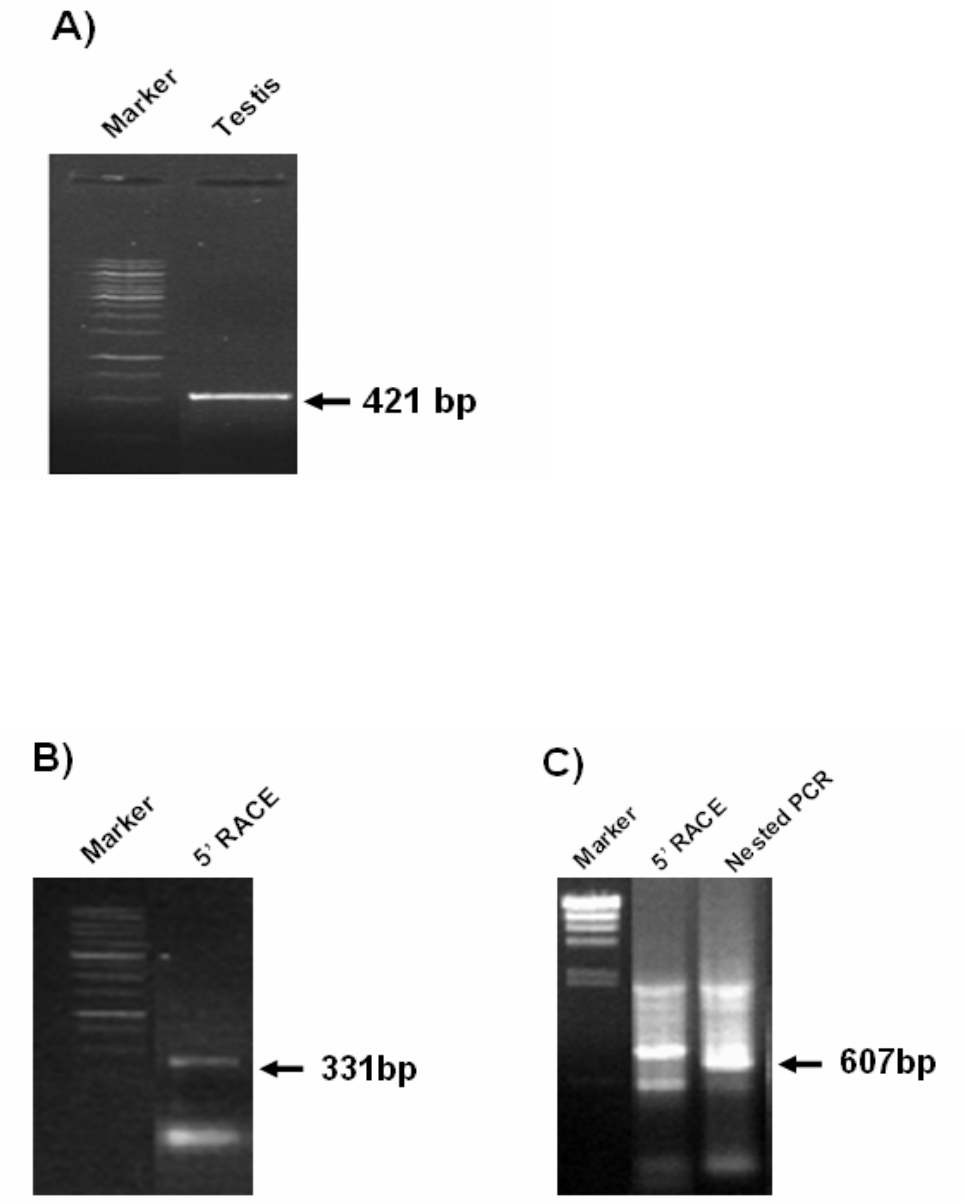

Fig. 1. 1% Agarose gels showing RT-PCR products. A) Partial cDNA fragment of vasa from catfish ovary. B) and C) 5' and 3' RACE products using gene specific primers to obtain full-length cDNA of vasa

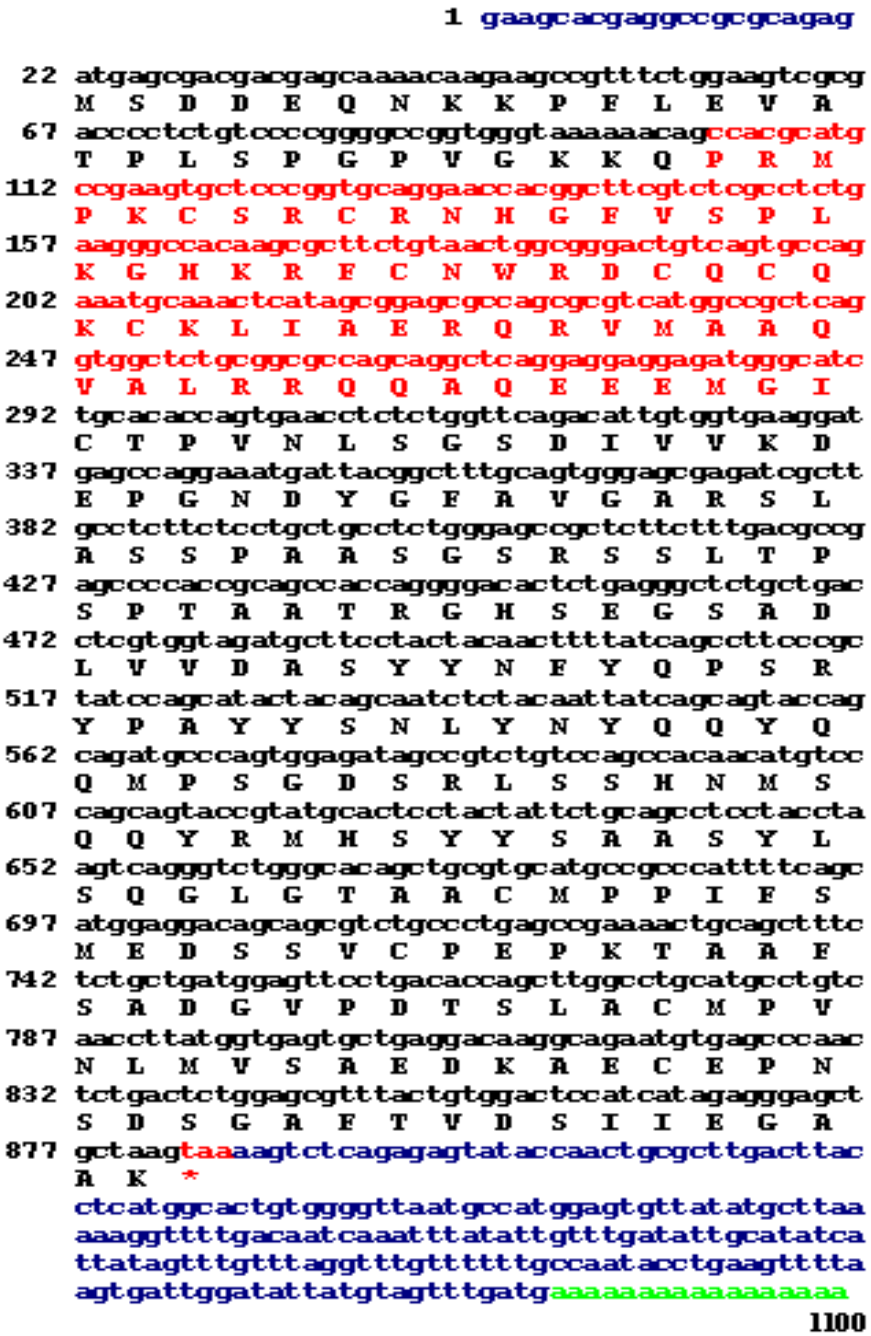

Fig. 2. Nucleotide sequence of catfish full-length Dmrt1a cDNA and its deduced amino acid sequence. 5' and 3' UTRs were shown in blue letters. The DM domain was labeled in red color, putative SY domain is underlined, *indicates stop codon.

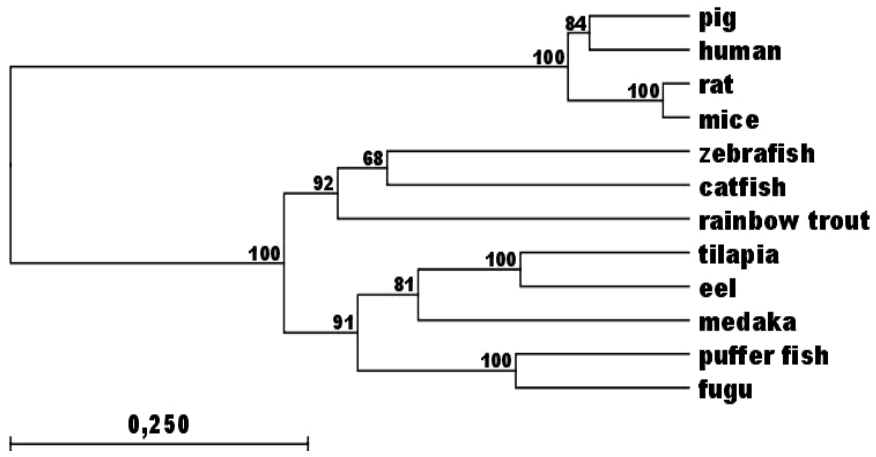

Fig. 3. Phylogenetic tree showing the evolutionary status of catfish Dmrt1. Bootstrap analysis with 100 replicates was used to assess the strength of nodes in the tree. Accession Nos.: Human NM_015826; Mice NM_021951.2; Rat NM_053706; Pig NM_214111; Zebrafish AY157562; Medaka AF319994; Rainbow trout NM_001124269; Eel AF421347; Fugu NM_001037949; Tilapia AF209095; Pufferfish AY135175; Catfish [Dmrt1a] FJ596554.

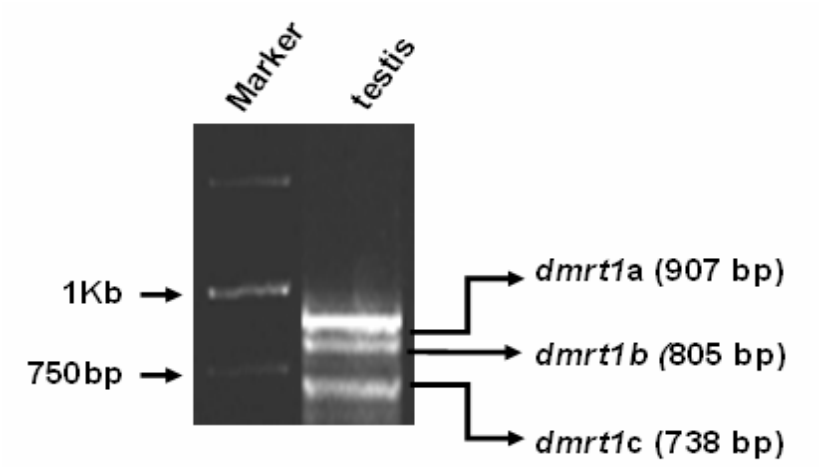

Fig. 4. Representative gel image showing multiple alternative spliced forms of catfish Dmrt1 amplified from adult testis by RT-PCR using ORF flanking primers.

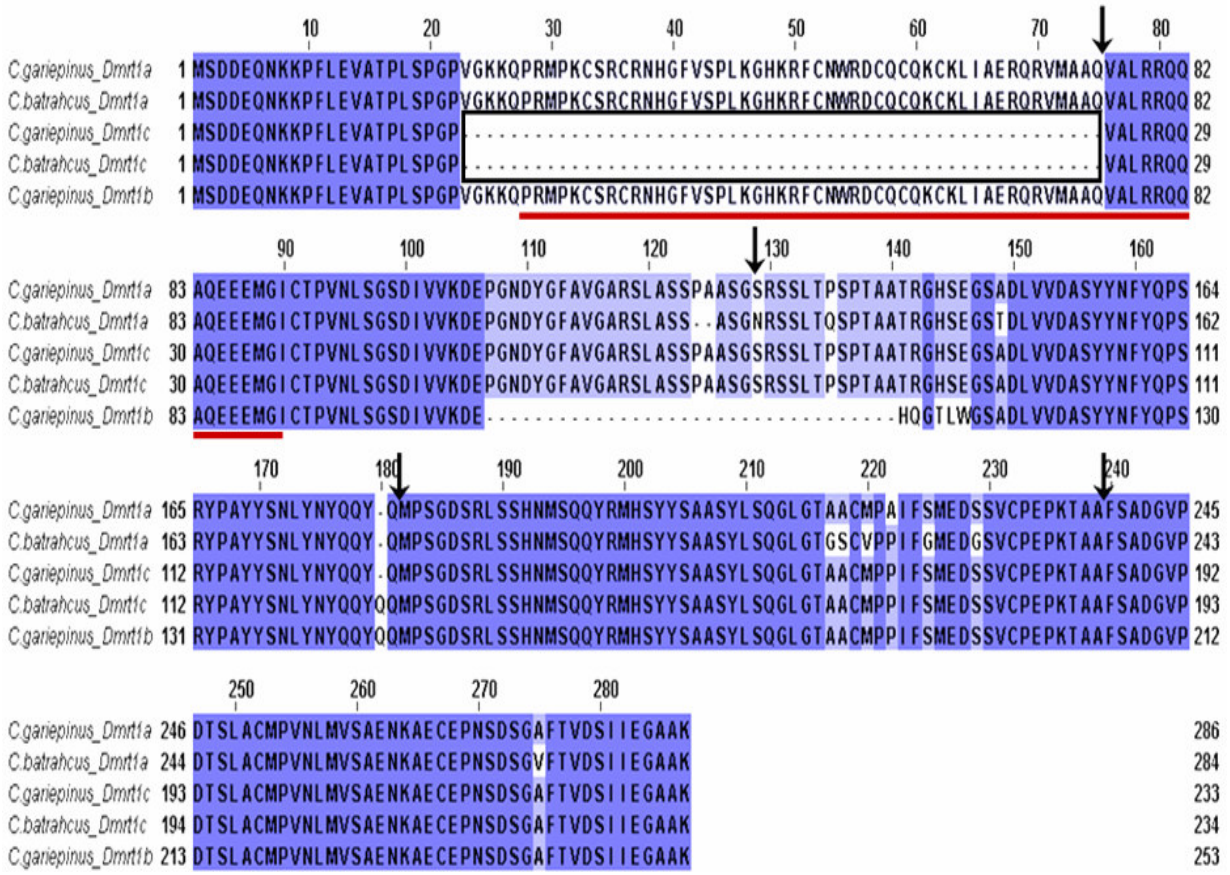

Fig. 5. Amino acid sequence alignment of multiple Dmrt1s from Clarias gariepinus and C. batrachus using EBI clustalW software. DM-domain is underlined in red color. Box indicates loss of DM-domain due to alternative splicing in Dmrt1c. Arrows showing splicing sites of the Dmrt1 intron-exon junctions.

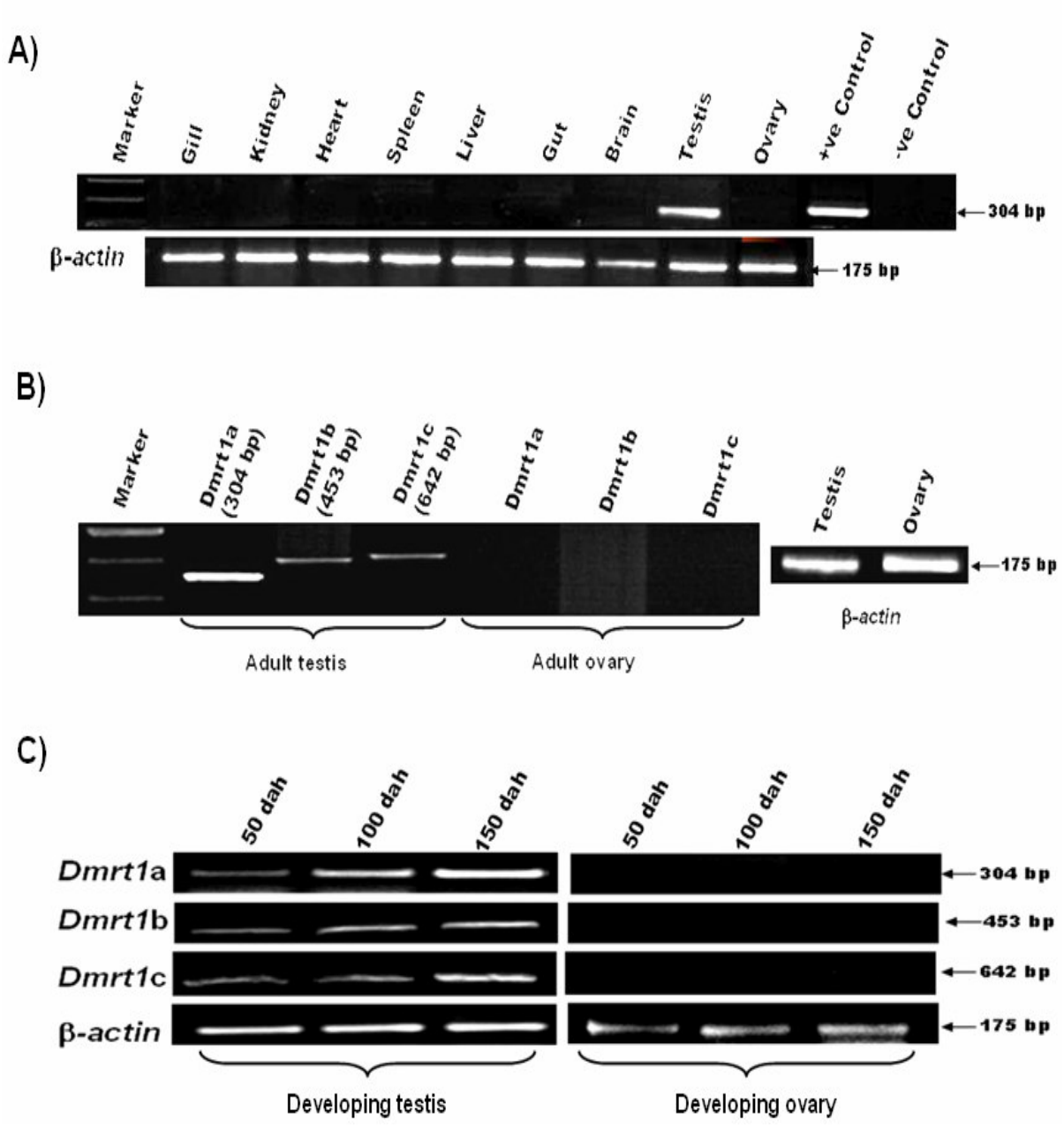

Fig. 6. A) RT-PCR analysis in different tissues of adult catfish. B) Semi-quantitative RT-PCR analysis (n=3) of multiple Dmrt1s in adult testis and ovary. C) Semi-quantitative RT-PCR (n=3) of multiple Dmrt1s in testis and ovary at different age groups of juvenile catfish (50, 100 and 150 days after hatching). β-actin was used as an internal endogenous control.

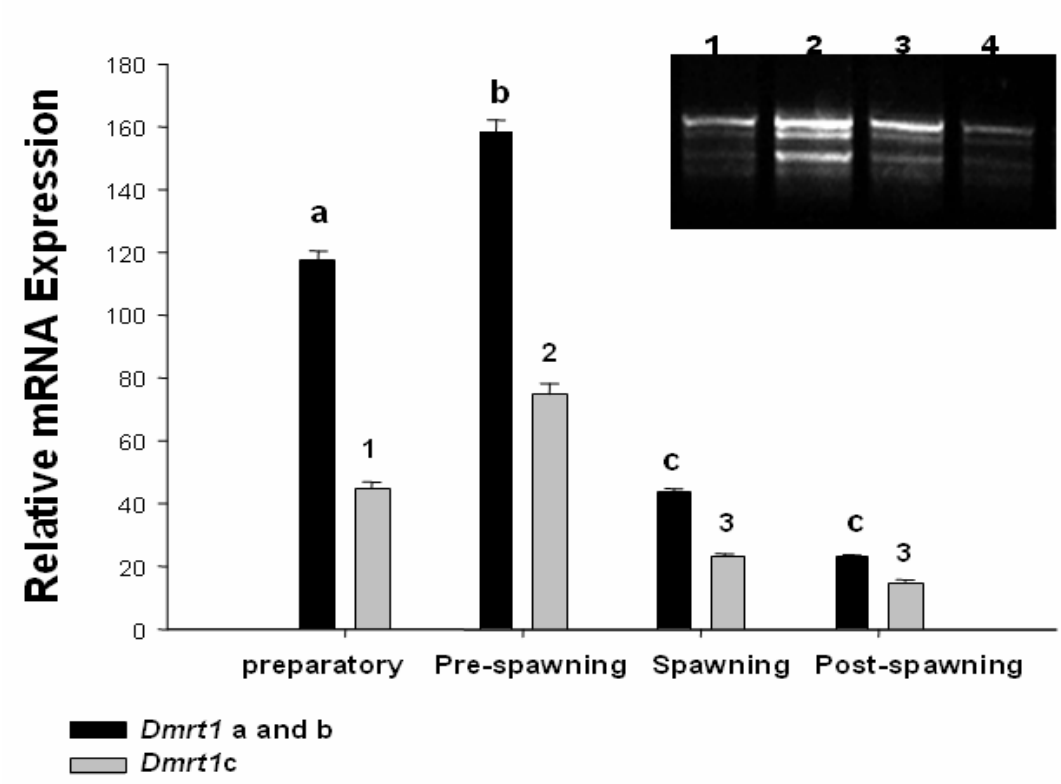

Fig. 7. Quantitative real-time RT-PCR analysis of multiple Dmrt1s during different phases of testicular cycle in catfish. Common letters/numbers indicate means that are not significantly different. Means with different letters/numbers differ significantly (P<0.05). Significance between groups was tested by ANOVA followed by SNK test using Sigma stat 3.0 software. Inlet of figure 4: Representative Gel image showing semi-quantitative RT-PCR expression of multiple Dmrt1s during different phases of testicular cycle (1. preparatory, 2. pre-spawning, 3. spawning and 4. post-spawning phases; n=3).

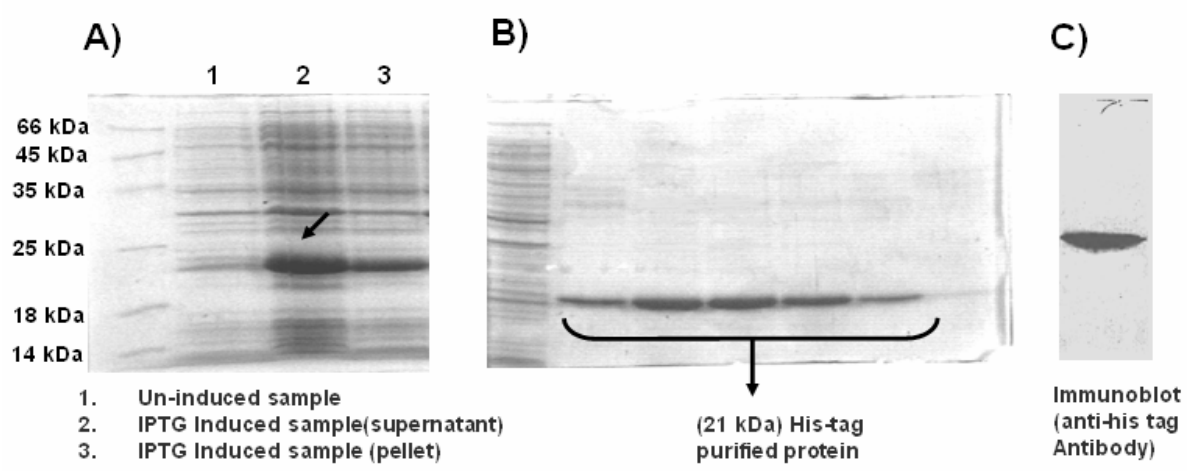

Fig. 8. A) 12% SDS-PAGE gel showing over-expression of 21 kDa recombinant protein (arrow) upon IPTG induction in E. coli. B) 10% SDS-PAGE gel showing purified 21 kDa Dmrt1 protein using Ni-NTA affinity chromatography column. C) Immunobot showing positive band using monoclonal His-tag antibody indicating purified protein is indeed recombinant Dmrt1 protein.

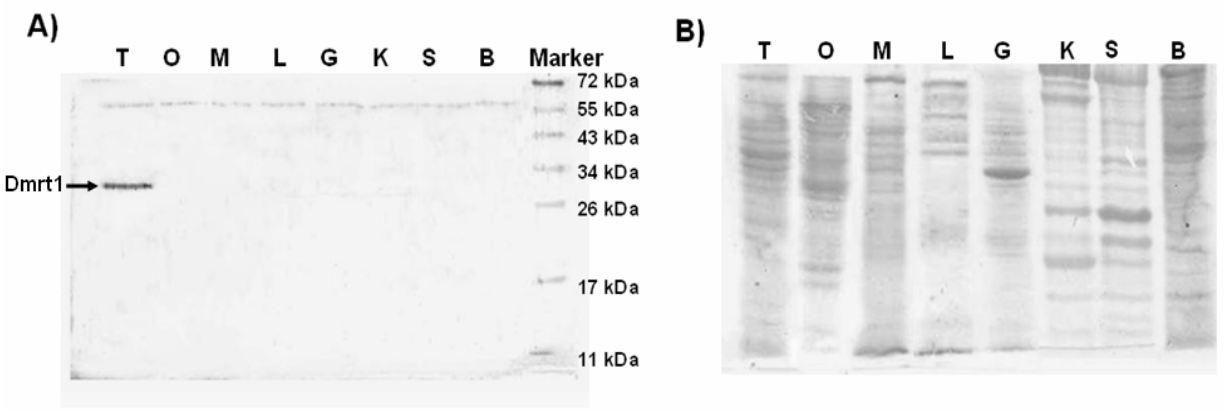

Fig. 9. Western blot analysis (left panel) of Dmrt1 protein expression in various tissues, such as heart (H), muscle (Mu), liver (L), kidney (K), spleen (S), brain (B), ovary (O) and testis (T) which revealed an approximate 31 kDa Dmrt1 protein band in testis (indicated by the arrow). Corresponding whole gel (right panel) showing different tissue samples in 12% SDS-PAGE.

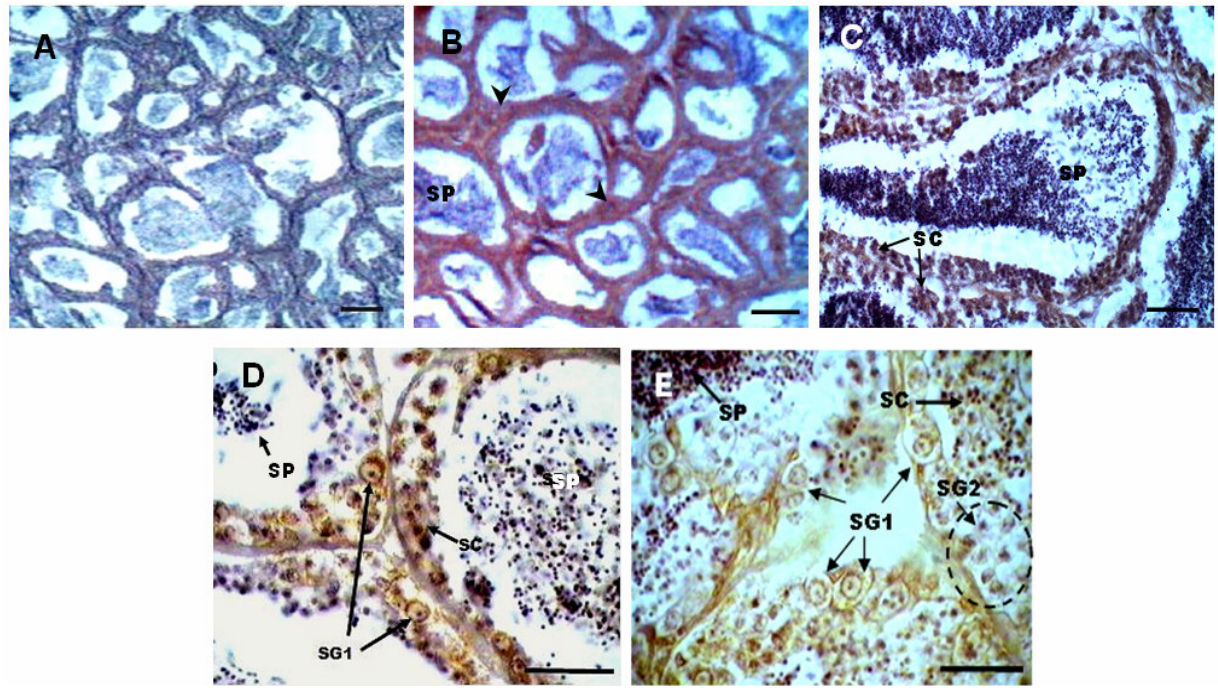

Fig. 10. Immunocytochemical localization of Dmrt1 in adult and juvenile testis using anti-catfish Dmrt1 serum. A) Adult testis section incubated with pre-absorbed antiserum, which was used as a negative control. B) Adult testis section showing Dmrt1 immunoreactivity in boundaries of lumens filled with spermatids/sperm (SP); Arrow heads showing DAB staining. C) & D) Adult testis section at higher magnifications. E) Juvenile catfish testis section at 200 dah. Arrows indicate the position of the cell types: primary spermatogonia (SG1), secondary spermatogonia (SG2), spermatocytes (SC), spermatid/sperm (SP). Counter stained with hematoxylin. All scale bars represent 50 µm.

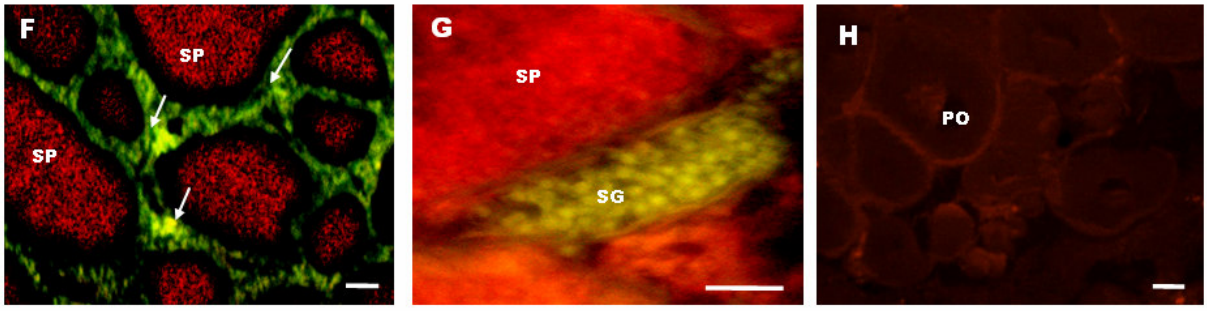

Fig. 10. Immunofluoresence localization of Dmrt1 in adult gonads. F) & G) Adult testis section: arrows indicate the position of the spermatogenic cells. H) Ovary section: previtellogenic oocyte (PO). Counter stained with propidium iodide. All scale bars represent 50 µm.

Discussion

Present report depicts cloning of multiple forms of *dmrt1* in *Clarias gariepinus*. We observed the expression pattern of *dmrt1* in developing testis and ovary by semi-quantitative RT-PCR and also during different phases of testicular cycle by real-time qRT-PCR. Among multiple *dmrt1*s, *dmrt1*a the dominant form was obtained using RACE. It showed high degree of homology with other vertebrate species at the amino acid level in the DM domain region which is conserved across different phyla (Raymond *et al.*, 1998). In addition to *dmrt1*a, we report the isolation of two different alternative spliced forms, *dmrt1*b and *dmrt1*c from adult catfish testis using RT-PCR amplification with ORF flanking primers. All splicing events occurred within the ORF region at 5' end. Interestingly, *dmrt1*c lacks major part of the DM-domain region. Multiple alternative splicing in *dmrt1* gene were familiar in diverse species (Burtis and Baker, 1989; Sreenivasulu *et al.*, 2002; Guo *et al.*, 2005; Cheng *et al.*, 2006; Lu *et al.*, 2007; Zhao *et al.*, 2007). To our knowledge 5' end spliced variants were shown for the first time in a lower vertebrate species (present study). Similar kind of splicing events was also reported in the gonads of mouse, where in four different of forms of *dmrt1* exists and one of the forms is lacking entire DM-domain region (Lu *et al.*, 2007). This shows that the multiple alternative splicing, which give rise to different isoforms, is a common feature of the *dmrt1* gene in vertebrates. The functional significance of alternative splicing events is unclear at present. Alternate spliced isoforms may provide various targets for different upstream and downstream interacting factors in sexual differentiation. Few targets that are regulated by DM-factors have been identified, for example, yolk protein genes in *Drosophila* and *Caenorhabditis elgans*, which is regulated by DSX and MAB-3 respectively (Yi and Zarkower, 1999; Yi *et al.*, 2000). The alternate spliced forms of *dmrt1*b or *dmrt1*c may regulate the activity of *dmrt1*a which is dominant. Though the isoform *dmrt1*c is lacking most of the DM domain region, presence of other regions (SY domain)

may prevent the DNA binding of dominant isoform especially *dmrt1*a, thus acting as a negative regulator. Further identification of new targets associated with *dmrt1* will provide more information on how these spliced forms operate and regulate each other.

Tissue distribution pattern indicated exclusive expression of *dmrt1*a in testis. This was also true when analyzed for Dmrt1 protein by Western blot. Semi-quantitative RT-PCR analysis of multiple *dmrt1*s further confirmed sexual dimorphism. Histological studies from our laboratory revealed that the gonadal sex differentiation in catfish starts around 40-50 dph. Based on this, we analyzed multiple *dmrt1*s expression pattern in developing gonads between 50 to 150 dph. Multiple forms of *dmrt1* exist in developing testis but not in developing ovary of catfish. Male-specific expression of *dmrt1* was noticed in some species like human (Moniot *et al.*, 2000), chicken (Nanda *et al.*, 1999), frog (Osawa *et al.*, 2005) garden lizard (Sreenivasulu *et al.*, 2002.) and few fish species (Fernandino *et al.*, 2006; Xia *et al.*, 2007; Kobayashi *et al.*, 2008; Ijiri *et al.*, 2008). In our study, we observed male-specific expression of multiple *dmrt1*s in developing and adult catfish (see below). Taken together, these results indicate an important role for *dmrt1*s in early testicular development and recrudescence (see below). In contrast, reports from zebrafish (Guo *et al.*, 2005) and rice field eel (Huang *et al.*, 2005) showed the expression of *dmrt1* in both male and female gonads. Nevertheless, the role of *dmrt1* in ovarian differentiation has not been defined properly in vertebrates.

Fate of *dmrt1* after testicular differentiation i.e. in adult testis has not been studied in detail so far in any of the lower vertebrates. In the present study, we quantified multiple *dmrt1*s at different phases of testicular cycle of catfish. Our real-time PCR data showed that *dmrt1*a and b was expressed abundantly during preparatory and pre-spawning phases where spermatogonia and spermatocytes are dominant (Swapna *et al.*, 2006), when compared to other phases of catfish testicular cycle. This indicates that in catfish, expression of *dmrt1* is higher during the period of

spermatogenesis and its expression decreases gradually thereafter during spawning/spermiation and post-spawning phases. In rainbow trout (Marchand *et al.*, 2000) and pejerry (Fernandino *et al.*, 2006), *dmrt1* expression was found to be high throughout spermatogenesis but decreased at spermiation. Seasonal changes in *dmrt1* expression may contribute for the maintenance of testicular cycle. Further, immunocytochemical and immunofluorescence data revealed that Dmrt1 was localized in primary spermatogonia, secondary spermatogonia and spermatocytes, while spermatid/spermatozoa did not show any immunoreactivity. Similar expression profile of Dmrt1 was observed in zebrafish (Guo *et al.*, 2005), red-spotted grouper (Xia *et al.*, 2007). Localization pattern and up regulation of *dmrt1* expression during testicular recrudescence indicates plausible role in spermatogenesis but not in spermiation/spawning.

In conclusion, we identified multiple forms of *dmrt1* from adult catfish testis. Stage-dependent elevation of *dmrt1*s in juveniles authenticates pivotal role for *dmrt1* in testicular differentiation. It is localized in SG and SC but not in SP. High expression of *dmrt1* during testicular recrudescence indicates that it might have a plausible role in the entraining of testicular cycle.

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Chapter 3

Isolation of sox9 duplicates in catfish: localization, differential expression pattern during gonadal development and recrudescence, and hCG-induced up regulation of sox9 in testicular slices

Abstract

In vertebrates, *sox9* is a transcription factor that plays a crucial role in testicular development and chondrogenesis. Here, we report cloning of duplicated copies of *sox9* (*sox9a* and *sox9b*) from air-breathing catfish, *Clarias gariepinus* that undergoes annual reproductive cycle. Tissue distribution pattern showed differential expression of *sox9* duplicates, where in both forms were highly expressed in brain and gonads. Further, we observed dimorphic expression pattern of *sox9a* and *sox9b* in both adult and developing gonads using RT-PCR indicating that *sox9a* retained its function in testis while *sox9b* might have a new role to play in ovary. Changes in *sox9* mRNA levels using real-time PCR (qRT-PCR) during seasonal reproductive cycle revealed that *sox9a* transcript in testis was abundant during testicular recrudescence (during spermatogenesis) and its expression significantly decreased during spawning and post-spawning phases. Further, treatments of human chorionic gonadotropin and 11-ketotestosterone, *in vitro* up regulated *sox9a* mRNA levels in the testicular slices at 12 and 24 h time points suggesting that gonadotropins might stimulate *sox9* expression. These results suggest that *sox9* might have plausible role in the entrainment of testicular cycle. In contrast, during ovarian cycle, *sox9b* mRNA was gradually declined from preparatory to post-spawning phases. Immunohistochemical (IHC) data showed that in testis, *sox9* is detectable in Sertoli and spermatogonial cell types except spermatid/spermatozoa. In the ovary, it is localized in the ooplasm of primary and pre-vitellogenic oocytes. These results were further confirmed by whole-mount IHC and qRT-PCR.

Introduction

SOX (*Sry*-related high mobility group box) gene family encodes several transcription factors that possess a DNA-binding motif known as the high mobility group (HMG) domain (Sinclair *et al.*, 1990). The HMG domain of all SOX proteins contains 79- amino acid residue, which is highly conserved across different phyla. *SOX9* was first identified as the gene responsible for autosomal XY sex reversal associated with campomelic dysplasia, a human skeletal malformation syndrome (Foster *et al.*, 1994; Wagner *et al.*, 1994). *Sox9* is the direct target of the testicular sex-determining gene, *Sry* (Sekido and Lovell-Badge 2008). *Sox9* transcription is up regulated in presence of *Sry* in developing pre-Sertoli cells during male development (Kent *et al.*, 1996; Sekido *et al.*, 2004). However, *Sox9* over expression in the genital ridges resulted in normal male development in transgenic mice lacking *Sry* (Bishop *et al.*, 2000; Qin and Bishop, 2005). In mammals, birds, and turtle, expression of *sox9* is up regulated in the developing male genital ridges during testicular differentiation (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996; Moreno-Mendoza *et al.*, 1999). In mice, ectopic expression of *Sox9* in XX gonads resulted in the formation of testis (Vidal *et al.*, 2001). These results together suggest for a crucial role of *sox9* in testicular development of several vertebrates. In addition to this, *sox9* is also involved in the regulation of many developmental events such as notochord maintenance (Barrionuevo *et al.*, 2006), prostate development (Thomsen *et al.*, 2008) and cartilage formation in vertebrate species, including teleosts (Bi *et al.*, 1999; Chiang *et al.*, 2001; Yan *et al.*, 2002).

To date *sox*9 gene has been identified in several vertebrate species like mammals (Foster *et al.*, 1994; Wagner *et al.*, 1994; Kent *et al.*, 1996), birds (Morais da Silva *et al.*, 1996), reptiles (Choudhary *et al.*, 2000; Western *et al.*, 1999; Moreno-Mendoza *et al.*, 1999), amphibians (Takase *et al.*, 2000), and fishes (Chiang *et al.*, 2001; Yokoi *et al.*, 2002 and Takamatsu *et al.*,

1997). Two different forms of s*ox9* genes have been identified in teleosts like zebrafish, rice field eel, fugu and medaka (Chiang *et al.*, 2001; Zhou *et al.*, 2003; Koopman *et al.*, 2004; Klüver *et al.*, 2005). These two forms of s*ox9* genes were found to be orthologues of the tetrapod *Sox9* gene that arose during a whole genome duplication event (Amores *et al.*, 1998; Postlethwait *et al.*, 1998). In rainbow trout, s*ox9* was expressed in testis (Takamatsu *et al.*, 1997) while in medaka s*ox9* was expressed in ovary (Yokoi *et al.*, 2002). In zebrafish, s*ox9a* was expressed in testis, as in mammals, and s*ox9b* in ovary (Chiang *et al.*, 2001). In rice field eel, two s*ox9a* genes (s*ox9a1* and s*ox9a2*) were expressed in testis, ovary, and ova-testis (Zhou *et al.*, 2003). In medaka, s*ox9a2* was isolated, and found to express at highest level in adult testis (Nakamoto *et al.*, 2005). In common carp, s*ox9b* was expressed in testis (Du *et al.*, 2007). These results suggest that the expression of s*ox9* in gonads seems to be somewhat diversified in teleosts. Most of these studies were done during early gonadal development in daily or fortnight breeders like zebrafish, medaka and tilapia. In this regard, catfish is an annual breeder, which undergoes seasonal pattern of gonadal attenuation and recrudescence that may provide interesting highlights to understand the role of s*ox9* not only during development but also during recrudescence. In the present study, we cloned full-length cDNAs of s*ox9a* and s*ox9b* from catfish gonads and analyzed their expression pattern in different stages of gonadal development and recrudescence. Extensive analysis was also made to localize Sox9 protein for the first time in developing and adult gonads. We also studied the expression pattern of s*ox9* in human chorionic gonadotropin (hCG) and 11-ketotestosterone (11-KT) treated testicular slices at different time intervals by an *in vitro* culture.

Materials and Methods

Animals and Sampling

Breeding and rearing of catfish (*C. gariepinus*) at different age groups was already described in chapter 1. Adult male and female gonads at different phases of reproductive cycle were collected and stored at –80^{o}C for total RNA extraction. Juvenile catfish fries at different age groups (50, 75, 100 and 150 days post hatch [dph]) were dissected and gonads (testis and ovary) were removed using a fine forceps under stereozoom microscope. Gonads of similar age group and same sex were pooled (5 fishes per each sample) to get sufficient amount of total RNA. Oocytes at different maturation stages were collected from prespawning female catfish. Oocytes were separated under stereozoom microscope in F.O. solution (NaCl 113 mM, KCl 1 mM, $CaCl_2$ 2 mM, Hepes 5 mM, pH 7.5) in four different stages: stage I, perinucleolar/primary oocytes (< 50 µm); stage II, pre-vitellogenic oocytes (50-100 µm); stage III, vitellogenic oocytes (150-300 µm); and stage IV post-vitellogenic oocytes (300-800 µm). After separation, total RNA was immediately prepared, without any freezing step.

Cloning of full-length cDNA of s*ox9a* and s*ox9b* from catfish gonads

A set of degenerate primers (Table 1) were designed by aligning the available *sox9* nucleotide sequences of other species from NCBI GenBank data base. Partial cDNA fragments were amplified from both testis and ovary cDNA templates by RT-PCR and cloned as per the methodology mentioned in chapter 1. The two partial cDNA fragment obtained from testis and ovary were found to be two isoforms of *sox9* (*sox9a* and *sox9b*) that was confirmed using NCBI-BLAST search. Later on 5' and 3' cDNA ends of sox9 duplicates were isolated using rapid amplification of cDNA ends (RACE) strategy as described in chapter 1. Gene specific primers

(GSP) for 5' and 3' RACE were designed based on the sequence information of partial cDNA fragments of s*ox9a* and *sox9b* (Table 1). The nucleotide and deduced amino acid sequences of *sox9a* and *sox9b* cDNAs were verified using the NCBI-BLAST search.

Tissue distribution and expression pattern of sox9 duplicates by RT-PCR

Tissue distribution pattern of *sox9a* and *sox9b* in adult catfish performed using specific primers (Table 1) by RT-PCR as described in chapter 1. To study the expression patterns of s*ox9a* duplicates in gonads of adult and juvenile catfish at different age groups (50, 75, 100 and 150 dph) RT-PCR was carried out. PCR amplification was done using a thermal cycler (Applied Biosystems) under following conditions: 94°C (1 min), 60°C (30sec), 72°C (1min), for 30 cycles using *sox9a* and *sox9b* specific primers (Table 1).

Real-time quantitative RT-PCR (qRT-PCR)

Expression of *sox9a* and *sox9b* was analyzed by relative qRT-PCR using SYBR Green detection method as described in chapter 1. Real-time specific primers for *sox9a*, *sox9b* and β-*actin* (Table 1) were designed using Primer Express software (Applied Biosystems). The *sox9* expression was normalized against β-*actin* expression, generating a ΔCt value (ΔCt = *sox9* Ct – β-*actin* Ct). Relative expression was then calculated according to the equation $2^{-\Delta Ct}$.

Primer Name	Nucleotide sequence (5′– 3′)	Usage
sox9dgFr	GC(AC)GA(GA)(CT)T(GC)AGCAAAAC(ATC)C(TG)G	Degenerate RT-PCR
sox9dgRv	CTGGA(TA)GC(TG)GG(CGA)A(AG)TCGG	Degenerate RT-PCR
sox9a5'GSP1	ACAAATGGCCGCTTCTCCCCTTC	5'RACE
sox9b5'GSP1	TGCGTGTGCTCGGGACCCTCTTCC	5'RACE
sox9a5'GSP2	AGCCGCCAGAGTTTGCCCCG	5'RACE
sox9b5'GSP2	TCCTTCGCCGTGGCTGGTACT	5'RACE
sox9a3'GSP1	AAGGGCGGCCAGGGTGAAACCG	3'RACE
sox9b3'GSP1	GCATCAGCCGGCCTCAGTACG	3'RACE
sox9a3'GSP2	AGCCCTACAGGGAGCCGACTC	3'RACE
sox9b3'GSP2	GAGTTCTAGCCAGAGGCCCATG	3'RACE
sox9aF	ACCCAGAGGCCCATGTACAC	Tissue distribution
sox9aR	CTGGTGGAGGGAGTGGAC	Tissue distribution
sox9bF	CGCAGTTACCGTTGGCTCTG	Tissue distribution
sox9bR	GCTGCAGGTTGAAGGAGCCA	Tissue distribution
β-*actinRTF*	GCAGAGCTCAGCAAAACCCGG	qRT-PCR
β-*actinRTR*	GCTGGAAGCGGGAGAGTCGG	qRT-PCR
sox9aRTF	TCTGGCGGCTGCTGAATGAAGG	qRT-PCR
sox9aRTR	CTCGGTATCCTCGGTTTCACC	qRT-PCR
sox9bRTF	GAGACCCAGTCAGGCCACAG	qRT-PCR
sox9bRTR	AGGGTCTCGATGTGGGCCA	qRT-PCR

Table1. List of primers used for cloning and expression analysis of sox9a and sox9b

In vitro culture of testicular slices in L-15 medium

Testes from catfish in preparatory phase were dissected under sterile conditions and kept in ice-cold Leibovitz (L15) culture medium (Sigma). Testicular slices of 20 μm thickness were prepared using McIlween tissue chopper (vibrotome). Testicular slices were individually transferred to tissue culture plates containing 2 ml of L-15 medium supplemented with 10 mM

Hepes and antibiotic (penicillin, 100 IU/ml, streptomycin, 0.1 mg/ml). Testicular slices were cultured for 24 h at 20-22° C in the presence 100 IU/ml of hCG or saline. At each time interval of 0, 6, 12 and 24 h slices were collected from both hCG- and saline-treated, washed with ice-cold phosphate buffer (pH 7.4) and total RNA was prepared using sigma TRI-reagent. The expression of s*ox9a* was quantified for each time interval using qRT-PCR. Likewise, the effect of 11-ketotestosterone (11-KT, Wako pure chemical industries ltd, Japan) on *sox9a* expression was also examined by treating the testicular slices with 100 ng/ml of 11-KT at different time points. For this purpose, 11-KT was first dissolved in absolute ethanol and later reconstituted in the L15 culture medium. The expression of s*ox9a* was then analyzed using qRT-PCR.

Western blot analysis

Western blot analysis was carried out to detect Sox9 protein in catfish gonadal tissue (testis and ovary) homogenates using polyclonal Sox9 antibody (LifeSpan Biosciences, Seattle, WA) raised against N-terminal region (1-150 amino acids) of human Sox9 protein that showed 88.5% and 78% homology with the N-terminal region of catfish Sox9a and Sox9b proteins, respectively. The procedure for performing Western blot was described in chapter 2.

Immunohistochemistry (IHC)

Both adult and juvenile gonads were fixed in 4% paraformaldehyde (PFA), processed and embedded in paraplast (Sigma). Sections were cut at 4 µm thickness using microtome (Leica, Germany). IHC was performed as described in chapter 2 using the human SOX9 polyclonal antibody or anti-Sox9 pre-absorbed with excess of synthetic peptide for overnight. Photomicrographs were taken using Olympus CX41 bright field microscope (Olympus, Japan).

Whole mount IHC

Whole mount IHC was performed according to the procedure reported earlier (Wang *et al.*, 2006) with few modifications for the detection of Sox9 protein in the ovary of juvenile and mature catfish. Gonads were fixed overnight at 4^{o}C in 4% PFA and dehydrated through a methanol series in PBS and kept in -80^{o}C for few a days. Samples were rehydrated through a reverse methanol series, and blocked with PBMT (3% skimmed milk powder, 0.1% Triton X-100/PBS) for overnight at 4^{o}C. Samples were then incubated overnight at 4^{o}C in PBMT containing 2% BSA and 1:200 dilution of polyclonal Sox9 antibody followed by 15 min washes in PBST (0.1% Triton X-100/PBS) for 5 times at 4^{o}C. As a negative control, Sox9 antibody pre-incubated with excess of Sox9 synthetic peptide overnight at 4^{o}C was used as primary antibody. Primary antibody was detected with 1:500 dilution of alkaline phosphatase conjugated anti-rabbit secondary antibody (Vector Laboratories) in PBMT overnight at 4^{o}C followed by PBST washes. For enzyme detection, samples were incubated with BCIP/NBT in NTMT buffer (0.1M NaCl, 0.1M Tris-HCL pH 9.5, 50mM $MgCl_2$ and 0.1% Tween20) for 15-30min. After optimal color development, the reaction was stopped by a PBST wash.

Statistical analysis

All the results were expressed as mean ± SEM. Differences between groups were analyzed for statistical significance by One-Way ANOVA followed by Students-Newman-Keuls (SNK) post hoc test using SigmaPlot 9.0 software. A probability of $P < 0.05$ is considered statistically significant.

Results

Cloning of s*ox9a* and s*ox9b* from catfish gonads

A distinct partial cDNA fragment of *sox9* (269 bp) was isolated from catfish testis using RT-PCR with degenerate primers (Fig. 1A). The 5' and 3' ends of these partial cDNA fragments were amplified using RACE PCR (Fig. 1B and C). The full-length catfish *sox9a* cDNA isolated (2065 bp) from testis had an ORF of 1383 bp encoding a putative protein of 460 amino acids residues. Catfish *sox9b* cDNA (2059 bp) obtained from ovary had an ORF of 1377 bp encoding a putative protein of 458 amino acids residues. ClustalW alignment of these two duplicates, *sox9a* and *sox9b* from catfish showed 62% homology between them at the amino acid level (Fig. 2). Multiple sequence alignment reveals that the HMG box domain is highly conserved, with more than 96% sequence identity with other vertebrates. Sequence homology among *sox9* from different species other than the HMG domain showed an overall 70–85% sequence identity among all other known vertebrate SOX9 proteins. Phylogenetic analysis using neighbor-joining method revealed that the catfish *sox9a* was closely related to rainbow trout, while catfish *sox9b* was clustered with *sox9b* of carp and zebrafish (Fig. 3). The nucleotide sequence data of *sox9a* and *sox9b* have been submitted to the GenBank. The accession number of catfish *sox9a* is HM149258 and *sox9b* is HM149259

Tissue distribution pattern of *sox9* duplicates by RT-PCR

Tissue distribution analysis by RT-PCR showed that the expression of *sox9a* (Fig. 4A) was confined to few tissues like brain, heart, gill, liver, kidney and testis. On the other hand, *sox9b* transcript (Fig. 4A) was detectable in brain, heart, liver, ovary and spleen. However in both cases

sox9 was abundantly expressed in brain and gonads. Interestingly, *sox9a* and *sox9b* depicted sexual dimorphism in gonads.

Dimorphic expression pattern of *sox9a* and s*ox9b* in adult and developing gonads

Further analysis of sexual dimorphism of *sox9* by semi-quantitative RT-PCR endorsed that *sox9a* was detectable in adult testis while *sox9b* was expressed in adult ovary (Fig. 4B). This kind of expression pattern of *sox9* duplicates was observed in developing gonads at different age groups (50, 75, 100 and 150 dph) in catfish, where in *sox9a* expression was seen in developing testis but not in developing ovary and the reverse is true for *sox9b* (Fig. 4C).

Expression of *sox9* in different phases of testicular and ovarian cycles

qRT-PCR analysis revealed that *sox9a* expression was significantly high during preparatory and pre-spawning phases when compared to spawning and post-spawning phases of testicular cycle (Fig. 5A). This indicates that *sox9a* transcripts are abundantly expressed during active spermatogenesis i.e. in preparatory and pre-spawning phases where in spermatogonia and spermatocytes are more in number (Swapna *et al.*, 2006). While s*ox9b* transcript was abundantly expressed during preparatory phase and later its expression was drastically reduced in pre-spawning phases, spawning and post-spawning phases of ovarian cycle (Fig. 5B).

Effect of hCG treatment, *in vitro* on s*ox9a* expression in the testicular slices

Expression of s*ox9a* was analyzed in testicular slices (late-preparatory phase of mature fish) that were collected at different time intervals after the treatment of hCG, *in vitro* using qRT-PCR.

Expression of *sox9a* was significantly increased at 12 h (*, $P < 0.05$) and peaked at 24 h (**, $P < 0.001$) after hCG treatment (Fig. 6).

Effect of 11-KT treatment, *in vitro* on *sox9a* expression in the testicular slices

Analysis of the effect of 11-KT treatment on *sox9a* expression in testicular slices (late preparatory phase of mature fish) at different time points using qRT-PCR revealed a significant rise at 12 h (*, $P < 0.05$) and 24 h (**, $P < 0.001$) when compared with 0 h (Fig. 7).

Expression pattern of *sox9b* in different stages of follicles

We collected four different stages of follicles (Fig. 8A) from catfish ovary during pre-spawning period: Stage I (perinucleolar/primary oocytes), stage II (pre-vitellogenic oocytes), stage III (vitellogenic oocytes) and stage IV (post vitellogenic oocytes). qRT-PCR was performed to quantify the *sox9b* transcript abundance in these four stages of oocytes. The real-time PCR results (Fig. 8B) showed that the transcript was abundantly expressed in stage I oocytes, moderately low in Stage II oocytes (*, $P < 0.05$) and its expression was drastically reduced in stage III and IV oocytes (**, $P < 0.001$).

Localization of Sox9 in developing and adult gonads of catfish

Western blot analysis revealed an expected band of ~ 51 kDa in testis and ovarian tissue homogenates of catfish. This band corresponds to the Sox9 protein, which encodes a 51 kDa putative protein (Fig. 9). Absence of immunoreactivity using the antibody pre-absorbed with Sox9 synthetic peptide confirms that the primary antibody is indeed specific to Sox9 protein in catfish (Fig. 10A). IHC analysis in the preparatory phase of adult testis revealed that Sox9

immunoreactivity was observed in Sertoli and spermatogonial cell types such as primary spermatogonia (SG1), secondary spermatogonia (SG2) and spermatocytes (SC) except spermatid/spermatozoa (SP; Fig. 10 B, C and D). In the case of adult ovary, the sox9 immunoreactivity was restricted to ooplasm of stage I and stage II oocytes (Fig. 10E). This was further confirmed by the whole mount IHC in different follicular stages (oocytes) where in the signal was confined to stage I and stage II oocytes (Fig. 10F and G). No signal was detectable in stage III and stage IV oocytes (Fig. 10G). No immunoreactivity was observed with pre-absorbed antibody was used as negative control (Fig. 10H). During critical period of gonadal differentiation in catfish, Sox9 protein was detectable in germ cells of undifferentiated gonad at 40 dph (Fig. 11A). The Sox9 immunoreactivity was prominent in the primary oocytes (stage I) and oogonia of differentiated ovary at 50 and in stage I and II oocytes of juvenile ovary at 100 dph (Fig. 11B and C). Where as in developing testis of juvenile catfish at 100 dph it was observed in the Sertoli and germ cells present in cysts (Fig. 11D).

Figures

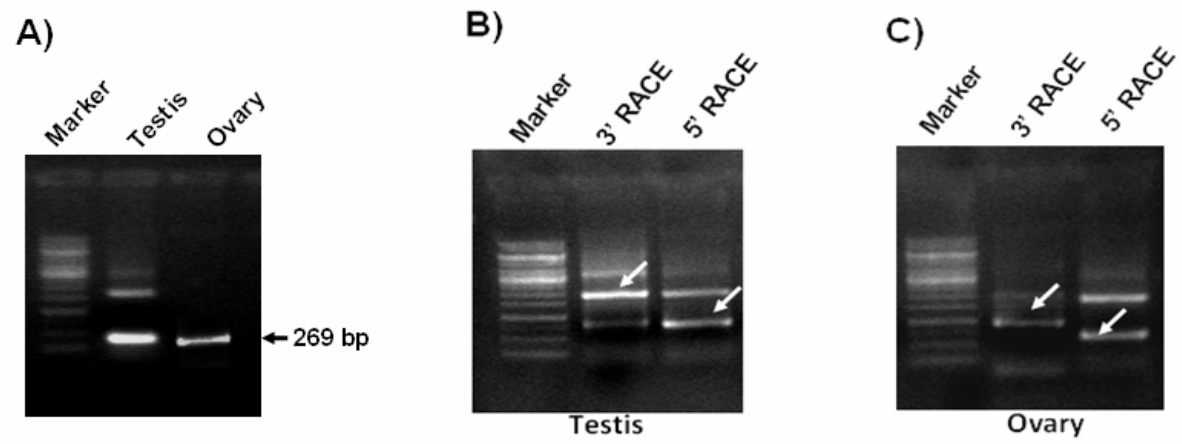

Fig.1. 1% Agarose gels showing RT-PCR products. A) Partial cDNA fragment of sox9 from catfish ovary. B) and C) 5' and 3' RACE products (indicated by white arrows) using gene specific primers to obtain full-length cDNA of sox9 from testis and ovary.

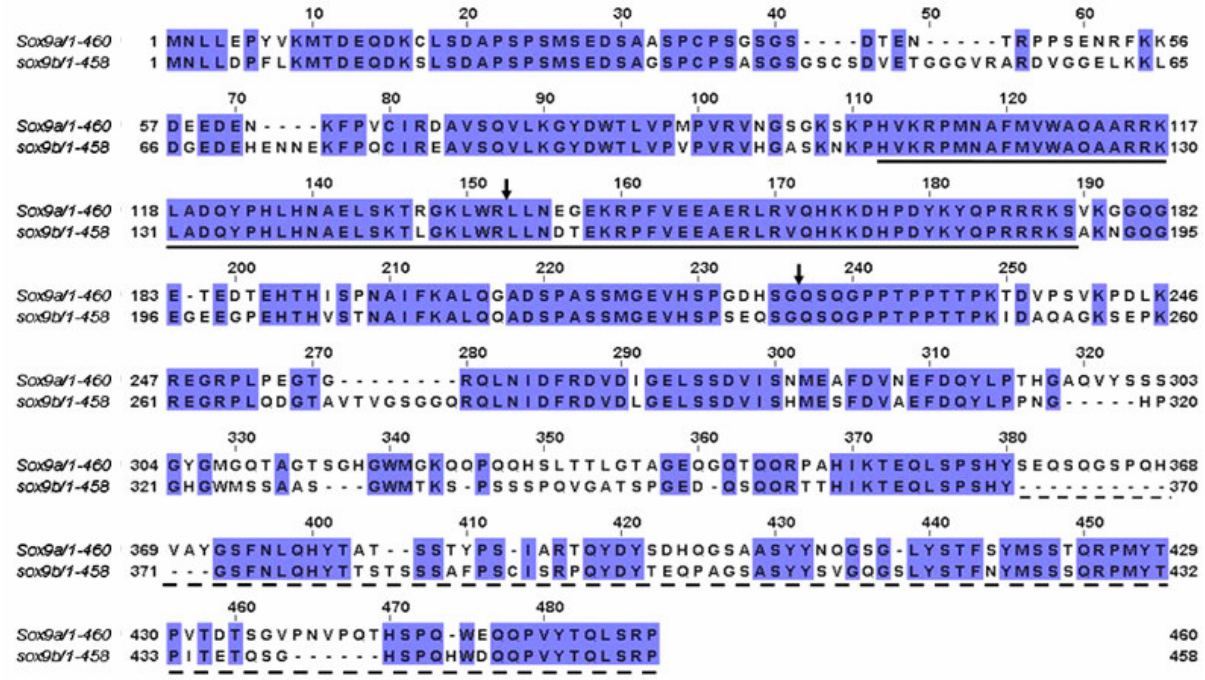

Fig. 2. ClustalW alignment of deduced amino acid sequences of catfish sox9a and sox9b. This alignment was created using the ClustalW alignment software version 1.82. Arrows indicate intron positions. Solid lines indicate conserved HMG box region, dotted lines indicate transactivation domain.

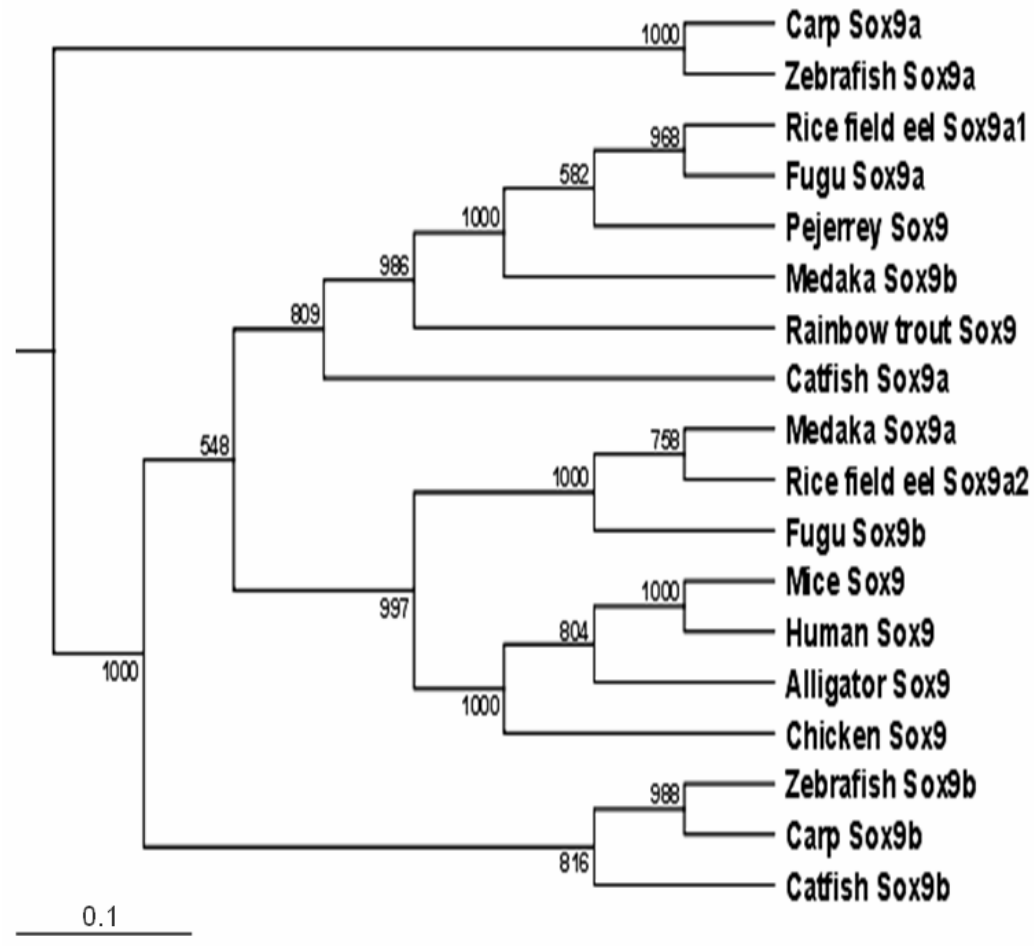

Fig. 3. Phylogenetic tree showing the evolutionary status of catfish sox9a and sox9b. The tree was constructed using the neighbor-joining method and a bootstrap analysis with 1000 replicates was used to assess the strength of nodes in the tree. Phylogenetic analysis was done using CLC Main workbench 5 software. GenBank accession numbers: human (CAA86598), mice (NP_035578), chicken (AAB09663), alligator (AAD17974), rainbow trout (BAA24365), pejerrey (AAP84605), zebrafish sox9a (AF277096), carp sox9a (AAZ07990), fugu sox9a (AAQ18507), medaka sox9a (AAX62152), rice field eel sox9a1 (AAK59254), zebrafish sox9b (AF277097), carp sox9b (AAX56088), fugu sox9b (AAQ18508), medaka sox9b (AAX62151), rice field eel sox9a2 (AAK59255).

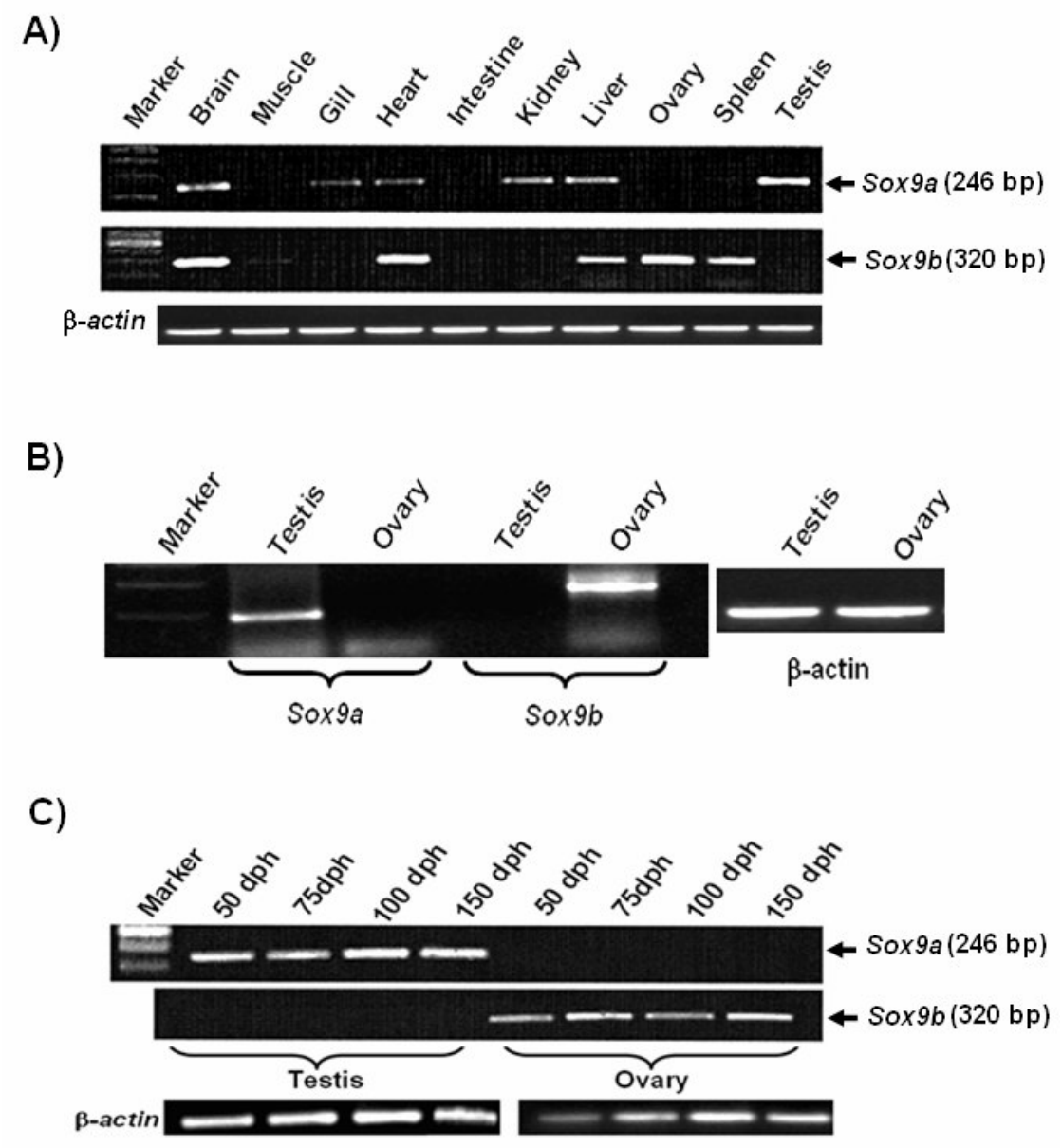

Fig. 4 A) RT-PCR analysis of sox9a and sox9b in different tissues of adult catfish. B) Semi-quantitative RT-PCR analysis (n=3) of sox9 duplicates in adult testis and ovary. C) Semi-quantitative RT-PCR (n=3) amplification of sox9a and sox9b in developing gonads at different age groups of catfish (50, 75, 100 and 150 days post hatching). β-actin was used as an internal endogenous control.

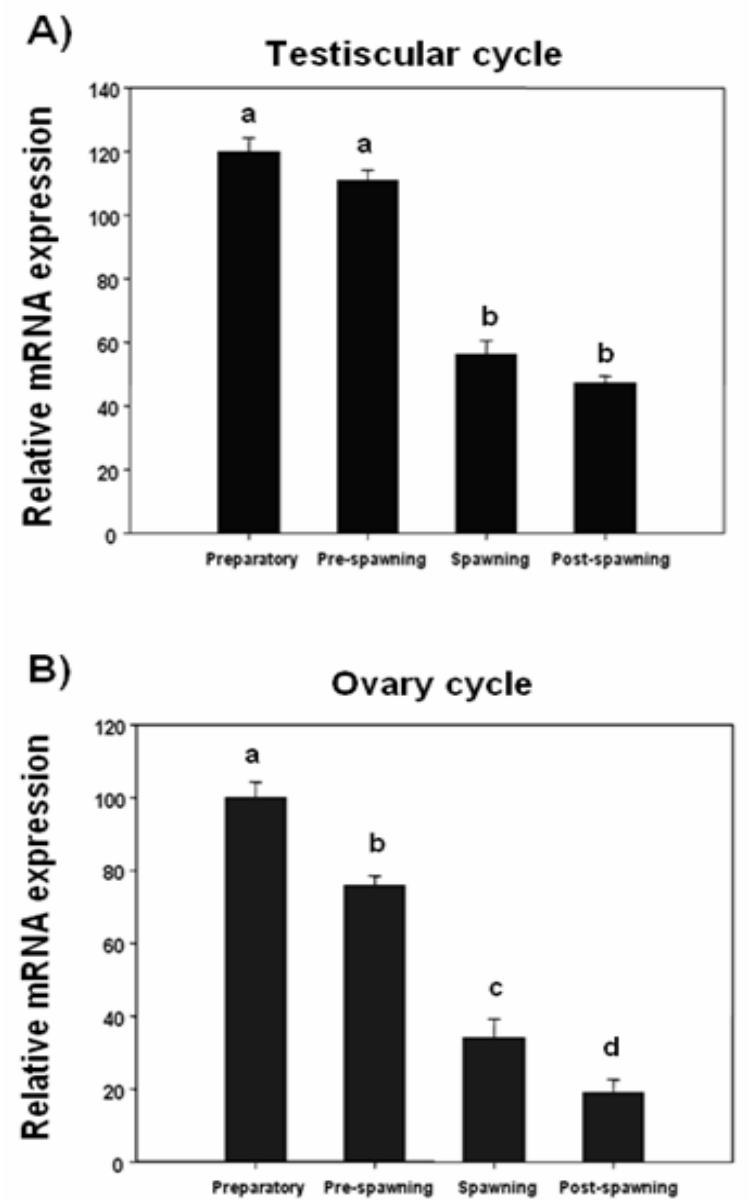

Fig. 5 A) qRT-PCR analysis of sox9a expression during different phases of testicular cycle in catfish. The relative expression of sox9a normalized with β-actin in samples was calculated using comparative Ct method. Data for real-time PCR were expressed as mean ± SEM (n=5). Different letters on the bars indicate significant increase in the sox9 mRNA levels in preparatory and pre-spawning phases from that of spawning and post-spawning phases ($P < 0.05$, ANOVA followed by SNK test). Same letters on the bars indicates the differences were not significant. B) qRT-PCR analysis of sox9b expression during different phases of ovarian cycle in catfish. Other details are as in Fig. 4A.

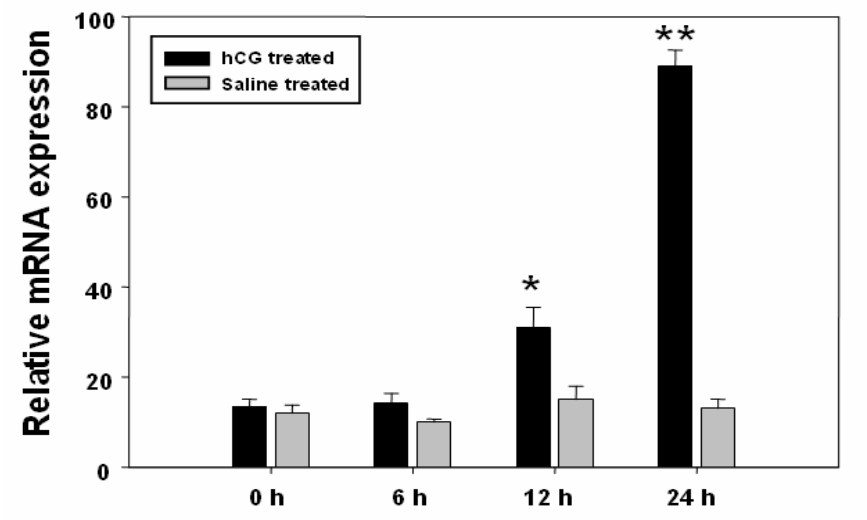

Fig. 6 qRT-PCR analysis showing sox9a mRNA levels in the testicular slices treatment with hCG, in vitro at different time intervals. sox9a mRNA levels were significantly increased after hCG induction, in vitro at 12 and 24 h time points compared to 0 h time point. *, P < 0.05; **, P < 0.001 (ANOVA followed by SNK test, n=3).

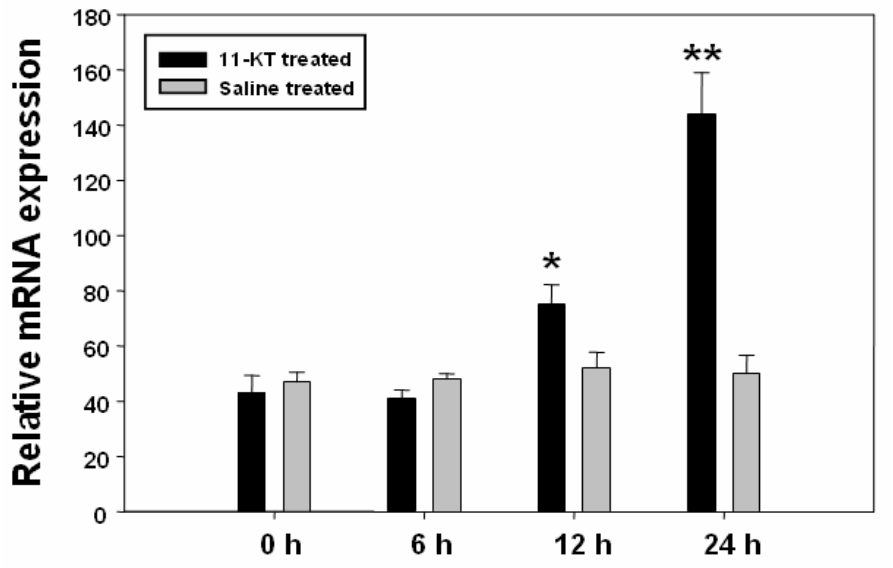

Fig. 7 qRT-PCR analysis showing sox9a mRNA levels in the testicular slices treatment with 11-KT (100 ng/ml), in vitro at different time intervals. sox9a mRNA levels were significantly increased after hCG induction, in vitro at 12 and 24 h time points compared to 0 h time point. *, P < 0.05; **, P < 0.001 (ANOVA followed by SNK test, n=3).

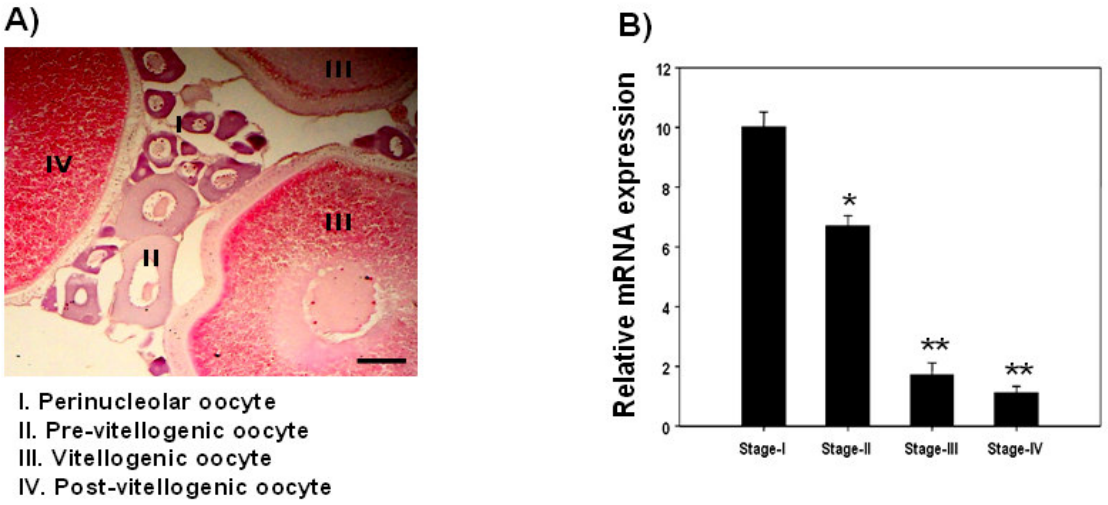

Fig. 8 A) Hematoxylin-Eosin stained section of mature catfish ovary showing different stages of oocytes. Scale bar represent 50 µm. B) qRT-PCR analysis showing the expression pattern of sox9b in different stages of oocytes. The relative expression of sox9b in different stages was calculated using comparative Ct method. Sox9b mRNA levels were significantly lower in stage II, III and IV oocytes, when compared with stage I oocytes. *, P < 0.05; **, P < 0.001 (ANOVA followed by SNK test, n=5).

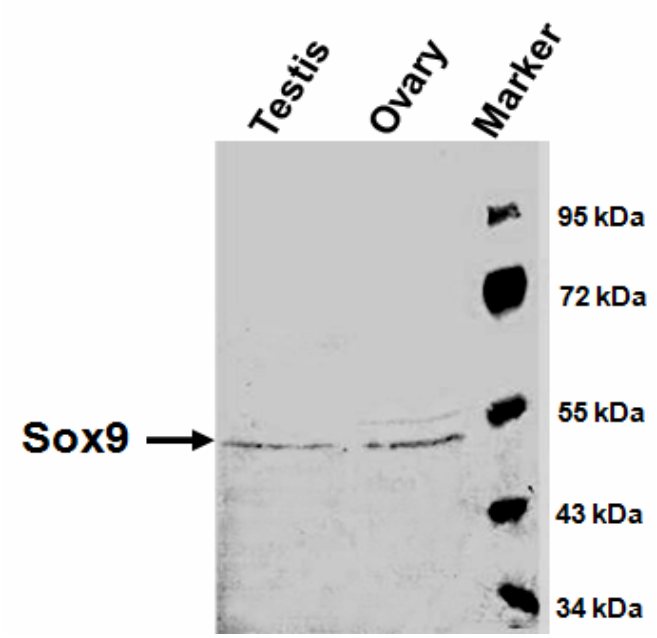

Fig. 9 Western blot showing Sox9 protein band (arrow) of molecular weight approximately 52 kDa detectable in testis and ovary of adult catfish, which is consistent with the size, calculated using the LaserGene software (51.6 kDa) from the deduced amino acid sequence of Sox9.

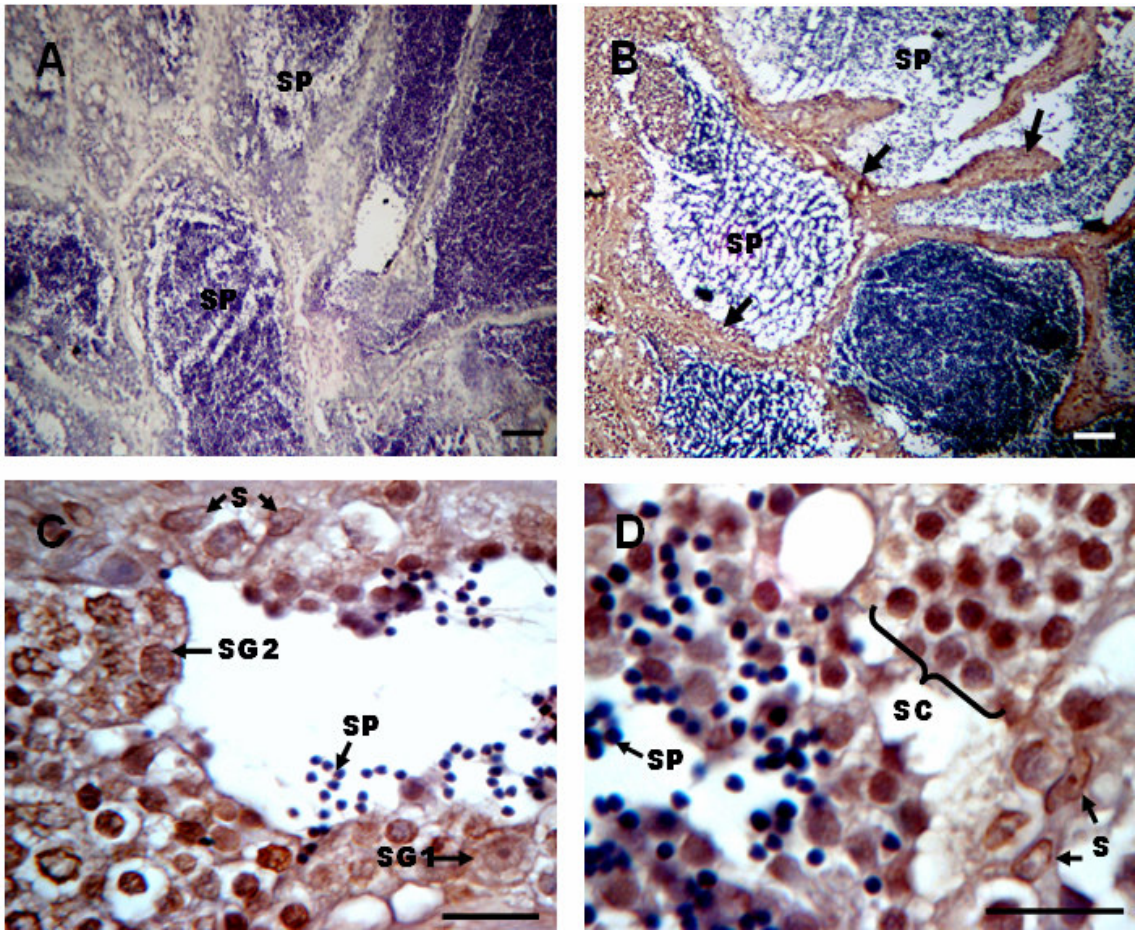

Fig. 10 Immunocytochemical (IHC) localization of Sox9 protein in adult testis using anti-human Sox9 antibody. A) Negative control showing Sox9 immunoreactivity was abolished when pre-absorbed antibody used as primary antibody. B) Adult testis section showing Sox9 immunoreactivity in boundaries of lumens filled with spermatids/sperm (SP); Arrow heads showing positive immunoreactivity. C) & D) Adult testis section at higher magnification showing Sox9 signal was detectable in Sertoli and spermatogonial cell types except spermatid/sperm (SP). Arrows indicate the position of the cell types: Sertoli cells (S), primary spermatogonia (SG1), secondary spermatogonia (SG2), spermatocytes (SC) and SP. Counter stained with hematoxylin. All scale bars represent 50 μm.

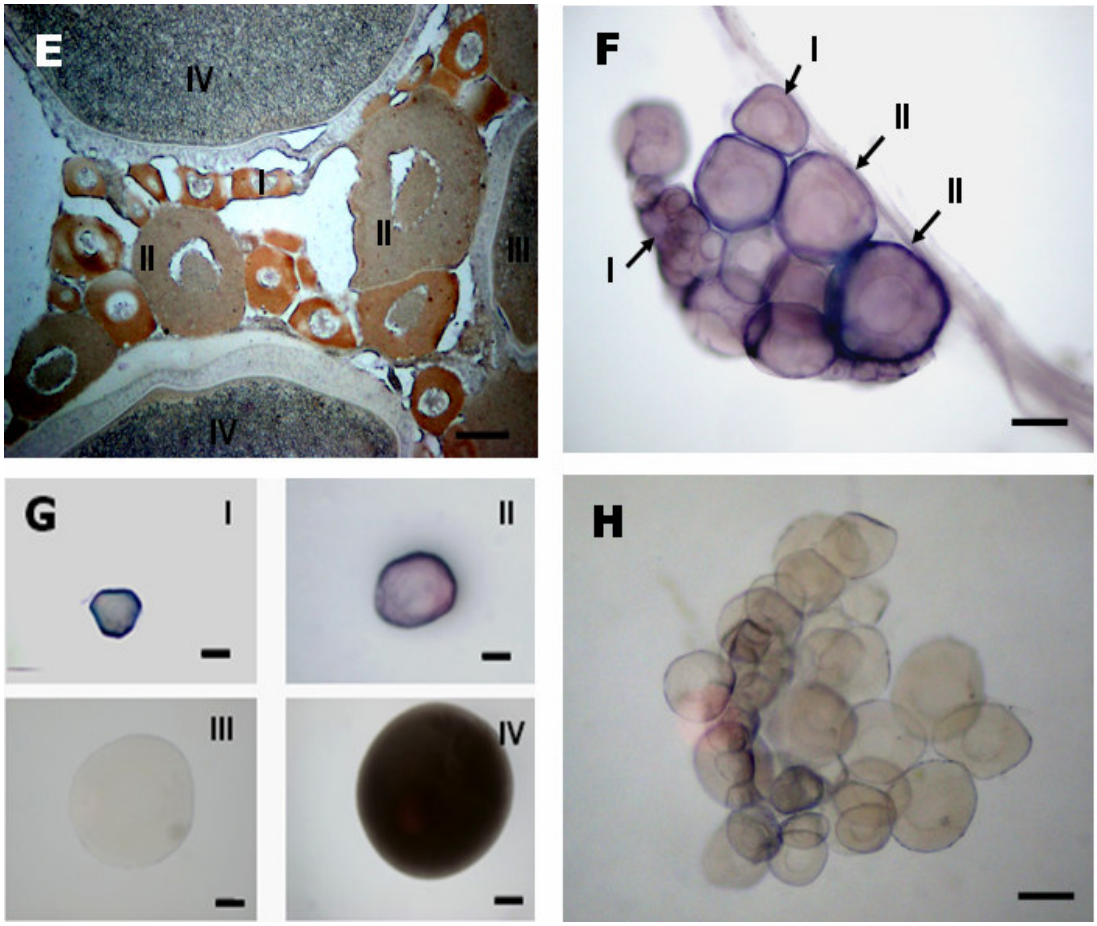

Fig. 10 E) IHC detection of Sox9 in different oocytes of mature female. Sox9 protein was detectable only in stage I (primary oocytes) and stage II (pre-vtellogenic oocytes) oocytes of mature ovary using IHC. There was no signal in stage III and stage IV oocytes. F) & G) Whole-mount IHC localization of Sox9 protein in different stages of oocytes. H) Negative control showing no signal when pre-absorbed Sox9 antibody was used as primary antibody. All scale bars represent 50 μm.

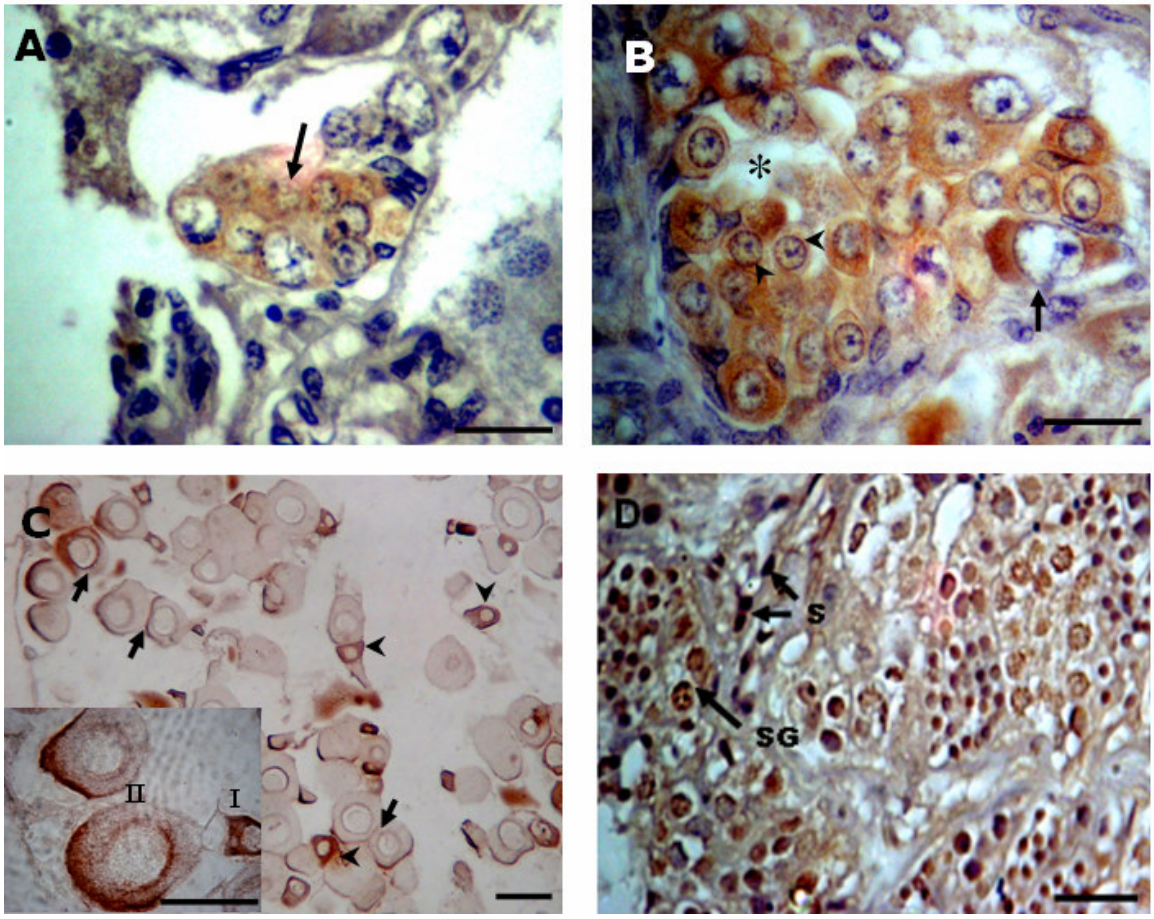

Fig. 11. A) Sox9 signal in germ cells (shown by arrow) of undifferentiated gonad at 40 dph. B) Differentiated ovary at 50 dph. Arrows indicating primary oocyte; arrowheads showing oogonial cells, * indicates ovarian cavity. C) Developing ovary at 100 dph. Arrows showing primary oocytes and arrowheads indicate pre-vitellogenic oocytes. Inlet of figure 9C showing immunoreactivity in stage I and stage II oocytes at higher magnification. D) Immature testis at 100 dph. Counter stained with hematoxylin. All scale bars represent 50 μm.

Discussion

In the present study, we report cloning of two duplicate orthologs of *sox9*, named as s*ox9a* and *sox9b*. Both the isoforms contain the *Sox* gene signature domain HMG, which is highly conserved across different vertebrates. Recent studies in teleosts like zebrafish, medaka, ricefield eel and carp also reported two forms of s*ox9* (Chiang *et al.*, 2001; Zhou *et al.*, 2003; Klüver *et al.*, 2005; Du *et al.*, 2007). Phylogenetic analysis using neighbor-joining method revealed that the isolated catfish *sox9* duplicates showed high homology to its counterpart with yellow catfish and zebrafish. Previously, Klüver *et al.*, (2005) reported in medaka that the two *sox9* genes are co-orthologs of the single tetrapod *Sox9*. This was reliable with whole genome duplication in the ray-fin lineage (Amores *et al.*, 1998; Postlethwait *et al.*, 2004) that might have promoted lineage divergence by partitioning different ancestral gene subfunctions among co-orthologs of tetrapod genes (Lynch and Force 2000; Cresko *et al.*, 2003). Zhou *et al.*, (2003) also reported that the s*ox9* gene was duplicated during evolution of rice field eel. The duplicated copies of the s*ox9* gene showed similar kind of expression pattern in testis, ovary and ova-testis. Tissue distribution analysis in catfish showed differential expression pattern of *sox9a* and *sox9b*. In catfish both the isoforms are expressed abundantly in brain and gonads. Semi-quantitative RT-PCR analysis in adult gonads revealed sexual dimorphic expression of *sox9a* and *sox9b* in adult and developing gonads, where in s*ox9a* was preferably expressed in testis, while *sox9b* was abundantly expressed in ovary. This type of differential expression pattern of duplicated genes may attribute to more flexible gene regulation and function during catfish development. Similar kind of expression pattern was also noticed in zebrafish using PCR Southern and *in situ* hybridization (Chiang *et al.*, 2001). Sexual dimorphic expression pattern of *sox9a* was observed 25 days after hatching in monosex population of Nile tilapia (Kobayashi *et al.*, 2008). In carp, s*ox9b* expressed

preferably in ovary (Yan *et al.*, 2007). This kind of dimorphic expression pattern of s*ox9* duplicates in fish gonads indicates that *sox9a* retained its function as testis specific gene while s*ox9b* might acquire a new role to play in ovarian development. Male specific expression pattern of s*ox9a* was also observed in fresh water and marine turtles suggesting its role in testicular differentiation (Moreno-Mendoza *et al.*, 1999; Spotila *et al.*, 1998). In contrast, there are a few reports where in s*ox9* duplicates did not show any dimorphic expression in the gonads of medaka and eel (Zhou *et al.*, 2003; Klüver *et al.*, 2005).

In mammals and birds, s*ox9* expression was down regulated during ovarian differentiation, but its expression was high in developing testis and remains throughout adulthood (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996). As *sox9* expression still persists after gonadal development its role during adulthood in teleosts is yet to be defined. Fate of *sox9* in different phases of adult gonads during reproductive cycle has not been studied in detail so far in any teleosts. To this end, we quantified s*ox9* transcript abundance in different stages of testicular and ovarian cycle. Real-time PCR analysis of s*ox9a* during testicular cycle showed that the transcript was higher during the period of spermatogenesis i.e. preparatory and pre-spawning phases and its expression decreased gradually thereafter during spermiation and post-spawning phases. This high expression during spermatogenesis is due to more number of germ cells expressing *sox9*. In contrast, during ovarian cycle, expression of *sox9b* transcript was abundant in preparatory phase when compared to pre-spawning phase, spawning and post-spawning phases. Recent report showed that gonadotropin-releasing hormone analogue treatment (*in vitro* and *in vivo*) up regulates *sox3* and *dmrt1* mRNA expression in immature black porgy (Shin *et al.*, 2009). In another study, it was observed that follicle-stimulating hormone could regulate *dmrt1* expression in postnatal rat testis (Chen & Heckert 2001). In light of this finding, role of gonadotropins, if

any, on the regulation of *sox9* mRNA expression is not known in lower vertebrates. Hence, we studied the expression of *sox9* after hCG induction specifically during testicular recrudescence as *sox9* expression tends to be at higher levels in preparatory and prespawning phases. We observed a significant increase in s*ox9a* mRNA in the testicular slices (prepared from testis in late-preparatory phase) upon treatment with hCG, *in vitro* at 12 and 24 h time points. This result tends to propose a role for gonadotropins in *sox9* regulation, at least in testis. In this regard, luteinizing hormone (LH)-like hCG is known to bind to LH receptors, which is expressed by Leydig cells to stimulate androgen production and acts on Sertoli cells to influence spermatogenesis (Skinner, 1991). In fish it is well known that hCG (functionally similar to LH), *in vitro* induces testosterone and 11-KT production (Wade and Van der Kraak, 1991). Hence, it is possible that hCG might have induced *sox9a* via androgen production. To test this contention, we also studied the effect of 11-KT on *sox9a* expression in testicular slices (*in vitro* culture). In this study, for the first time we demonstrated that the treatment of 11-KT significantly elevated the expression of *sox9a* in testicular slices. This may be one of the modes of action for gonadotropins. Although the androgen receptor is present in Sertoli cells, but the specific mechanism of how androgens act on Sertoli cells remains to be elucidated. Further, there are reports which clearly show that gonadotropins like FSH can directly act on Sertoli cells and regulate the expression of other genes by activating other transcription factors like cAMP response element binding protein (CREB), cAMP response element modulator (CREM) (Sassone-Corsi, 1995). It has been reported that FSH can regulate the expression of *Dmrt1* in Sertoli cells via cAMP-PKA pathway by activating CREB or CREM (Chen & Heckert 2001). In humans, it has been shown that SOX9 promoter is regulated by CREB (Piera-Velazquez *et al.*,

2007). These results altogether suggest that gonadotropins can regulate *sox9* expression in Sertoli cells either directly or indirectly.

IHC data in male gonad revealed that Sox9 was localized in Sertoli cells and different stages of germ cells, while spermatid/spermatozoa did not show any signal. Similar kind of localization pattern in germ cells was noticed for Dmrt1 in zebrafish, red-spotted grouper and mice (Guo *et al.*, 2005; Xia *et al.*, 2007) and Sox3 in grouper (Xia *et al.*, 2007) that are related to testicular development as that of Sox9. This contention requires more validation. Nevertheless, we used highly specific antibody that detects only Sox9 protein in Sertoli cells of human testis (as per antibody characterization data from the manufacturer). Hence, the observation of Sox9 protein in Sertoli and germ cells in catfish is acceptable technically and we presume the detection of Sox9 may be a feature observed in siluriforms as that of Dmrt1. However, in zebrafish the s*ox9* expression was confined only to Sertoli cells of testis (Chiang *et al.*, 2001). Presence of Sox9 protein in oogonia during ovarian differentiation is shown for the first time in any teleosts. This further validates that Sox9 protein is also detectable in germ cells. However, the difference in subcellular localization of male and female germ cells of the protein recognized by the anti-sox9 antibody is quite striking as it is found in the nucleus of male germ cells while there is little to no signal in the nucleus of developing oogonia. The apparent reason for low detection of this transcription factor (Sox9 protein) in the nucleus of female germ cell is not clear at the moment. In accordance to our findings, *sox9* transcripts were shown to be detectable mostly in the cytosol of immature oocytes of zebrafish by *in situ* hybridization (Chiang *et al.*, 2001). In mature female catfish, *sox9* expression was restricted to perinucleolar stage of developing oocytes and pre-vitellogenic oocytes, but not in mature oocytes. In the whole mount IHC, Sox9 signal was more in the periphery with less intensity in ooplasm. On the other hand, in the cross section of

mature ovary the immunoreactivity was more clearly evident in ooplasm. The decrease in *sox9* transcript in mature oocytes may be due to the dilution of transcripts with increasing mass/size of mature oocytes that moreover accumulate large amount of vitellogenin and maternal RNAs. These results together indicate that s*ox9* may have an important role in early gonadal development and recrudescence.

In conclusion, we identified/isolated *sox9* duplicates (s*ox9a* and *sox9b*) in catfish gonads. Dimorphic expression pattern of *sox9* duplicates in developing gonads authenticates an important role for the correlates in gonadal development. Sox9 protein could be localized in Sertoli and germ cells of catfish testis. Its expression was prominent in immature oocytes while it is negligible in mature oocytes. High expression of s*ox9* during testicular recrudescence indicates its importance in the entraining of testicular cycle in teleosts, which may be regulated by gonadotropins.

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Chapter 4

Gender differences in tryptophan hydroxylase mRNA, serotonin and 5-hydroxytryptophan levels in brain during sex differentiation of catfish: Plausible role of tryptophan hydroxylase in early male brain development through serotonergic system

Abstract

Tryptophan hydroxylase (*tph*) is the key regulator in serotonin (5-HT) biosynthesis which has stimulatory effect on the release of GnRH and gonadotropins by acting at the level of hypothalamo-hypophyseal axis. Brain form of *tph* is neuron-specific and expressed predominantly in preoptic area-hypothalamus (POA-HYP) region in teleosts. Therefore, in the present study we isolated *tph* from catfish brain to evaluate its expression pattern in male and female brain during early development. *tph* cloned from catfish brain is 2.768 Kb in length which encodes predicted protein of 488 amino acid residues. The characterization of recombinant *tph* was done by transient transfection in CHO cells. Tissue distribution of *tph* revealed ubiquitous expression in many tissues except ovary. Real time PCR analysis in discrete regions of adult brain revealed that *tph* mRNA was abundant in the POA-HYP and thalamus (TH). We observed differential expression of *tph* at mRNA and protein levels in the POA-HYP and TH regions of male and female brains during development that further correlate with the 5-hydroxytryptophan (5-HTP) and 5-HT levels measured using HPLC method in these regions of male and female brain. *tph* immunoreactive neurons were observed in different regions of brain at 50 days post hatch using catfish specific *tph* antibody. Changes in *tph* mRNA expression, 5-HTP and 5-HT levels in the POA-HYP + TH region of methyltestosterone and para-chlorophenylalanine treated fish brains during development further endorse our results. Based on our results we tend to propose that the serotonergic system may favor male brain sex differentiation in teleosts.

Introduction

Serotonin (5-hydroxytraptamine, 5-HT) is one of the important momoamines in the central nervous system (CNS) regulating variety of physiological processes. Tryptophan being the precursor for 5-HT production where in the homotetrameric enzyme tryptophan hydroxylase (*tph*; EC1.14.16.2) hydroxylates tryptophan into 5-hydroxytryptophan by utilizing tetrahydrobiopterin and O_2 as co-substrates, and Fe^{2+} as a cofactor. Later 5-hydroxytryptophan is immediately decarboxylated by hydroxytryptophan decarboxylase to 5-HT (Lovenberg *et al.*, 1967; Jequier *et al.*, 1969; Walther and Bader, 2003). *Tph* belongs to a gene family of aromatic amino acid hydroxylases (AAAHs). The signature domains such as small ligand binding domain and biopterin-dependent AAAH catalytic domain characteristic to all aromatic AAAHs (Ledley *et al.*, 1985; Fitzpatrick, 1999). Two distinct forms of *Tph* (*Tph*1 and *Tph*2) were first reported by Walther *et al.* (2003). *Tph*1 was first cloned from rabbit pineal gland yet found in gut and other peripheral locations outside CNS (Grenett *et al.*, 1987). The second form, *Tph*2 which was newly discovered is neuron-specific and expressed primarily in serotonergic neurons of the brain (Walther *et al.*, 2003). Later both forms of *tph* have been cloned from few other vertebrate species like human, mouse, rat and zebrafish (Kim *et al.*, 1991; Stoll and Goldman, 1991; Bellipanni *et al.*, 2002; Carkaci-Salli *et al.*, 2006).

In brain, 5-HT as a neurotransmitter also regulates the secretion of various reproductive neurohormones and sexual behavior in vertebrates (Peter *et al.*, 1991; Khan and Thomas, 1992; Hull and Dominguez, 2007). In teleosts, 5-HT neurons regulating reproductive neuroendocrine functions are localized mainly in the hypothalamus. 5-HT is a stimulatory monoamine for the release of GnRH and gonadotropins (GTHs) by acting at the level of hypothalamo-hypophyseal

axis. (Peter *et al.*, 1991; Khan and Thomas, 1992; Goos *et al.*, 1999; Senthilkumaran *et al.*, 2001; Wong *et al.*, 2004; Nakamura and Hasegawa, 2007).

Sex differentiation in teleosts show sexual plasticity and it can be influenced by factors like genetic sex, temperature and exogenous steroids. Steroid hormones play a crucial role in the process of gonadal development which in turn regulate the expression pattern of sex-specific genes during development. For example, estradiol is known to promote ovarian differentiation in teleosts (Nakamura *et al.*, 2003; Nagahama, 2005). Since, brain has got a potential role in the neuroendocrine mediated regulation of reproduction, we intend to know the occurrence of sexual dimorphism (brain sex differentiation), if any in teleosts. Recently, we demonstrated sexual dimorphic expression of *tph* in the brain of XX and XY Nile tilapia during early development (Sudhakumari *et al.*, 2010). Previous studies showed that para-chlorophenylalanine (pCPA) treatments, a *tph* blocker mimicked like estradiol during early sex differentiation period in the Nile tilapia which resulted in more number of females (Tsai and Wang, 1999; Tsai *et al.*, 2000). It is imperative to know how pCPA mimics the action of estradiol during early development. However, to date there is no clear understanding of the role of *tph* vis-à-vis 5-HT and 5-hydroxytryptophan (5-HTP) in brain in relation to male and female sex development. The aim of the present study was cloning, cDNA characterization, localization (by immunohistochemistry) of *tph* in the brain of catfish, *Clarias gariepinus*. We studied the expression pattern of *tph* in different regions and also in the whole brain of male and female during development in correlation with 5-HT and 5-HTP levels. We also compared the expression of *tph*, 5-HT and 5-HTP levels in the brains of control fishes with that of fishes exposed to methyltestosterone (MT) and pCPA.

Materials and methods

Animal and Sampling

Breeding and rearing of catfish (*C. gariepinus*) at different age groups was already described in chapter 1. The catfish hatchlings were collected at different age groups: 0, 5, 10, 20, 30, 50, 75, 100 and 200 dph and brains were dissected using a fine forceps under stereo zoom microscope (Leica, Germany) except for 0 day where in whole body was used. Adult male catfish brain was dissected into five different regions: olfactory bulb (OB), Telencephalon (TEL), preoptic area-hypothalamus (POA-HYP), thalamus (TH) region comprising of optic tectum and cerebellum and medulla oblongata (MO). All these brain samples were stored in -80°C until used for total RNA preparation for analyzing expression of *tph*.

Cloning of full-length cDNA of *tph* from catfish brain

Total RNA was prepared from brain of catfish using the Sigma TRI-reagent method (Sigma, USA) with manufacturer's protocol. Reverse transcription was carried out using superscript-III reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo $d(T)_{18}$ primers and 5 µg of total RNA at 50°C. PCR amplification was done at 94°C (1min), 55°C (30sec), 72°C (1min), for 35 cycles using the following set of degenerate primers sense primer 5' CA(AG) TTC TC(AC) CA(AGT) GAA ATT GG 3' and antisense primer 5' TGA CTT T(AG)T C(GT)T T(AT)G C(AG)T CTT C 3' designed based on the available *tph* nucleotide sequences from DDBJ/EMBL/GenBank data base. PCR amplified cDNA fragment was gel purified, cloned in pGEM-T Easy vector and sequenced. The catfish brain cDNA library was constructed using 5µg of poly(A)+ RNA with λZAP vector system (Stratagene, La Jolla, CA) and packaged into UNI-ZAP XR using Gigapack II Gold packaging extract (Stratagene) as per the method described earlier (Senthilkumaran *et al.*,

2002). Screening for *tph* clones was carried out by hybridization under high stringency conditions using RT-PCR amplified partial cDNA fragment of *tph* as probe. The probe was labeled by random-priming with (α P^{32}) dCTP by using Random hexamer labeling kit (Fermentas). After three rounds of screening, three positive clones were obtained. Single clone excision and rescue of pBluescript phagemids were performed according to the manufacturer's protocol (Stratagene). All the clones were sequenced and analyzed using LaserGene software version 3.05 (DNASTAR, Madison, WI).

Transient transfection of catfish recombinant Tph in CHO cells

Open reading frame of catfish *tph* was cloned into pCDNA3.1^{+}TOPOV5-His mammalian expression vector (Invitrogen) and the sequence was verified bi-directionally. Approximately 1 x 10^5 CHO cells were plated onto a 6-cm tissue culture dish with 2.5ml of DMEM containing 10% (w/v) fetal calf serum (GIBCO-BRL, Gaithersburg, MD). The cells were cultured at 37°C in 5% CO_2 until they reached 75% confluent. Then, 1-1.5µg of recombinant plasmid DNA was transiently transfected into the CHO cells using TfxTM-20 (Promega, USA) according to the manufacturer's protocol and cultured for 24h. Later, the cells were harvested by scraping, followed by centrifugation at 1500 xg and lysed in 0.1 M sucrose using a sonicator for the measurement of enzyme activity.

Enzyme assay of recombinant catfish brain Tph

Tph activity, *in vitro* was determined using cell extracts (lysate) as per the methodology described earlier (Sudhakumari *et al.*, 2010). In brief, the complete reaction mixture volume of 0.3 ml contains the following components: 50 mM of Tris-acetate, pH 7.6, DTT 2 mM, catalase

50 µg, L-tryptophan 250 µM, cell lysate 0.1 ml, ferrous ammonium sulfate 0.1 mM, glucose-6-phosphate 20 mM, NADH 2 mM, excess of glucose-6- phosphate dehydrogenase and dihydropteridine reductase. The reaction was initiated by the addition of 250 µM tetrahydropterin and incubation at 37^{o}C with shaking for 30 min. After incubation, the reaction was stopped by the addition of 0.03 ml of 70% perchloric acid. The reaction mixture was centrifuged and 5-HTP in the supernatant fraction was measured by using reverse phase HPLC-ECD system (Waters, Milford, MA) as described by Murai *et al.* (1988).

Tissue distribution pattern of *tph* by RT-PCR

Tissue distribution pattern of *tph* in adult catfish performed using specific primers by RT-PCR as described in chapter 1. PCR amplification of *tph* was carried at 94°C (1 min), 60°C (30sec), 72°C (1min) for 30 cycles using specific primers (sense primer: 5' CAG TTC TCC CAG GAA ATT GGC C 3' and antisense primer: 5' CGA AAC TCT CTG AGA CAA AGT 3').

Real-time quantitative RT-PCR (qRT-PCR)

tph mRNA levels in different regions of mature brain and during development at different age groups of catfish were quantified by relative qRT-PCR using TaqMan MGB probes (Applied Biosystems, Foster city, CA). Real-time PCR specific primers and TaqMan MGB probe for *tph* (forward primer: 5' ACAGCACTGACCCACTCTACA 3'; reverse primer: 5' ATGGCCCAGGAGCTCATG 3'; probe: 5' 6FAM-CACCAGAGCCGGACACG-MGBNFQ 3') were custom designed using primer express 2.0 software of Applied Biosystems. 18S rRNA was used as endogenous control. Primers and probes for 18S rRNA (Assay ID: Hs99999901_s1) were obtained as pre-designed TaqMan endogenous control assay kit (Applied Biosystems).

Reverse transcription was carried out with 1 µg total RNA and random hexamer primers using superscript-III (Invitrogen) according to manufacture's protocol. Real-time PCR was carried out in 25 µl reaction volume in triplicates containing the following components: 1 µl of cDNA template (1:10 diluted), 1 µl of taqman probe mix and 12.5 µl of Fast Taqman master mix (Applied Biosystems). A two-step real-time PCR amplification was then performed in an ABI-7500 fast real-time PCR machine (Applied Biosystems) for 40 cycles under the following condition: Initial hold at 95^{o}C for 20sec and then two-step RT-PCR for 40 cycles at 95^{o}C (3sec) and 60^{o}C (30sec). During each cycle of the PCR, fluorescence accumulation resulting from DNA amplification was analyzed and converted in to Cycle threshold (Ct) by the ABI 7500 sequence detection system software (Applied Biosystems). Ct values were obtained from the exponential phase of PCR amplification. The transcript levels were calculated using relative quantification method where in the expression of *tph* was normalized against the expression of 18S rRNA, generating a ΔCt value (ΔCt = Ct value of *tph* – Ct value of 18S rRNA). The Relative mRNA expression for each sample group was then expressed according to the equation $2^{-\Delta CT}$.

Western blot analysis

Western blot analysis was carried out to analyze the levels of Tph protein in the POA-HYP and TH regions of male and female brains at 50 dph using polyclonal catfish specific Tph antibody. Tph antibody was raised in rabbit against the purified recombinant Tph protein of 159 amino acids. Detailed procedure for raising the polyclonal antibody was described in chapter 2. The specificity of the Tph antibody was determined by immunoblot using the whole brain (50 dph male fish) homogenate as protein sample (75 µg of protein). For negative/blocking control, immunoblot was performed using the same protein sample, but probed with Tph antibody

(1:1000 dilution) preadsorbed with excess of antigen (purified Tph recombinant protein) for overnight at 4°C using a shaker incubator. We dissected out the POA-HYP and TH regions of male and female brain at 50 dph and homogenized in 250 µl of homogenization buffer using a Sigma hand homogenizer. The homogenates were centrifuged at 10,000x*g* for 10 min at 4°C and the supernatants were used for Western blot analysis was carried out as described in chapter 2.

Immunohistochemistry (IHC)

Brain of juvenile catfish at 50 dph was fixed in 4% paraformaldehyde, processed and embedded in paraplast (Sigma) to localize Tph using IHC as per the methodology described in chapter 2. Photomicrographs were taken using Olympus CX41 bright field microscope (Olympus, Japan).

Estimation of 5-HTP and 5-HT levels in developing brains by HPLC method

Catfish male and female brains at 50 and 75 dph were dissected out. POA-HYP and TH regions were isolated from the dissected brains and homogenized immediately in 10mM perchloric acid containing 1mM EDTA. The homogenate samples were centrifuged down and supernatants were filtered using 0.45 mm filters for quantification of 5-HTP and 5-HT levels using reverse phase HPLC-ECD system (Waters).

Quantitative measurement of Tph mRNA, 5-HTP and 5-HT levels in methyltestosterone, pCPA treated and control fishes

Catfish hatchlings were treated alternative days with methyltestosterone (MT), and pCPA. Treatments were started from day one (after hatching) onwards till 50 dph by exposing the hatchlings with different compounds in fresh water for 6 hr. The dosages used for treatments

were as follows: MT (100 µg/L) and pCPA (500 µg/L). After treatments, we calculated the percentage male and female population. Brains were dissected out from these treated fishes and stored at –80^{o}C for total RNA extraction. Expression of *tph* in the POA-HYP + TH region of treated and control fish brains were analyzed using qRT-PCR. We also measured the 5-HTP and 5-HT levels in the POA-HYP + TH region of treated and control fish brains using HPLC-ECD method.

Statistical analysis

All the results were expressed as mean ± SEM. Data was analyzed by Student's *t*-test and one way ANOVA followed by Student-Newman-Keuls (SNK) post hoc test using SigmaStat 3.1 software. A probability of $P < 0.05$ is considered statistically significant.

Results

Cloning of catfish Tph from brain

Partial cDNA fragment (298 bp) of *tph* from catfish brain was amplified by RT-PCR using degenerate primers (Fig. 1). The full-length *tph* cDNA was isolated from brain by screening the catfish brain cDNA library under high stringent conditions using the *tph* partial cDNA fragment as probe. The sequence analysis of the positive plasmid clones revealed a full-length *tph* cDNA of 2.768 Kb in size and an ORF of 1464 bp long, which encodes a putative protein of 488 amino acid residues (Fig. 2). The 3' UTR of *tph* was 1212 bp long and contains two poly-adenylation signals 28 bp and 299 bp upstream from the beginning of poly-A^+-tail. The 5' UTR of *tph* was 92 bp long. Cloned catfish *tph* nucleotide sequence has been submitted to GenBank and the accession number is GU290195. Based on the phylogeneitc tree analysis, the cloned catfish *tph* showed high homology and formed a separate clade with teleost *tph* isoform2 (Fig. 3). Tilapia *tph* seems to be ancient among teleosts and it formed a separate clade before the distinction of *tph1* and *tph2*. Catfish *tph* showed 86% and 78% identity with zebrafish and human *tph2* sequences at protein level, respectively. On the other hand, it showed 66% and 69% identity with zebrafish and human *tph1* sequences at protein level, respectively.

Transient expression and characterization of recombinant Tph in CHO cells

The recombinant pCDNA3.1 construct was transiently transfected into the CHO cells and cultured for 24 h. The enzymatic activity of recombinant catfish *tph* was verified by measuring the 5-HTP formed *in vitro* after incubation of transfected cell extracts with L-tryptophan and all other necessary cofactors mentioned above. The recombinant *tph* showed a conversion of 52.6 ±

1.7% (n=5) while the vector without insert (1.9 ± 0.8%) and mock vector having insert locked in reverse direction (2.4 ± 1.1%) transfected cell lysates showed no enzyme activity (Fig. 4).

Tissue distribution pattern of Tph in catfish

RT-PCR analysis of spatial expression pattern of *tph* showed that the transcript was expressed ubiquitously in all tissues like brain, muscle, gill, kidney, liver, testis and ovary except heart and adipose tissue (Fig. 5). However, the transcript was high in brain, kidney, liver, heart and testis.

Tph mRNA expression in different regions of adult brain

A diagrammatic representation of the discrete regions of adult catfish brain used for real-time PCR was shown in Fig. 6A. Expression pattern of *tph* was quantified in the five different regions of male brain (OB, TEL, POA-HYP, TH and MO) using real-time PCR. Real-time PCR data showed that the *tph* mRNA was abundant in POA-HYP and TH regions of adult brain when compared to OB, TEL and MO regions of the brain (Fig. 6B).

Differential expression pattern of Tph mRNA during brain ontogeny

qRT-PCR analysis revealed that the *tph* transcript in whole brain was evident from 5 days post hatching (dph) onwards and it was higher during the period of gonadal sex differentiation (Fig. 7A). The critical period of gonadal sex differentiation in catfish is around 40 -50 dph (for more details refer next chapter). The expression of *tph* in brain at 0 dph was negligible. Levels of *tph* mRNA were significantly higher in 50 and 75 dph male (*, $P < 0.05$) brains when compared to female brains at the same age group (Fig. 7A) There was no significant difference in expression of *tph* in the brain of male and female catfish after 75 dph. Since the expression of *tph* was more

in the POA-HYP and TH regions of adult brain (Fig. 7B). We further quantified the *tph* mRNA expression in the POA-HYP and TH regions of the male and female brains at 50 and 75 dph using qRT-PCR. We observed significantly higher levels of *tph* mRNA in the POA-HYP and TH regions of male brain (Fig. 7B) when compared to POA-HYP and TH regions of female brain at 50 and 75 dph (*, $P < 0.05$).

Tph protein expression in the POA-HYP and TH regions of male and female brains by Western blot analysis

SDS-PAGE gel picture showing over-expression of 18 kDa recombinant Tph protein using bacterial pET BLUE2 vector system and later then was purified using Ni-NTA column (Fig. 8A and 8). The purified protein was confirmed by immunoblot using monoclonal His-tag antibody (Fig. 8C). Western blot analysis using this antibody revealed an expected Tph protein band of approximately 56 kDa in the homogenates prepared from (Fig. 9A). This band was not detected following preadsorption of the antibody with excess antigen (Fig. 10A). Later the expression pattern of Tph protein was analyzed in the POA-HYP and TH regions of brain of 50 dph juvenile fish (Fig. 9B). Densitometry analysis showed significantly higher expression of Tph protein in the POA-HYP region of brain was in males when compared to females at 50 dph (densitometric graph; *, $P < 0.05$). Similarly, a moderately higher (densitometric graph; *, $P < 0.05$) expression of *tph* protein in the TH region of male brain when compared to TH region of female brain at 50 dph was observed (Fig. 9C).

Changes in levels of 5-HTP and 5-HT in the POA-HYP and TH regions of male and female brains of catfish during development

The levels of 5-HTP and 5-HT measured using HPLC-ECD were high in POA-HYP and TH regions of male brain when compared to female brain in those regions at 50 and 75 dph (Fig. 10A-D).

Changes in levels of Tph mRNA, 5-HTP and 5-HT in the POA-HYP + TH region of brain of MT and pCPA treated fishes

Under normal laboratory conditions (23-25˚C ambient temperature) usually more number of females (60-65% out of total mixed population) were obtained when compared to males in catfish. Exposure of hatchlings to exogenous steroids like MT can skew the population towards males (Nagahama, 2005). In the present study, treatment of MT resulted in more number of male fishes, which is around 90 ± 1.2%. Further, as reported by Tsai *et al.* (2000) in the present study also we obtained more number of females (83 ± 3.5%) when hatchlings were treated with pCPA. To further validate our results, we quantified the *tph* mRNA levels in the POA-HYP and TH regions together in control, MT and pCPA treated fishes at 50 dph by qRT-PCR. We observed significantly higher levels of *tph* mRNA in brains of MT treated fishes when compared to control male fishes (Fig. 11A). In contrast, we obtained lower levels of *tph* mRNA in brains of pCPA treated fishes when compared to control male fishes (Fig. 11A). We also measured the 5-HTP and 5-HT levels in the POA-HYP + TH region of brain of control, MT and pCPA treated fishes. We observed a significant increase in 5-HTP and 5-HT levels in the MT treated fishes when compared to control fishes (Fig. 11B and C). As expected there was significant decline 5-HTP and 5-HT levels in the pCPA treated fishes when compared to control fishes (Fig. 11B and C).

Localization of Tph in early developing brains using IHC

We studied the distribution of Tph immunoreactivity (-ir) in different regions of brain at 50 dph using IHC. Tph -ir was not detected in the TEL region of brain when preadsorbed antibody was used as primary antibody (Negative control, Fig. 12A). IHC results showed Tph -ir cell groups in the different regions of the brain: OB, TEL, diencephalon and posterior region of the hypothalamus (HYP). Tph -ir cells were found in the dorsal region of TEL (Fig. 12B and C) and OB region (Fig. 12D) of the brain. Prominent Tph -ir cell bodies were seen in the pre-optic area (Fig. 12E and F) and in the relatively large area of posterior hypothalamic region (Fig. 12G - I) along the wall of the nucleus recessus lateralis (NRL) and paraventricular organ (PVO). Transverse section of frontal diencephalon (Fig. 12J) showed a small group of Tph -ir cells lining the third ventricle in the region of nucleus preoptic (NPO) and periventricular preoptic nucleus (NPP). Tph –ir cell bodies were also localized along the wall of third ventricle at the level of nucleus ventromedialis thalami (NVM) and nucleus posterior periventricularis (NPPv) (Fig 12K). Schematic representation of distribution of Tph -ir cells in different regions of brain at 50 dph was shown in Fig. 13.

Figures

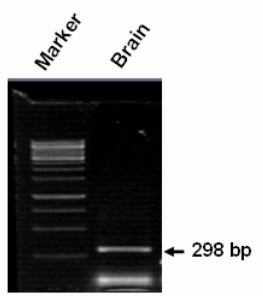

Fig. 1. 1% Agarose gels showing partial cDNA fragment of tph from catfish brain by RT-PCR

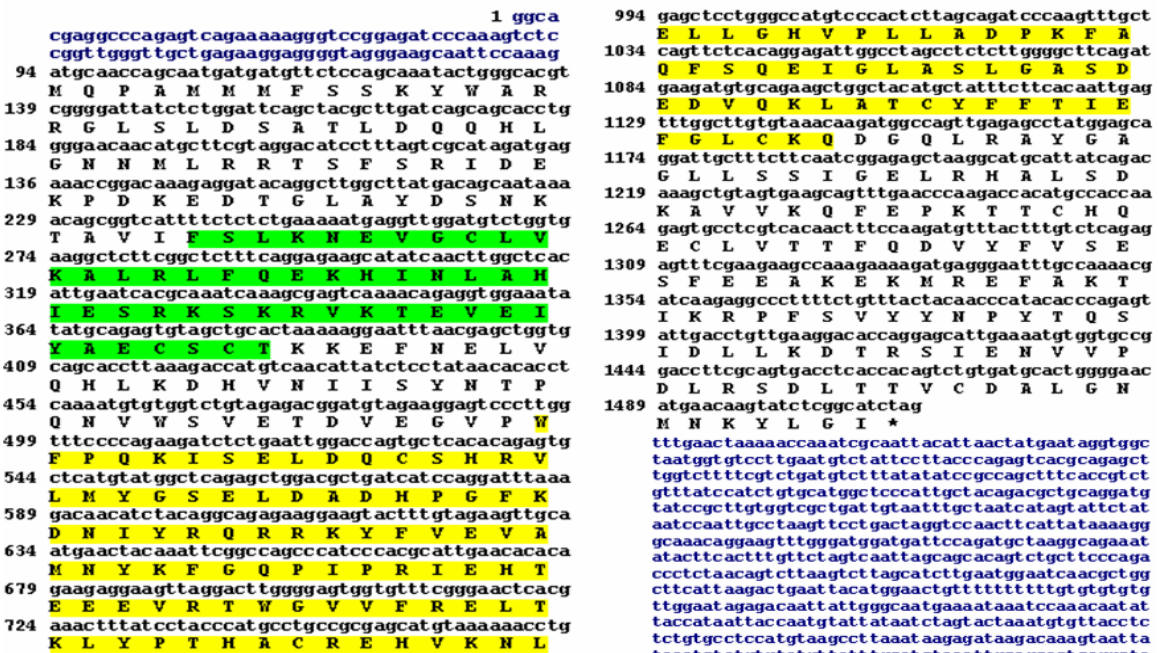

Fig. 2. Nucleotide and deduced amino acid sequence of brain form of Tph from the Catfish (Clarias gariepinus). 5' and 3' UTRs were shown in blue letters. Green color region indicates small ligand

binding domain and yellow color region indicates biopterin-dependent aromatic amino acid hydroxylase domain. (*) indicates stop-codon.

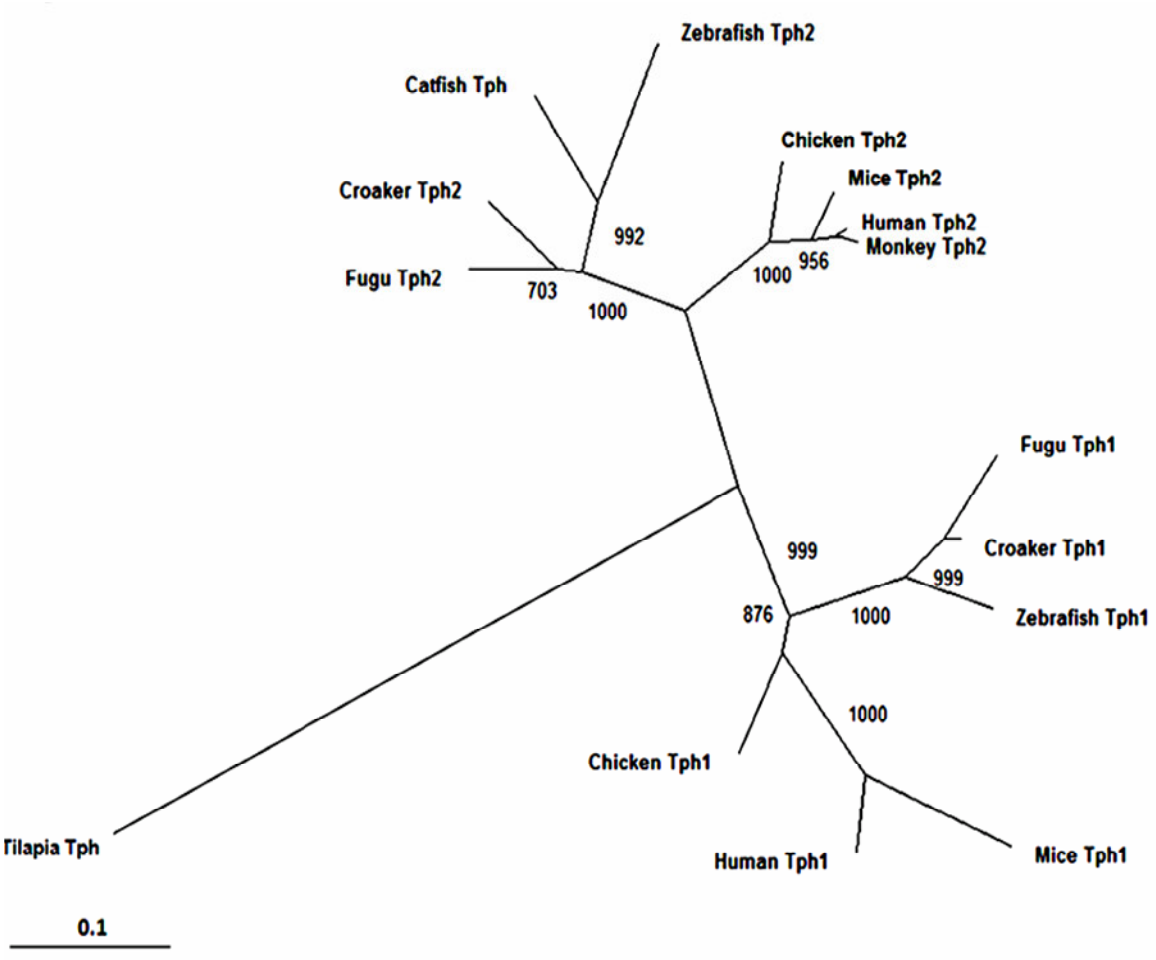

Fig. 3. Phylogenetic tree of catfish Tph. Phylogenetic analysis was performed using the neighbor-joining method and a bootstrap analysis with 1000 replicates was used to asses the strength of nodes in the tree using clustalW program (http://clustalw.ddbj.nig.ac.jp). Phylogenetic tree was constructed using TreeView software package version 1.4. GenBank accession numbers of the sequences used in clustalw alignment were as follows: Catfish (Tph, GU290195), Atlantic Croaker (Tph1, EU730759; Tph2, EU730760), Fugu (Tph1, AY616188; Tph2, AY616189), Zebrafish (Tph1, BC059550; Tph2, AY616135), Human (Tph1, NM_004179; Tph2, NM_173353), Mice (Tph1, BC072582; Tph2, NM_173391), Chicken (Tph1, NP_990287; Tph2, ABC94730) and Monkey (Tph2, NM_001039946), Tilapia (Tph FJ8008390). Domain in dotted box indicates small ligand binding domain and domain in black box indicates biopterin-dependent aromatic amino acid hydroxylase domain.

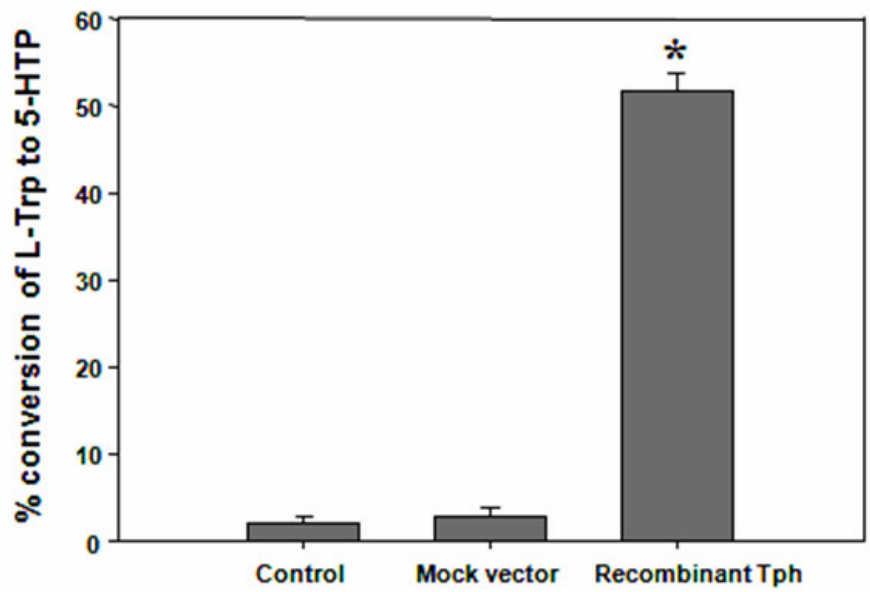

Fig. 4. Enzymatic activity of recombinant catfish Tph protein expressed transiently in CHO cells. Graph showing percentage conversion of L-tryptophan to 5-hydoxy tryptophan by the Tph recombinant protein expressed using pCDNA 3.1 TOPO vector and mock vector where the insert was locked in reverse orientation. * indicates P < 0.001 (ANOVA followed by the SNK test, n=3).

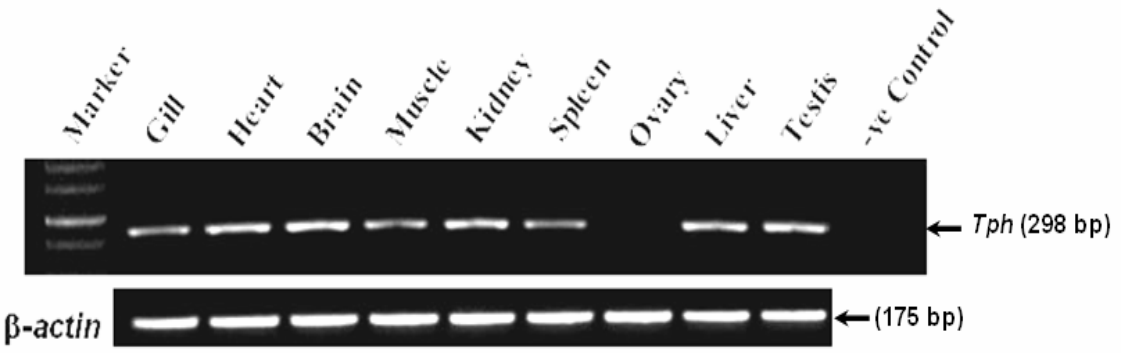

Fig. 5. RT-PCR analysis (representative gel; n=3) of tissue distribution pattern of catfish Tph (upper panel) and β-actin (lower panel).

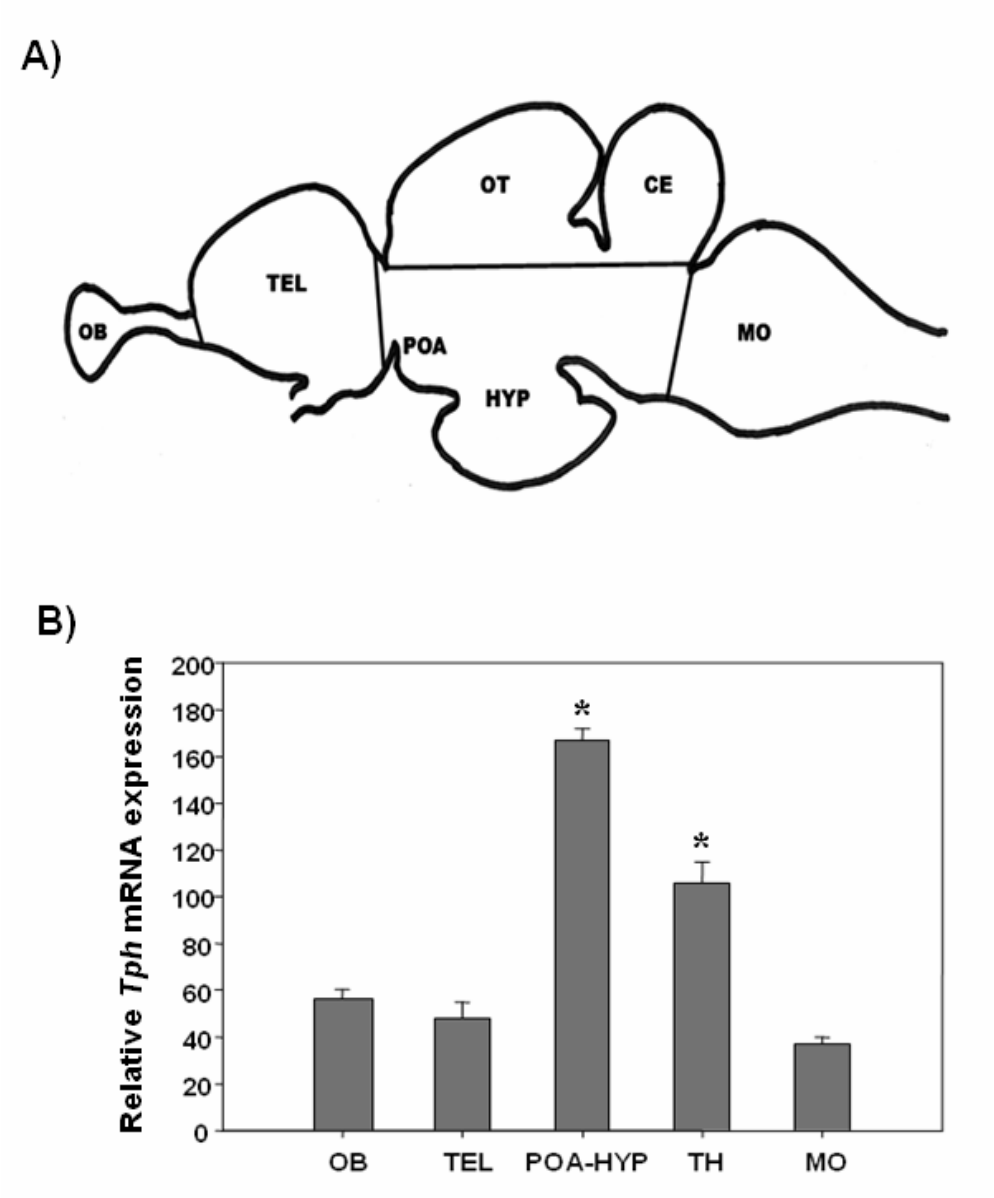

Fig. 6. A) A diagrammatic representation of the different regions of adult catfish brain used for the quantification of Tph mRNA using real-time PCR. B) qRT-PCR analysis (n=5) showing Tph expression pattern in different regions of the catfish adult brain. * indicates significantly high expression of Tph in the POA-HYP and TH regions when compared with other regions of brain (* P < 0.05; ANOVA followed by the SNK test). OB, olfactory bulb; TEL, telencephalon; POA, pre-optic area; HYP, hypothlamus; thalamus region comprising of both OT, optic tectum and CE, cerebellum; Mo, medulla oblongata.

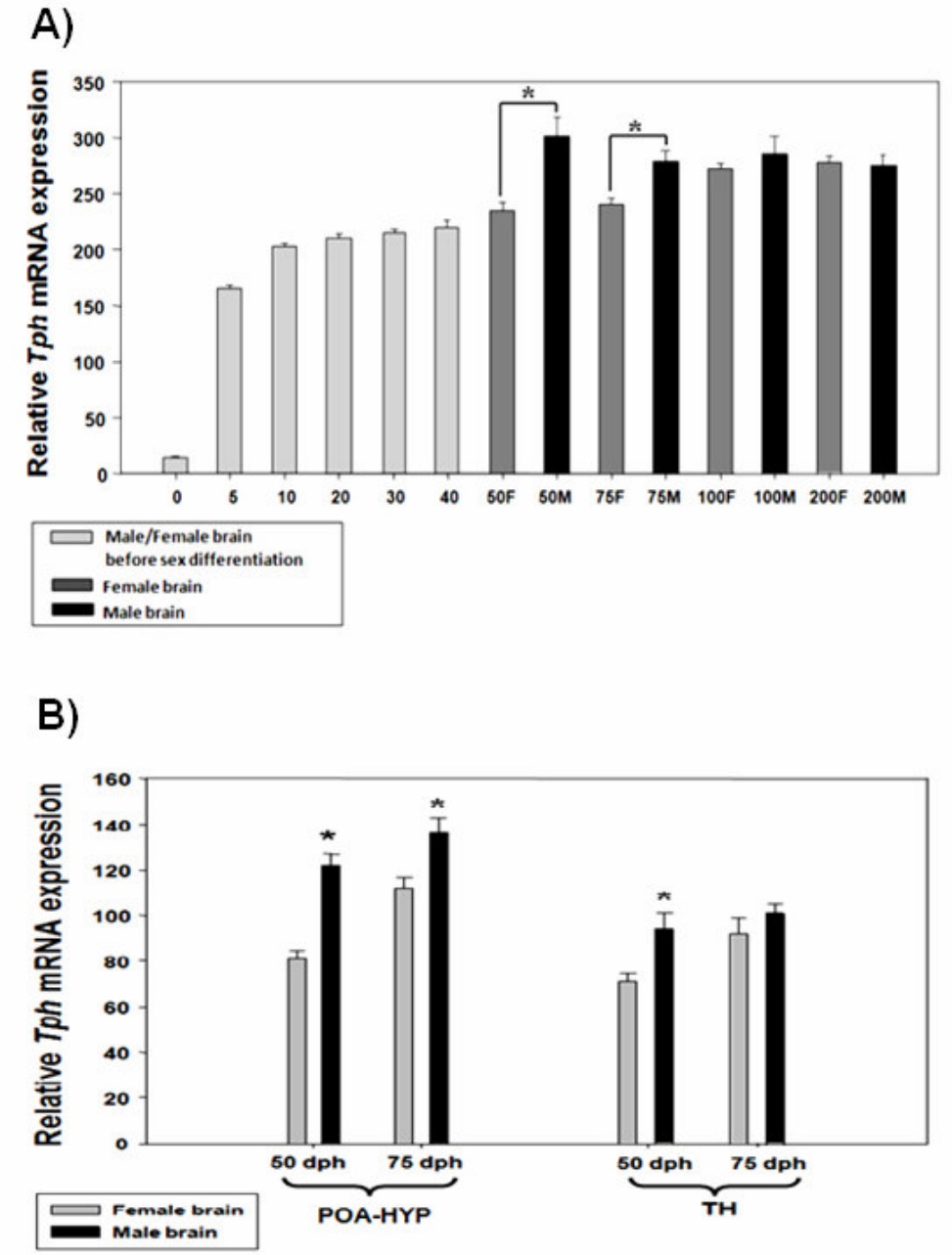

Fig. 7. A) qRT-PCR expression pattern of Tph during brain ontogeny, ie. from 0 dph onwards to 200 dph. * indicates means with significantly higher Tph mRNA levels in male brain at 50 and 75 dph when compared with female brain of the same age group, respectively. P < 0.05 (ANOVA followed by the SNK test, n=3). B) Differential expression of Tph in the POA-HYP and TH regions of male and female brains at 50 and 75 dph using qRT-PCR (* P < 0.05; ANOVA followed by the SNK test, n=5). POA-HYP, Pre-optic area-hypothlamus; TH, thalamus.

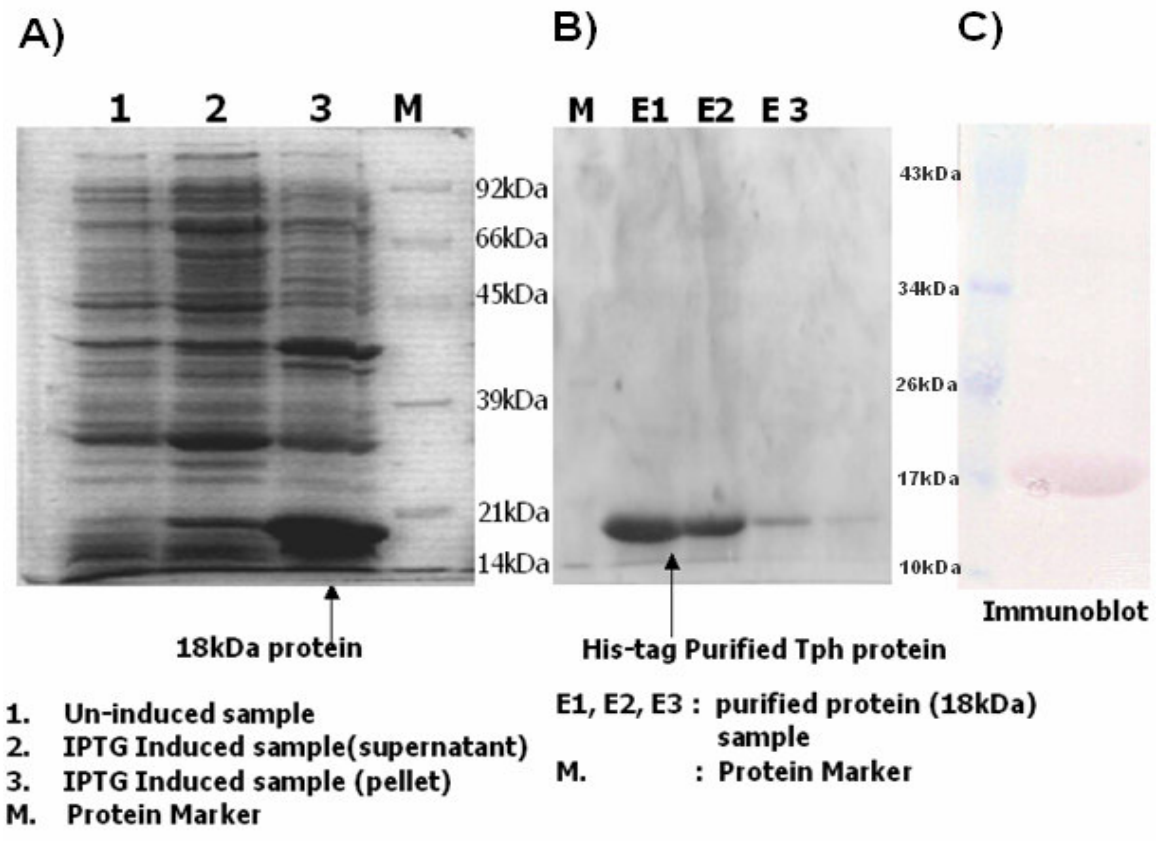

Fig. 8. A) 12% SDS-PAGE gel showing over-expression of 18 kDa Tph protein upon IPTG induction in E. coli. B) 12% SDS-PAGE gel showing purified 18 kDa Tph protein using Ni-NTA affinity chromatography column. C) Immunoblot showing positive band using monoclonal His-tag antibody indicating purified protein is indeed recombinant Tph protein.

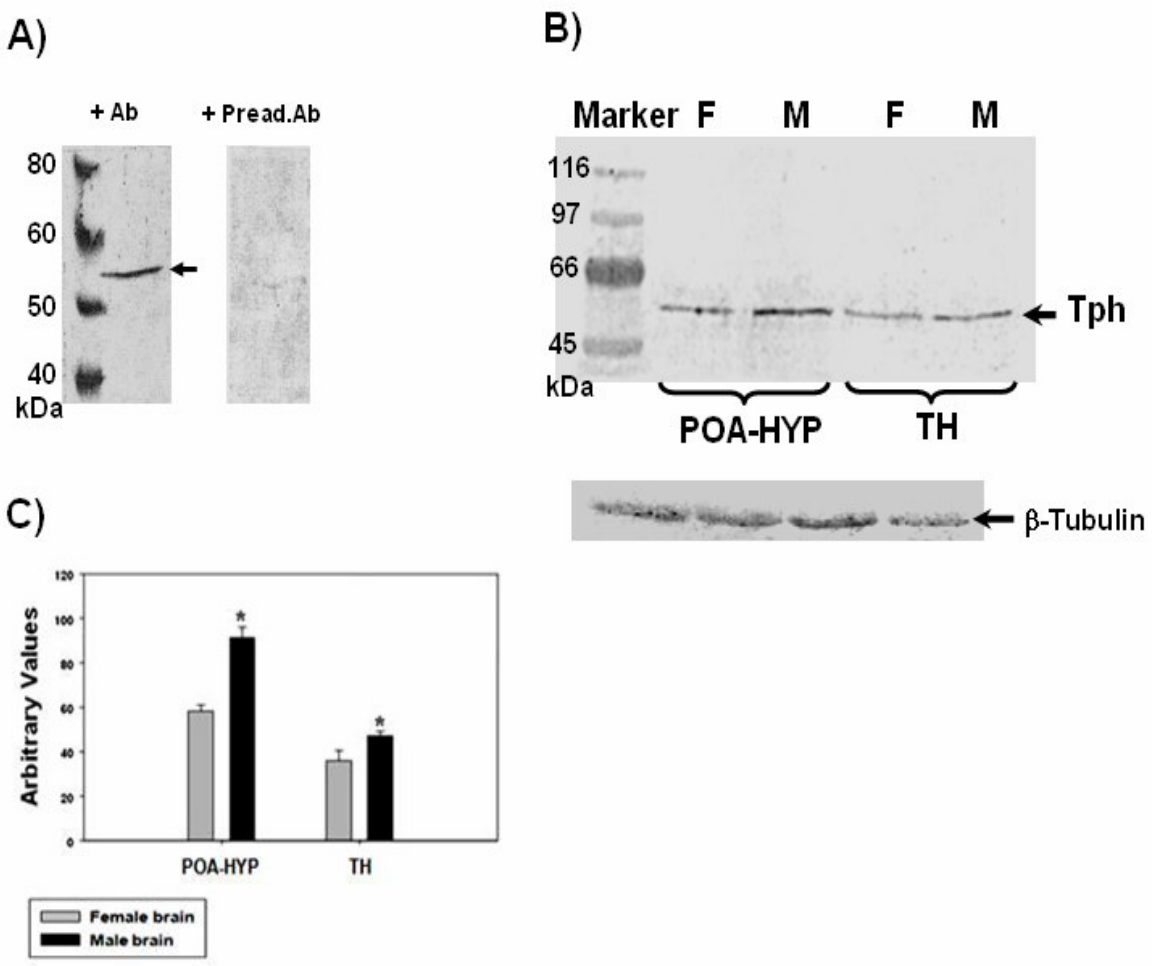

Fig. 9. Western blot analysis showing Tph protein expression. A) Controls showing specificity of catfish Tph antibody. Immunoblot with whole brain homogenate of 50 dph male fish (indicated by arrow) revealed an approximate band of 56 kDa (+Ab), which was abolished following preadsorption with excess antigen (+Pread. Ab). B) Tph protein expression in the POA-HYP and TH regions of male and female brains at 50 dph using western blot. β-Tubulin was used as endogenous control. C) Densitometric graph showing expression of Tph protein in male and female brains at 50 dph (lower panel). * indicates significantly higher Tph protein expression in male brain when compared to female brain at both age groups (P < 0.05; Student's t-test, n=5). POA-HYP, Pre-optic area-hypothlamus; TH, thalamus.

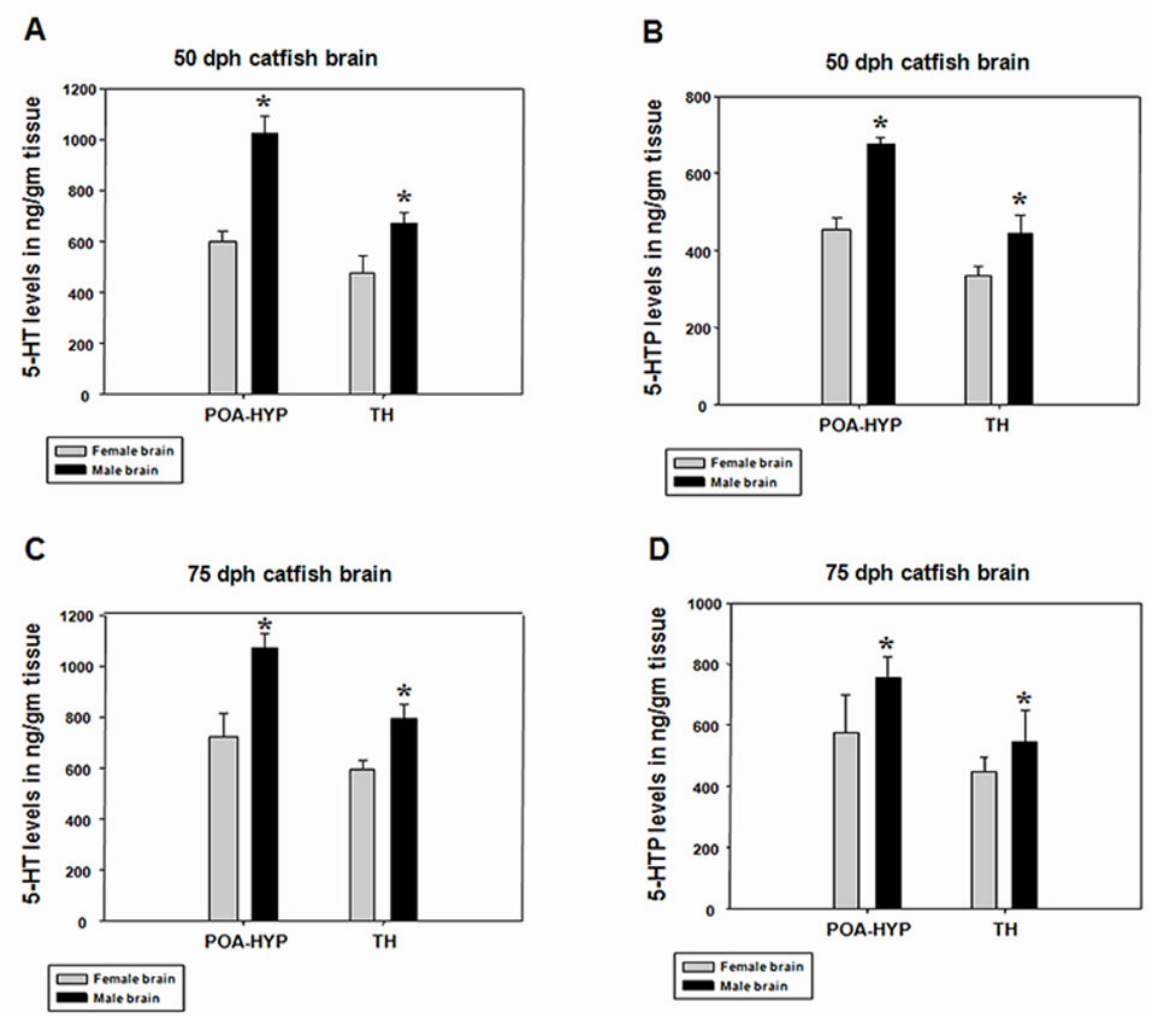

Fig. 10. Quantitative estimation of 5-HT and 5-HTP levels in POA-HYP and TH regions of male and female brain of catfish during development. A) 5-HT levels in male and female brains at 50 dph. B) 5-HTP levels in male and female brains at 50 dph. c) 5-HT levels in male and female brains at 75 dph. D) 5-HTP levels in male and female brains at 75 dph. * indicates significant change in 5-HT and 5-HTP levels between male and female brains at both age groups (P < 0.05; Student's t-test, n=6). POA-HYP, Pre-optic area-hypothlamus; TH, thalamus.

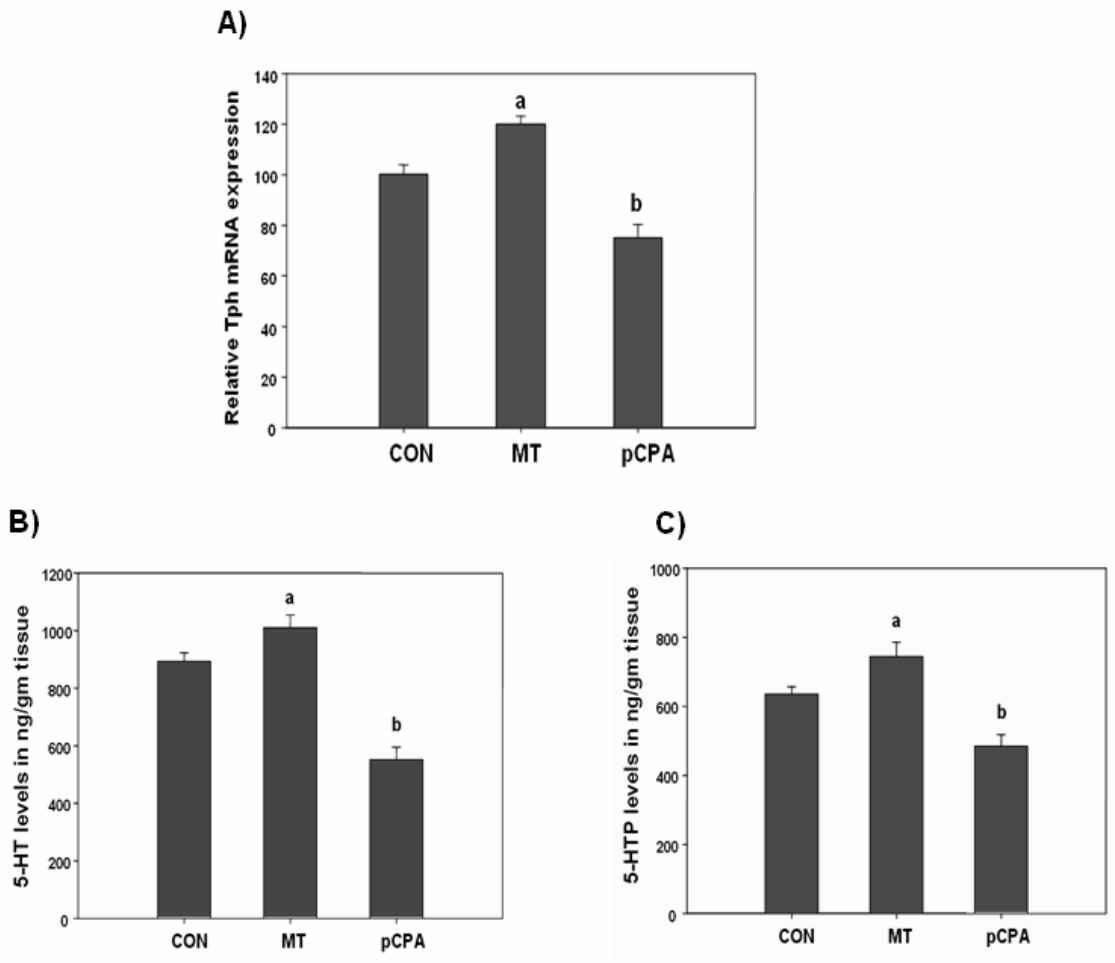

Fig. 11. A) qRT-PCR analysis of Tph expression in POA-HYP + TH region of catfish brains of MT, pCPA treated and control fishes at 50 dph. Alphabets 'a' and 'b' indicates significantly high levels of Tph mRNA in the brains of MT and pCPA treated fishes when compared with control fishes. (P < 0.05; ANOVA followed by the SNK test, n=5). B) Changes in the levels of 5-HT in POA-HYP + TH region of catfish brains of MT, pCPA treated and control fishes at 50 dph. C) Changes in the levels of 5-HTP in POA-HYP + TH region of catfish brains of MT, pCPA treated and control fishes at 50 dph. a and b indicates significantly high levels of 5-HT and 5-HTP in the brains of MT and pCPA treated fishes when compared with control fishes. (P < 0.05; ANOVA followed by the SNK test n=5).

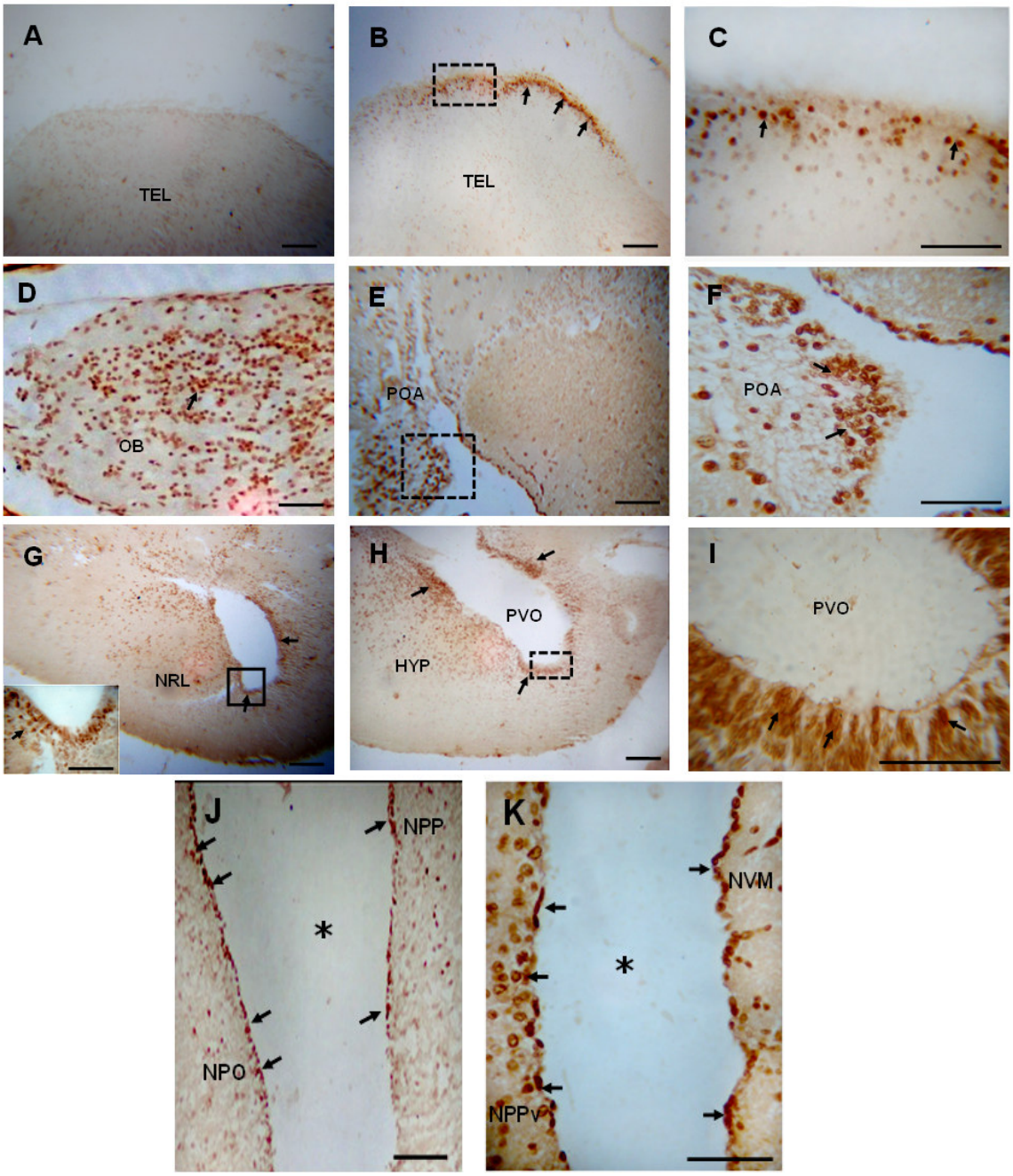

Fig. 12. Immunohistochemical distribution of Tph immunoreactivity (-ir) in different regions developing catfish brain at 50 dph and in adult brain: A) Sagittal section of TEL showing no Tph -ir when preabsorbed antibody was used for negative control. B) Sagittal section of TEL showing Tph-ir cells in the dorsal region of telencephali. C) Sagittal section of TEL at higher magnification (dotted box in fig. 10B). D) Tph -ir cell group in sagittal section OB region of brain. D) Sagittal section of OB showing Tph -ir cell. E) Sagittal section of diencephalon showing Tph -ir cell group in the POA region.

F) POA region at higher magnification (dotted box in fig. 10E) showing Tph -ir cells. G) Sagittal section of the posterior hypothalamus region showing Tph -ir along the wall of the NRL. H) Sagittal section of PVO in the posterior hypothalamus showing Tph -ir. Inlet showing magnified picture of that area indicated in black box. I) High magnification area (dotted box in fig. 10H) showing Tph -ir cell group. J) Transverse section of diencephalon showing Tph -ir in the NPO and NPP region. K) Transverse section of diencephalon showing Tph -ir in the NVM and NPPv. OB, olfactory bulb; TEL, telencephalon; POA, preoptic area; PVO, paraventricular organ; NRL, nucleus recessus lateralis; NPO, nucleus preopticus; NPP, nucleus preopticus periventricularis; NVM, nucleus ventromedialis thalami; NPPv, nucleus posterior periventricularis; HYP, Hypothalamus. Arrows indicate Tph-ir cells. Asterisks indicate third ventricle. All scale bars represent 50 μm.

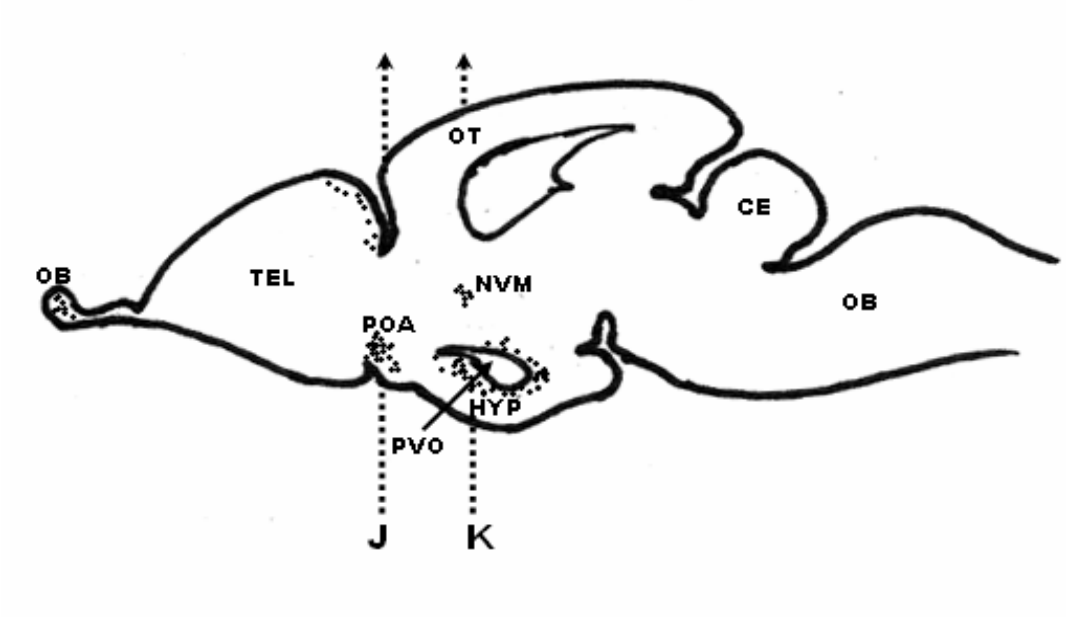

Fig. 13. Schematic representation of the distrution of 5-HT- ir cells (indicated as dots) in developing brain (50 dph). Dotted arrows in the figure indicate transverse sections of the brain shown in Fig. 10 J and K. OB, olfactory bulb; TEL, Telencephalon; OT, optic tectum; CE, cerebellum; medulla oblongata, MO; POA, preoptic area; PVO paraventricular organ; NVM, nucleus ventromedialis thalami; HYP, Hypothalamus.

Discussion

In this report, we have cloned full-length cDNA of *tph* from catfish brain and characterized the recombinant Tph protein by transient transfection in CHO cells. Expression analysis of *tph* mRNA in specific regions of catfish male and female brain revealed gender specific differences during early development that further correlated with 5-HTP and 5-HT levels. Our results suggest that sexual differences exist at level of serotonergic system during early brain development in teleosts.

In the present study, we obtained a 298 bp partial cDNA fragment corresponding to *tph* isoform from catfish brain by RT-PCR using degenerate primers and then we isolated the full-length *tph* cDNA. The NCBI-BLAST search and ClustalW alignment showed high homology for catfish *tph* with the mammalian and fish *tph* isoform2. Catfish *tph* was having high homology with zebrafish *tph*2. The catfish contained the signature domains characteristic to all aromatic AAAHs such as small ligand binding domain and biopterin-dependent AAAH catalytic domain (Ledley *et al.*, 1985). We have functionally characterized the cloned *tph* by transiently expressing the recombinant *tph* protein in CHO cell lines. The recombinant *tph* protein was effective enough to convert tryptophan to 5-HTP in the presence of co-factors and co-substrates.

Tissue distribution pattern of *tph* revealed that its expression was evident in brain and also in several other tissues such as liver, kidney, testis, gill and muscle. We showed using qRT-PCR that *tph* was abundantly expressed in POA-HYP region of the brain when compared to other regions. Similar kind of expression pattern of *tph* was also recently shown by Rahman and Thomas (2009) in the Atlantic croaker using RT-PCR. This kind of high expression in the entire hypothalamus is of particular importance as 5-HT neurons are concentrated in this region which exerts a stimulatory action on GTH secretion in fishes. In teleosts, it has been well established

that 5-HT neurons controlling reproductive neuroendocrine functions are localized primarily in the hypothalamus and pre-optic area, separate from those regulating other neural functions of 5-HT in the raphe nucleus (Peter *et al.*, 1991; Khan and Thomas, 1993; Senthilkumaran *et al.*, 2001). This also corroborates well with IHC results showing Tph -ir cells (a key enzyme in 5-HT biosynthesis) were distributed in different regions of brain where the expression was noticed in the OB, dorsal region of the TEL, POA, diencephalon region, hypothalamic periventricular wall including PVO. Previous report (Sudhakumari *et al.*, 2010) from our laboratory in the Nile tilapia using *in situ* hybridization showed *tph* expression in OB, TEL and POA regions of brain at 11 dph. We also showed by IHC (using catfish specific Tph antibody) that Tph -ir was seen in the OB, TEL, NPO, OT, CE, HYP and dorsal raphe nucleus regions of developing brains (15 dph and 30 dph). In zebrafish, *tph* expression and 5-HT -ir was seen in the POA, posterior tuberculum and caudal hypothalamus regions of early developing brain. However, the authors did not find any Tph expression in the raphe region of brain, although they observed 5-HT -ir neurons in the anterior raphe (Bellipanni *et al.*, 2002). Similarly, in the catfish and other teleosts, 5-HT expressing neurons have been localized in different regions of the brain (Kah and Chambolle, 1983; Khan and Thomas, 1993; Chiba *et al.*, 1999). However, in mammals like human and rodents, Tph (isoform 2) expression was not seen in the hypothalamus. 5-HT neurons expressing Tph2 are concentrated in the dorsal raphe nuclei which regulate variety of neural functions in addition to reproduction (Patel *et al.*, 2004; Hull and Dominguez, 2007; Zill *et al.*, 2007). Taken together, the mediation of serotonin to enact brain sex differentiation might be different in fishes when compared to mammals, partially due to differential localization. Present study concentrated more on POA-HYP and TH regions of the catfish brain during development as these regions are well known for their inputs to regulate reproduction (Peter *et al.*, 1991; Khan

and Thomas, 1993; Goos *et al.*, 1999; Senthilkumaran *et al.*, 2001). We have analyzed *tph* expression pattern during brain ontogeny, which showed that its expression was evident in the brain from 5 dph onwards till adulthood. Further we observed that the transcript was significantly high in male brains at 50 and 75 dph when compared to female brains of the same age group. Results from real-time PCR further revealed differential expression of *tph* mRNA in POA-HYP and TH regions of male and female brains during the period of sex differentiation. This was further confirmed by Western blot where in the protein expression was abundant in the POA-HYP and TH regions of male brain at 50 dph when compared to female brain. Taken together, *tph* expression is predominantly high during early development of male catfish brain.

Earlier reports (Tsai and Wang, 1999; Tsai *et al.*, 2000) showed that p-CPA (a *tph* blocker) treatments resulted in more number of females during early sex differentiation in the Nile tilapia. It was hypothesized that the gonadal steroids influence central 5-HT synthesis (Goos *et al.*, 1999) and aromatase activity (Tsai *et al.*, 2000) during the critical period of sex differentiation. The authors showed that the reduction in 5-HT levels and changes in aromatase activity in brain due to the treatment of pCPA resulted in more female population. Recently in the Nile tilapia, we observed dimorphic expression pattern of *tph* during critical period of sex determination where in the transcript was detected in brains of XY males around 5 - 25 dph but not in XX females of same age groups (Sudhakumari *et al.*, 2010). Unlike tilapia, we observed only gender differences in the expression of *tph* mRNA in catfish brain during development. This may be partially due to the analysis of *tph* mRNA in catfish after sex determination. However, the expression pattern of *tph* mRNA in direct correlation with 5-HTP and 5-HT levels in the male and female brain during early development has not been analyzed in teleosts. Hence, it is worthwhile to measure 5-HTP and 5-HT in the brain of male and female catfish during early development. We observed high

levels of 5-HTP and 5-HT in the POA-HYP and TH regions of male brain when compared to female brain at those regions during catfish development (50 dph) and the effect being more pronounced in POA-HYP. However, we found no changes in levels of 5-HTP and 5-HT in TEL and MO regions of male and female brains during development.

In order to get more concrete evidence, we further quantified the *tph*, 5-HTP and 5-HT levels in the POA-HYP + TH region of brain of juvenile fishes at 50 dph after completion of MT and pCPA treatments during early development. Treatment of MT promoted testicular differentiation resulting in more number of males (see chapter 5). On the other hand, pCPA treatments skewed fish population towards females by initiating ovarian differentiation as reported earlier by Tsai *et al.* (2000). We observed an increase in *tph* transcript levels which correlate with the high levels of 5-HTP and 5-HT in the brain of MT treated fish when compared to control fishes. Taken together, the levels of Tph mRNA and 5-HT showed gender-specific difference during sex differentiation in teleosts (Sudhakumari *et al.*, 2010; present study). This was clearly evident from the results obtained under normal and MT treated groups where in the levels of *tph* and 5-HT was high in the brain of male fishes during development. However, we cannot delineate the role of sex-specific genes like *dmrt1* and *sox9* during testicular differentiation. Interestingly, in the present study pCPA treatment has decreased the levels of 5-HT and *tph* in brain which resulted in more female population which is in accordance with the report of Tsai *et al.* (2000). The authors cited changes in aromatase activity might be one of the reasons for this effect. Taken together, it is plausible to suggest that gonadal sex differentiation may perhaps entrains brain sex differentiation, if any. Interestingly, earlier report by Holdway *et al.* (1988) showed high levels of brain 5-HT in adult male flag fish. These results together suggest that *tph* vis-à-vis central 5-HT system may favor male brain sex differentiation in teleosts.

In summary, the present study provided direct evidence for the sex-specific differential expression of *tph* in early stages of developing male and female brain of catfish which in turn correlated well with the levels of 5-HT and 5-HTP. Present work opens up a new area of research for in depth analysis of brain sex differentiation in lower vertebrates. Additional studies on momoaminergic system in detail may add new dimension to this area of research.

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Chapter 5

Histological analysis of gonadal sex differentiation in catfish and effect of exogenous steroids, methyltestosterone and ethynyl estradiol on developing gonads of catfish hatchlings

Abstract

The objective of the present study is to observe the effect of exogenous steroids, methyl testosterone (MT) and ethynyl estradiol (EE_2) on gonadal differentiation and analyze its effect on the expression of few genes during testicular and ovarian differentiation in juvenile catfish. Histological observations indicated initiation of gonadal sex differentiation in catfish around 40-50 days post hatch (dph). The MT- and EE_2-treatments skewed the hatchlings towards male and female development, respectively. This was further validated by specific expression of *dmrt1* and *sox9a* in the gonads of MT-treated group but not in the EEL-treated group at 50 dph. The reverse is true for the expression of *cyp19a1* and *foxl2*. Interestingly, the expression pattern of *3β hydroxy steroid dehydrogenase* did not show any change in EE_2- and MT-treated fish. The present study also provides a strategy to study sex differentiation, for those species where genetic sex population is unavailable.

Introduction

Sex differentiation in the fish show high plasticity as it can be influenced by several factors like genetic sex, temperature and exogenous steroid hormones (Nagahama *et al.*, 2004). In teleosts, sex steroids affect the development of germ cells and other cell-types during the process of gonadal sex differentiation (Devlin and Nagahama, 2002). Genetic sex can be reversed functionally by exposure to androgens or estrogens during critical period of sex differentiation or at juvenile stage (Piferrer *et al.*, 1993; Nagahama *et al.*, 2004). Monosex population can be obtained by crossing experimentally produced YY or XX males with normal females (Scholz *et al.*, 2003). This approach has been widely used in fishes like the Nile tilapia, medaka and rainbow trout in order to study the expression of several genes during gonadal and sex differentiation (Scholz *et al.*, 2003; Matsuda *et al.*, 2002; Sudhakumari *et al.*, 2003). Such an approach was not attempted in subtropical annual breeders like catfish. The manipulation of sex in fishes with exogenous steroids has been extensively studied to understand the effect of steroid during differentiation process. Androgen treatment of fishes in most cases is very effective in inducing masculinization of fish. The most common employed androgen in sex-reversal studies is 17α-methyltestosterone (MT), while estradiol 17β or ethynylestradiol (EE_2) is the most regularly used estrogen to induce feminization. For example, 100% masculinization can be achieved in chinook salmon with only a single immersion in MT at 400 μg/l administered at the time of hatching (Piferrer *et al.*, 1993). In contrast, estradiol 17β and ethynylestradiol induced complete feminization in chinook salmon (Piferrer and Donaldson, 1993). Treatment of zebrafish with estrogens during sexual differentiation causes feminization, and with testosterone, it induces masculinization (Yamazaki, 1976). Similarly, in medaka, estrogen treatment during the sexual differentiation period leads to permanent feminization

(Nakamura *et al.*, 1998).

To study the progression and critical period of gonadal sex differentiation, it is essential to track down the histological changes and expression pattern of sex-specific genes like *dmrt1*, *sox9a*, *foxl2* and *cyp19a1*. *Dmrt1* is expressed specifically in testis during gonadal development in most of the teleosts, suggesting its importance in testicular differentiation (Nagahama *et al.*, 2004). Two isoforms of *sox9* (*sox9a* and *sox9b*) have been identified, where *sox9a* is more specific to testis and required for testicular development. Though two forms of cytochrome P450 aromatase (ovarian [*cyp19a1*] and brain [*cyp19a2*] forms) have been identified in tilapia and other species, ovarian aromatase seems to play a major role in ovarian differentiation (Sudhakumari *et al.*, 2003). More recently, in addition to *cyp19a1*, *foxl2* is found to be more critical gene to impart ovarian differentiation and function (Wang *et al.*, 2004). The gonadal differentiation period is different in each species based on its breeding and developmental pattern. These variations create immense diversity on the ontogeny of various sex specific genes. In the present study, we aimed to study the expression pattern of sex-specific genes after driving catfish hatchlings to testicular and ovarian differentiation by treating them with exogenous steroids. We also intend to analyze the expression of *3β hydroxy steroid dehydrogenase* (*3βhsd*) to monitor steroidogenic activity, as it is one of the markers for the gonadal steroidogenesis.

Materials and Methods

Animals and Sampling

Catfish fingerlings were obtained by in vitro fertilization using mature spermiating male and hCG treated gravid female. The newly hatched catfish fries were fed with live tube worms. Exogenous treatments of methyl testosterone and ethynyl estradiol were given by immersing the steroids in fresh water containing hatchlings from the 2 days after hatching (dph) onwards. Then the treatments were intermittently in 6 durations (4, 8, 12, 16, 21 dph) for 3 h and after that the treated water was replenished with fresh water. The dosage used for MT and EE_2 was 500 µg/L and 100 µg/L, respectively. We used high doses of MT and EE_2 for short period of time as low doses were found to be ineffective in our pilot studies. 100 hatchlings were used in each group, where 10% mortality rate in MT-treated group and 30% mortality rate in EE_2-treated group was observed during treatment. After treatments the gonads were collected from the MT, EE_2 control group. The MT-treated fishes were reared till they reach maturity and after (one to two years) to see the morphology of mature gonads.

Histology

The gonadal regions of catfish hatchlings at 20, 30, 50, 75, 100, 150 dph were fixed in Bouin's solution, dehydrated, and embedded in paraplast (Sigma). Similarly the gonads of treated fishes at 50 dph fixed in Bouin's solution and finally embedded in paraplast. For light microscopy, 4 µm thick sections were cut and stained with hematoxylin-eosin. All the photomicrographs for histological analysis were taken using Olympus light microscope.

Semi-quantitative RT-PCR as described by Kwon *et al.* (2001) was carried out to study the expression patterns of few sex specific genes like *dmrt1*, *sox9a*, *foxl2* and *cyp19a1* in the gonads of treated fishes. For this, total RNA was prepared from developing gonads collected from the treated juvenile fishes at 50 dph. RT was performed as mentioned above. PCR amplification was done using a thermal cycler (Applied Biosystems) under following conditions: 94°C (1 min), 60°C (30sec), 72°C (1min), for 30 cycles using specific primers designed for the amplification of above mentioned genes (Table 1). Catfish β-*actin* was PCR amplified at 94°C (1 min), 60°C (30sec), 72°C (1min) for 28 cycles using *sox9a* and *sox9b* specific primers (Table 1) as an internal control.

Primer Name	Nucleotide Sequence (5′– 3′)	Usage
dmrt1F	**ATGCCGAAGTGCTCCCGGTGC**	**Semi-quantitative RT-PCR**
dmrt1R	**AGCGGCTCCCAGAGGCAGC**	**Semi-quantitative RT-PCR**
sox9aF	**GCAGAACTCAGCAAAACCCGG**	**Semi-quantitative RT-PCR**
sox9aR	**GCTGGAAGCGGGAGAGTCGG**	**Semi-quantitative RT-PCR**
foxl2F	**TGCGAGGACATGTTTGAGAAGGG**	**Semi-quantitative RT-PCR**
foxl2R	**TCCCAGTATGAGCAGTGCATCAT**	**Semi-quantitative RT-PCR**
cyp19a1F	**TTGGATCGGGAATTGGGACAGC**	**Semi-quantitative RT-PCR**
cyp19a1R	**AGCTTTAGCGAAGTAGCTGCG**	**Semi-quantitativeRT-PCR**

Table1. List of primers used for the expression analysis of dmrt1, sox9a, foxl2 and cyp19a1

Results

Histological observation of gonadal development in catfish under normal conditions

Conventional histological methods were used to study the onset of gonadal sex differentiation in catfish. A primitive gonad with primordial germ cells (PGCs) surrounded supporting cells was observed in the abdomen region near coelomic cavity at 20 and 30 dph (Fig. 1A and B). At 40 dph formation of two bipotential gonads were observed protruding out from the mesonephros (Fig. 1C). Higher magnification of bipotenial gonad showed development of germ cell surrounded by somatic/supporting cells (Fig. 1D). The first signs of morphological differentiation of gonads was evident by the formation of ovarian cavity in the developing female gonad around 45-50 dph (Fig. 2A and B). The ovarian differentiation was clearly observed by the presence of few meiotic oogonia and perinucleolar/primary oocytes (Fig. 2A and B). At 75 and 100 dph female gonads were completely filled with pre-vitellogenic ooctytes and few primary oocytes (Fig. 2C and D). Female gonad at 150 dph showed growing oocytes at different stages (Fig. 2E). Testicular differentiation in catfish begins around 50 dph where developing germ cells surrounded by supporting/sertoli cells were noticed (Fig. 3A). Male gonads at 75 and 100 dph showed the proliferation and differentiation of spermatogonia and oocytes (Fig. 3B and C). The completely developed male gonadal sections at 150 dph showing different stages of spermatogonial cells, spermatocytes and few spermatozoa/free sperm in the lumens indicated the progress of spermatogenesis (Fig. 3D). These results suggest that the testicular differentiation was somewhat delayed and it takes longer time when compared to ovarian differentiation. Generally under normal developmental conditions, we always obtain more number of females (65% of total mixed population) when compared to males, which is evident by both morphological and histological observations.

Histological observation of gonadal development in catfish treated with MT and EE_2

Morphological and histological analysis of gonads of treated fishes at 50 dph revealed that MT treatments skewed the hatchlings towards male development resulting in complete masculization (Fig. 4A and B). However, we obtained very few fishes (intersex) that have both testis and ovary tissues which is also evident by external morphology and HE staining (Fig. 4E and F). On the other hand EE_2 treatments promoted ovarian differentiation resulting in all females (Fig. 4C and D) and became mature (gravid) females after 1 year. When we reared some of the MT-treated fishes till they reach maturity (one year), most of the fishes were turned out to be complete males having testes. Interestingly, we obtained few intersex/bisexual fishes which contribute 20% of total male skewed population having both type of gonads or ova-testis (Fig. 5A). Histological examination of ova-testis gonad showed the presence of both testicular and ovarian tissues fused together (Fig. 5B). At higher magnification we could able find lumens containing spermatogonial cells, sperm and oocytes (Fig. 5C).

Expression pattern of few sex specific genes in developing gonads of MT- and EE_2-treated fishes

After the treatment of exogenous steroids, we studied the expression pattern of few sex specific genes (*dmrt1*, *sox9a*, *foxl2* and *cyp19a*) in gonads of treated juvenile fishes (50 dph) to check whether the treatments has completely induced masculization or feminization. We observed specific expression pattern of *dmrt1* and *sox9* in gonads of MT-treated group but not in EE_2-treated group at 50 dph (Fig. 6A and B). In contrast, the *cyp19a1* expression was evident in the EE_2-treated group but not in MT-treated group (Fig. 6C). While *foxl2* expression was high in

gonads of EE_2-treated group with very low expression in the gonads MT-treated group (Fig. 6D). The expression pattern of *3β-hsd* did not show any change in gonads MT and EE_2-treated group (Fig. 6E).

Expression pattern of few sex specific genes in mature gonads of adult MT- and EE_2-treated fishes

We reared few steroid treated juvenile fishes till maturity (1 year) to check the expression of sex specific genes. Ova-testis gonad of MT-treated fish showed expression of all the genes tested, i.e., *dmrt1, sox9a, foxl2, cyp19a1* (Fig. 7) as it contains both oocytes and spermatogenic cells. On the other hand, MT-treated fishes with mature testes showed the expression of *dmrt1* and *sox9a* only (Fig. 7). The expression of *foxl2* and *cyp19a1* were restricted to mature ovary of EE_2-treated fish, which possess pre-vitellogenic and mature vitellogenic follicles (Fig. 7).

Figures

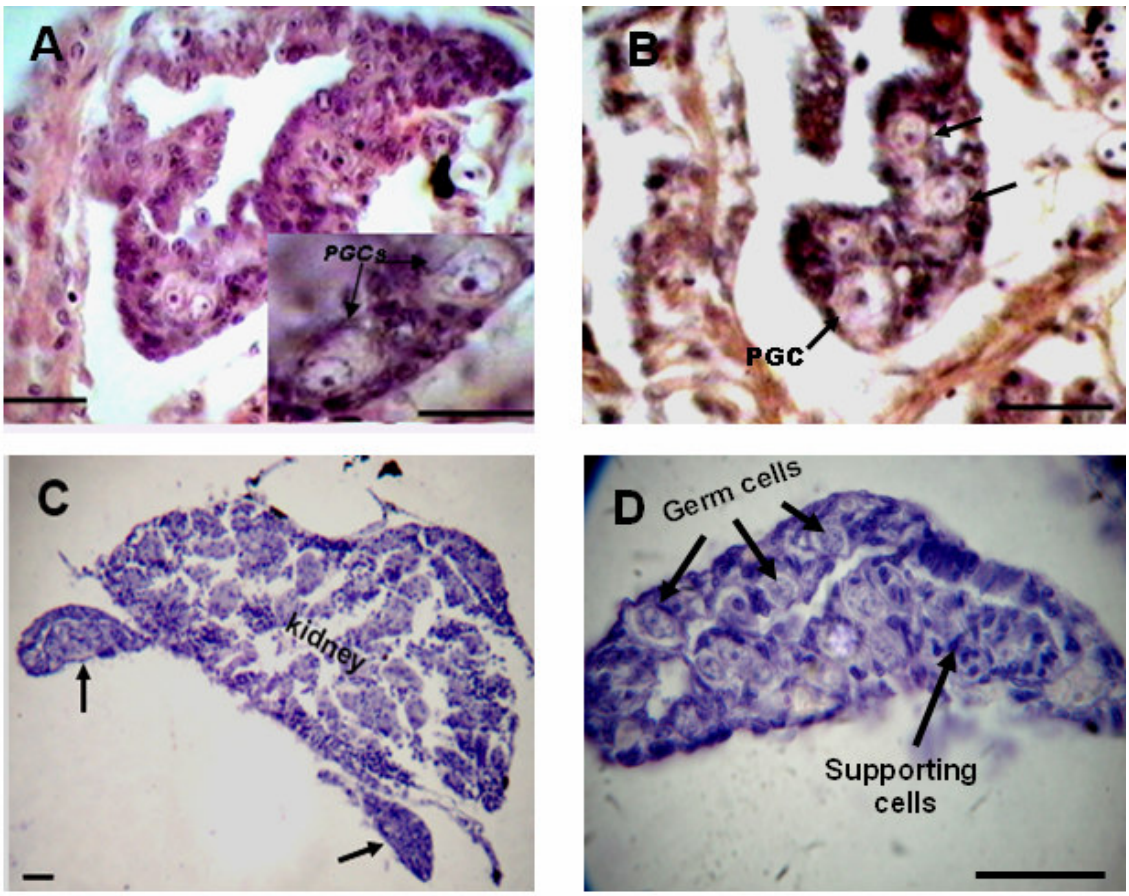

Fig. 1. Histology of indifferent gonads of catfish

A) Gonadal primordia at 20 dph showing primordial germ cells (PGCs). Inlet of fig.1A showing two PGCs at higher magnification. B) Primitive gonad at 30 dph showing PGCs surrounded by somatic/supporting cells. C) Gonadal section at 40 dph showing two bipotential/indifferent gonads attached to the mesonephros. D) Section of bipotential gonads at high magnification showing germ cells surrounded by supporting cells. All scale bars indicate 50 μm.

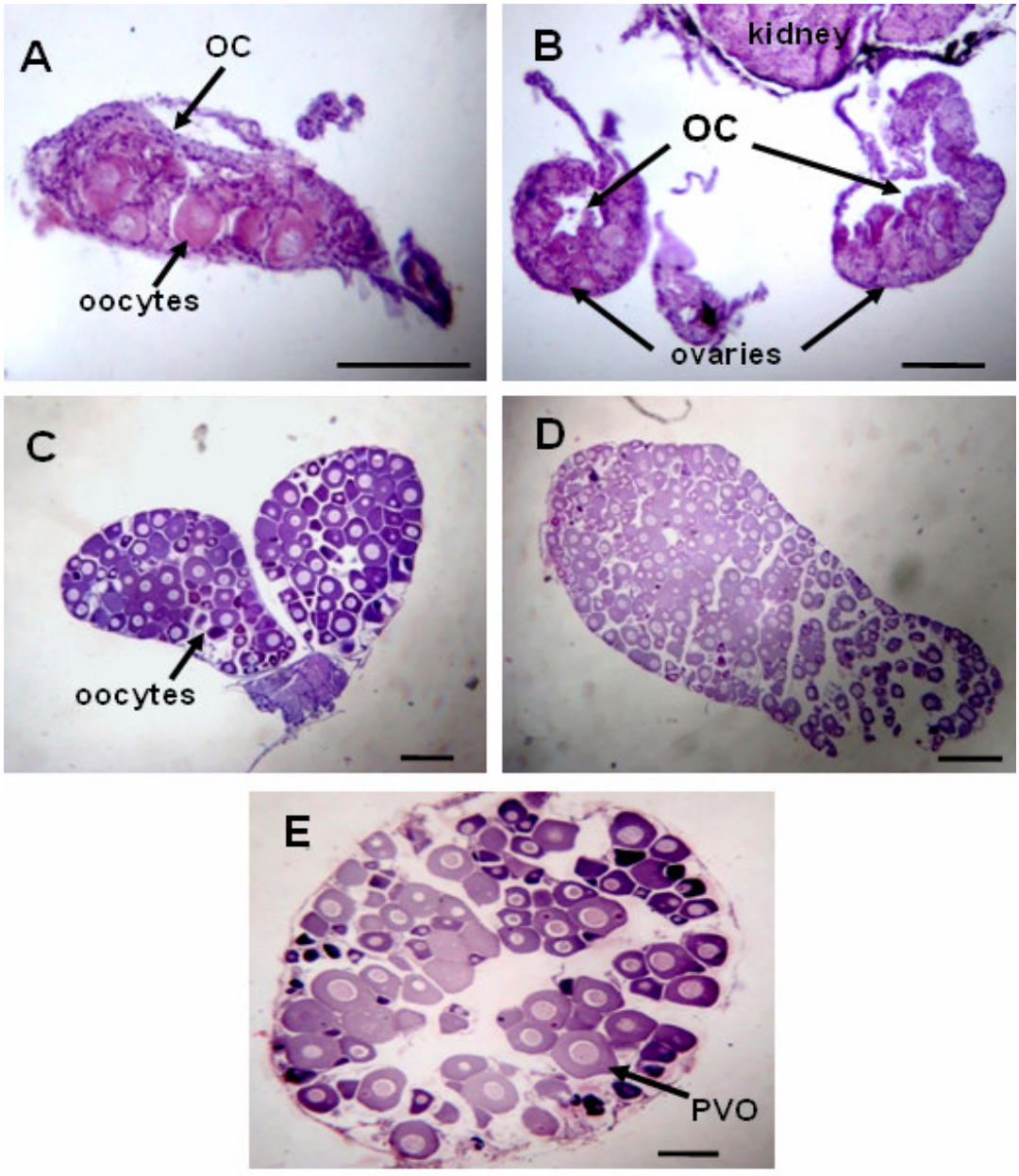

Fig. 2. Histology of developing female gonads of catfish

A) Differentiating female gonads at 45 dph. (OC) ovarian cavity is the first sign of ovarian differentiation. B) Differentiated ovaries at 50 dph showing primary oocytes. C) Developing ovary at 75 dph filled growing oocytes. D) and E) 100 and 150 dph ovary showing primary and pre-vitellogenic oocytes. (PVO) pre-vitellogenic oocytes. All scale bars indicate 50 μm.

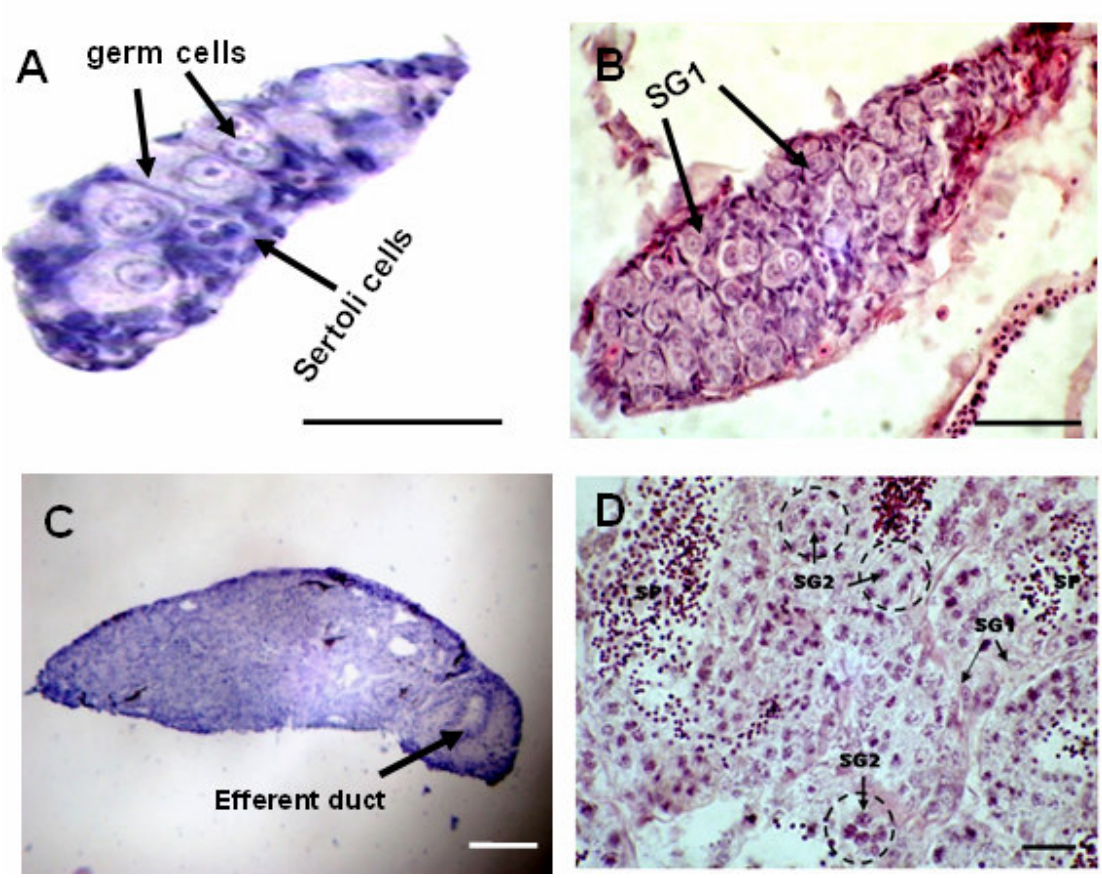

Fig. 3. Histology of differentiating female gonads of catfish

A) 50 dph differentiating testis having spermatogonial cells surrounded by Sertoli cells. B) 75 dph developing testis showing many proliferating primary spermatogonia. C) Testis at 100 dph with different spermatogonial cell types. D) 150 dph testis showing lumens filled with spermatozoa indicating progression of spermatogenesis. Primary spermatogonia (SG1), secondary spermatogonia (SG2) spermatocytes (SC), spermatid/spermatozoa (SP). All scale bars indicate 50 µm.

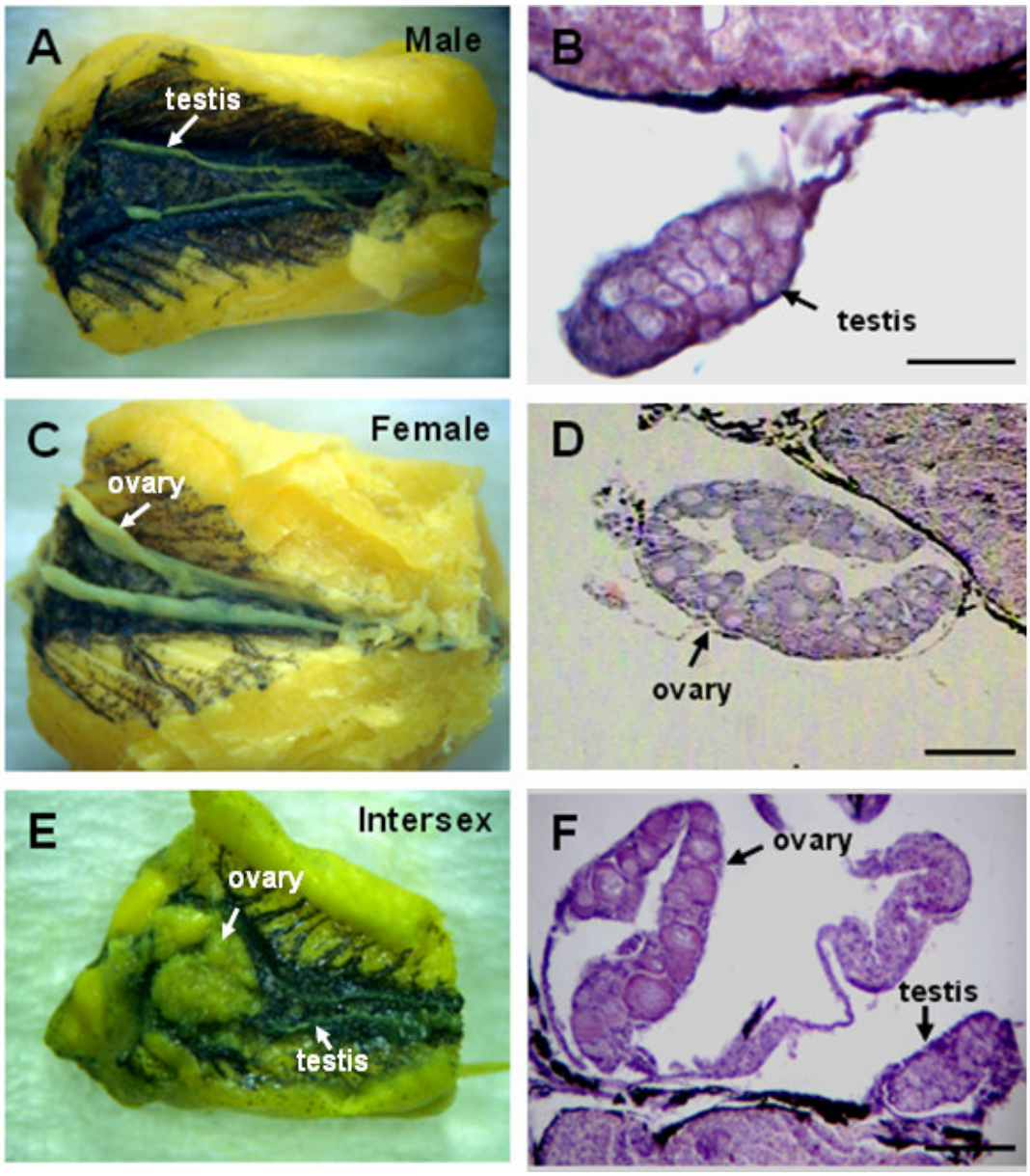

Fig. 4. External morphology of gonads of treated fishes: A) MT-treated fish showing testes, C) EE_2 treated fish showing developed ovaries and E) MT-treated fish which became intersex/bisexual having both testis and ovary.

Histology of gonads of treated fishes: B) Testis of MT-treated fish D) Ovary of EE_2-treated fish. E) Gonadal section of intersex fish showing both developing ovary and testis. All scale bars indicate 50 μm.

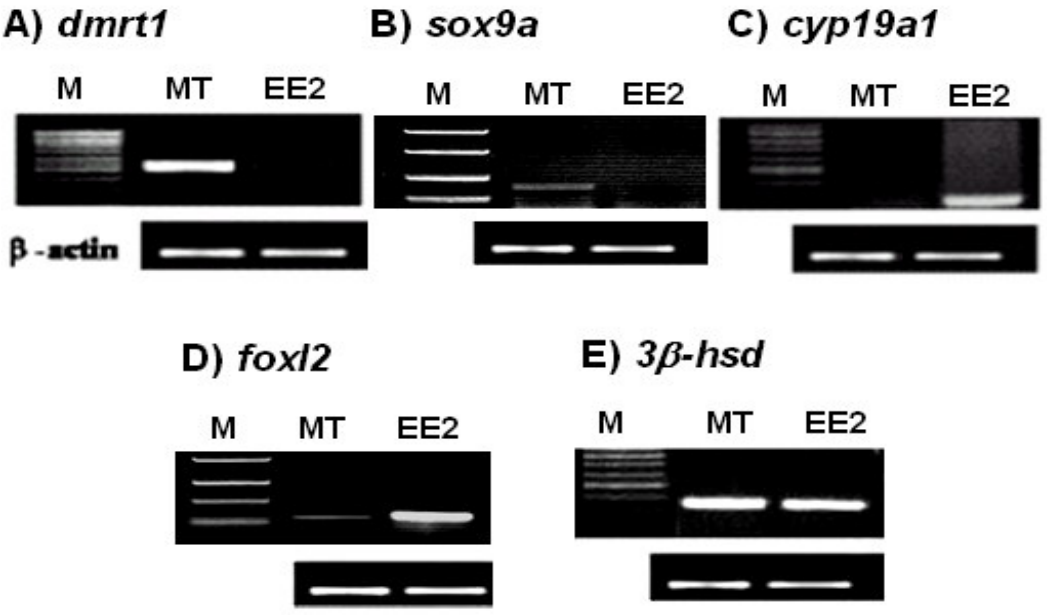

Fig. 5. Semi-quantitative RT-PCR analysis of sex-specific genes in gonads of treated fishes at 50 dph. A) Expression of dmrt1, B) Expression of sox9a, C) Expression of cyp19a1, D) Expression of foxl2, E) Expression of 3β-hsd.

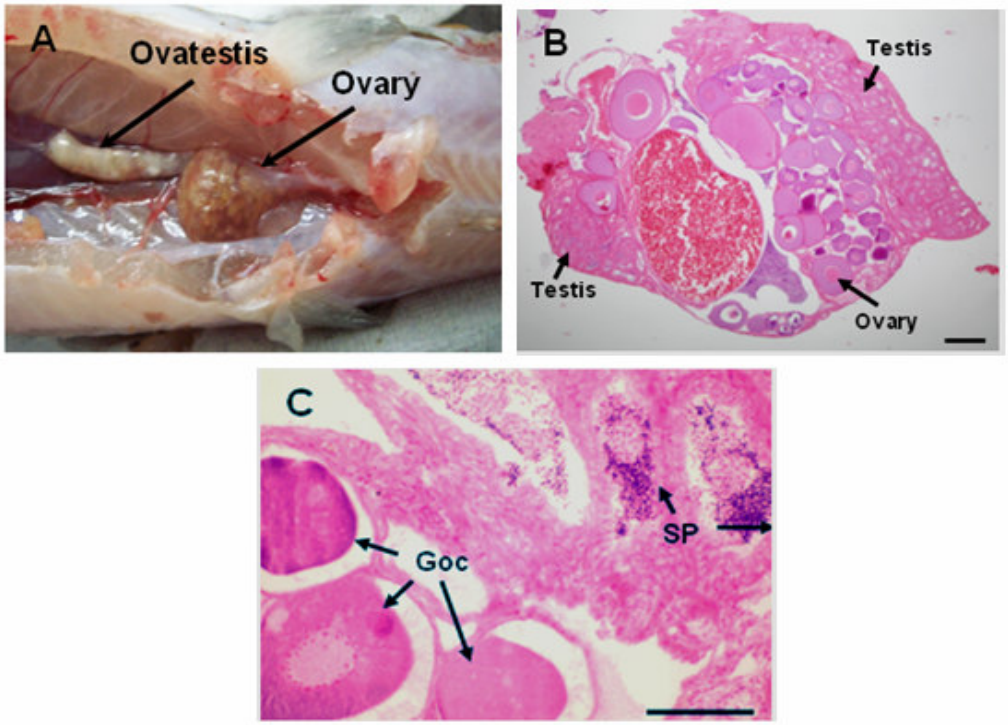

Fig. 6. A) Adult MT-treated intersex/bisexual fish showing morphology of ova-testis and developed ovary. B) Section of ova-testis showing testicular tissue on either side of the ovarian tissue. C) Ova-testis gonadal section at higher magnification showing lumens filled with both oocytes and sperms. Growing oocytes (Goc). All scale bars indicate 50 μm.

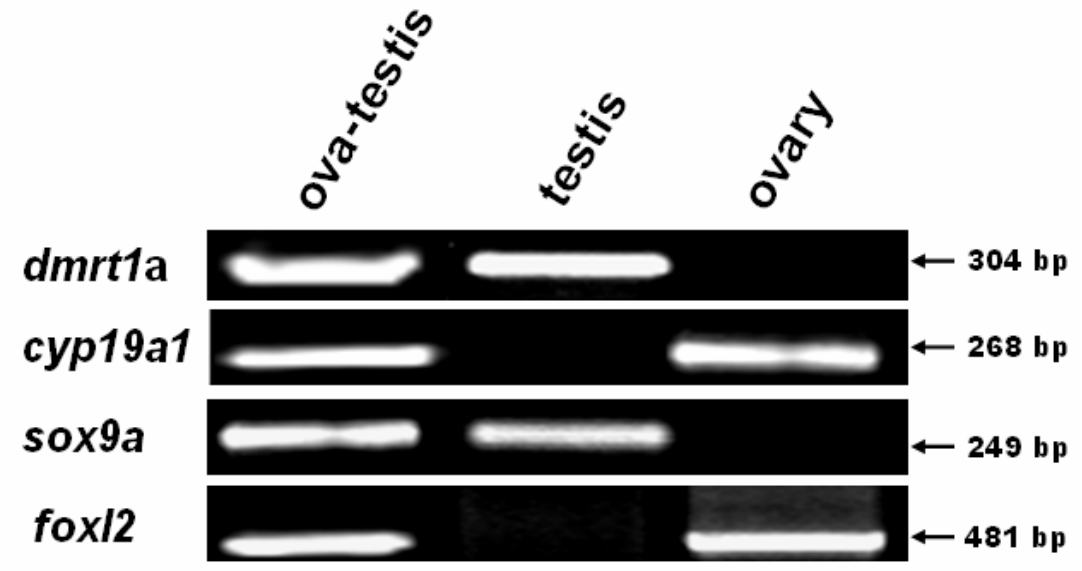

Fig. 7. Representative gel image showing expressions of dmrt1a, sox9a, foxl2 and cyp19a1 in ova-testis, mature ovary and mature testis of treated fishes which became adult (1 year old).

Discussion

In the present study, we depicted the onset of gonadal sex differentiation in catfish using simple histological observations of gonadal development at different age groups. We also studied the effect of exogenous steroids, MT and EE_2 on gonadal differentiation and analyze the expression of few sex-specific genes in gonads of treated fishes.

Based on our preliminary histological observations in catfish, the initiation of gonadal sex differentiation begins around 40 - 50 dph. This was also evident by the specific expression of genes like *dmrt1*, *sox9a*, *sox9b* (see chapter 2 and 4) and *cyp19a1* (Rasheeda *et al.*, 2010) during this period. Under normal developmental conditions in catfish, we always obtain more number of females when compared to males which was evident by histology. MT and EE_2 treatments given to catfish hatchlings before sex differentiation has got influence on the sex of the population. The MT treatments skewed the catfish fingerlings towards male development by initiating testicular differentiation, which was evident by the histology and expression of male sex specific genes like *dmrt1* and *sox9a*. On the other hand, the development of primary oocytes and expression of *cyp19a1* and *foxl2* in treated EE_2 developing gonads suggest that EE_2 treatment has promoted ovarian differentiation in catfish. These results together indicate dimorphic expression pattern of these genes during gonadal development in catfish. Exogenous hormone treatments (MT and EE2) before the critical period of sex differentiation resulted in complete sex reversal in the XX and XY genetic sex population of Nile tilapia (Kobayashi *et al.*, 2008). They also observed similar kind of sexual dimorphic expression of *dmrt1* and s*ox9a* during gonadal differentiation and hormone-induced sex reversal in tilapia. In rainbow trout estrogen treatment up-regulates female specific genes but does not suppress all male specific genes during male to female gonadal trans-differentiation (Vizziano-Cantonnet *et al.* 2008).

However, in the present study, we obtained both musculanized and intersex/bisexual fishes due to MT treatments. The expression of both male (*dmrt1* and *sox9a*) and female (*foxl2* and *cyp19a1*) sex-specific genes was observed in ova-testis gonad of MT-treated fish. In previous reports treatment of MT (1000 ng/L) in zebrafish and long term exposure to EE_2 (5 ng/L) in eel during gonadal development resulted in intersex fish (Orn *et al.*, 2003; Nash *et al.*, 2004).

In conclusion, the critical period of gonadal differentiation in catfish is around 40-50 dph. The exogenous steroid treatments effectively skewed the catfish hatchlings either towards male or female as per the nature (property) of the steroid. This strategy provides a scope to study gonadal differentiation/development in an annual breeder like catfish where genetic sex population is not available.

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Consolidated Summary

Present study examine histological changes in gonads during sex differentiation and expression analysis of *vasa*, *dmrt1* and *sox9* not only during gonadal development but also during recrudescence (reproductive cycle) in catfish. Since, brain has got a potential role in the neuroendocrine mediated regulation of reproduction, we intend to know the occurrence of sexual dimorphism (brain sex differentiation), if any in teleosts. To this end, we analyzed the expression of *tryptophan hydroxylase* in male and female brains during development. The present study also provides a strategy for steroid-induced manipulation of sex in catfish during development.

Initially we cloned the full-length cDNAs of *vasa*, *dmrt1* and *sox9a* from gonads of catfish using 5' and 3' RACE strategy. *Vasa* cDNA was isolated from both testis and ovary. We identified and isolated three isoforms of *dmrt1* (*dmrt1a*, *dmrt1b* and *dmrt1c*), from catfish testis of which *dmrt1*b and c are alternative spliced forms of *dmrt1*a. In the case of *sox9*, we obtained two isoforms *sox9a* from testis and *sox9b* from ovary which were not identical except in the conserved domain, HMG box. Tissue distribution analysis of these genes revealed that *vasa* was exclusively expressed in both male and female gonads. While *dmrt1* and *sox9* duplicates showed dimorphic expression pattern in gonads. Multiple forms of *dmrt1* were exclusively expressed in testis during gonadal development and in adult suggesting that *dmrt1* is testis-specific. *Sox9a* expression in adult and developing gonads was confined to testis, while *sox9*b was detectable only in ovary. This kind of dimorphic expression pattern of *sox9* duplicates in fish gonads indicates that *sox9a* retained its function as testis-specific gene while *sox9b* might acquire a new role to play in ovarian development. Expression analysis during gonadal development by real-time PCR showed that *vasa* was expressed from embryonic stage to adulthood. We also studied the fate of these genes in mature adult gonads during recrudescence (reproductive cycle) by real-time PCR. Quantification of *vasa*, multiple *dmrt1*s and *sox9*a transcripts by real-time PCR showed higher expression during preparatory and pre-spawning phases compared to spawning and post-spawning/regressed phases

of testicular cycle. This clearly suggests that the transcripts were abundant during period of spermatogenesis and thereafter decreases gradually during spawning/spermiation. While during ovarian cycle the *vasa* and *sox9b* transcripts were more in preparatory phase (during oogenesis) when compared to other phases. Both *vasa* and *sox9*b was abundantly expressed in stage I and II (immature) oocytes when compared to stage III and IV (mature vitellogenic) oocytes. We performed localization studies of *dmrt1* and *sox9* proteins in both juvenile and adult gonadal sections using immunohistochemistry (IHC) technique. IHC localization studies showed that *dmrt1* was detectable in germ cells (spermatogonia) but not in spermatozoa. While *sox9* expression was abundantly seen in Sertoli cells of testis and moderate expression was also noticed in germ cells of both developing and adult testis. This was also evident by the expression of *sox9* in oogonial cells in differentiating ovary at 50 days post hatch (dph). Its expression was in mature female gonad was seen only in stage I and II oocytes, but not in mature oocytes. In this study we also extended our work to understand the effect of gonadotropins on the expression pattern of *vasa* and *sox9* using hCG (functionally equivalent to LH in teleosts). We report up-regulation of *vasa* mRNA in a time-dependant manner due to hCG- induction in oocytes (*in vivo*) and testicular slices (*in vitro*). Treatment with hCG in the testicular slices showed a significant increase in expression of *sox9a* at 12 and 24 h time points, which suggest a role for gonadotropins in *sox9* regulation in testis. In fishes it is well known that hCG, *in vitro* induces testosterone and 11-KT production. Hence, it is possible that hCG might have induced *sox9a* via androgen production by acting upon Leydig cells. Therefore, we also intend to know the effect of 11-KT on *sox9a* expression in testicular slices, *in vitro*. In this study, for the first time we have shown that treatment of 11-KT significantly elevated the expression of *sox9a* in testicular slices.

Tryptophan hydroxylase (*tph*) is the rate-limiting enzyme in serotonin (5-HT) biosynthesis which has stimulatory effect on the release of gonadotropin releasing hormone and gonadotropins by

acting at the level of hypothalamo-hypophyseal axis. In the present study, we cloned full-length cDNA of *tph* from catfish brain and characterized the recombinant Tph protein by transient transfection in CHO cells. Real time PCR analysis in discrete regions of adult brain revealed that *tph* mRNA was abundantly expressed in the preoptic area-hypothalamus (POA-HYP) and thalamus (TH). Expression analysis of *tph* mRNA and protein in POA-HYP and TH of catfish brain revealed gender specific differences during early development (at 50 dph) which further correlated with 5-HTP and 5-HT levels. Changes in *tph* mRNA expression, 5-HTP and 5-HT levels in the POA-HYP region of methyltestosterone and para-chlorophenylalanine treated fish brains during development further endorse our findings. Our results suggest that sexual differences exist at the level of serotonergic system during early brain development in teleosts.

Histological observations of gonads during ontogeny/development revealed that the onset of gonadal sex differentiation in catfish is around 40-50 dph. This was further confirmed by the specific expression of genes like *dmrt1*, *sox9a* and *sox9b* in developing gonads during ontogeny. The first sign of morphological differentiation of gonads was evident by the formation of ovarian cavity in female differentiating gonads at 45 and 50 dph. Testicular differentiation in catfish begins around 50 dph where developing germ cells surrounded by supporting/Sertoli cells were noticed. Both male and female gonads of juvenile catfish around 100-150 dph showed the proliferation and differentiation of spermatogonia and oocytes, respectively. We also studied the effect of exogenous steroids, methyltestosterone (MT) and ethynyl estradiol (EE_2) on gonadal differentiation and analyze its effect on the expression of few sex-specific genes in gonads of treated fishes. Treatments of MT and EE_2 were given to catfish fingerlings before the critical period of gonadal differentiation to skew the sex of the population either towards male or female. Histological analysis revealed that MT treatment given to catfish hatchlings skewed towards male development during the course of gonadal differentiation while EE_2 treatment promoted ovarian differentiation

resulting in all females. This was further confirmed by the specific expression of *dmrt1*, *sox9a*, *foxl2* and *cyp19a1* (ovarian aromatase) in the gonads of MT-treated and EE_2-treated fishes, respectively. However, when we reared some of the MT-treated fishes till they reach maturity (one year), interestingly we obtained few intersex/bisexual fish which contribute 20% of total male skewed population. Histological examination of ova-testis showed the presence of spermatogenic cells, sperm and developing oocytes. Expression of both male (*dmrt1* and *sox9a*) and female (*foxl2* and *cyp19a1*) sex-specific genes was observed in ova-testis gonad of MT-treated fish. On the contrary, dimorphic expression pattern of male and female sex-specific genes were observed in completely developed testis and ovary. Histological observations and expression analysis of sex-specific genes during gonadal development in catfish revealed that the critical period of gonadal sex differentiation occurs around 40-50 dph and ovarian differentiation precedes testicular differentiation. Based on the present study and also from that of our laboratory we intend to propose a molecular mechanism of sex differentiation in catfish (refer Fig. 1).

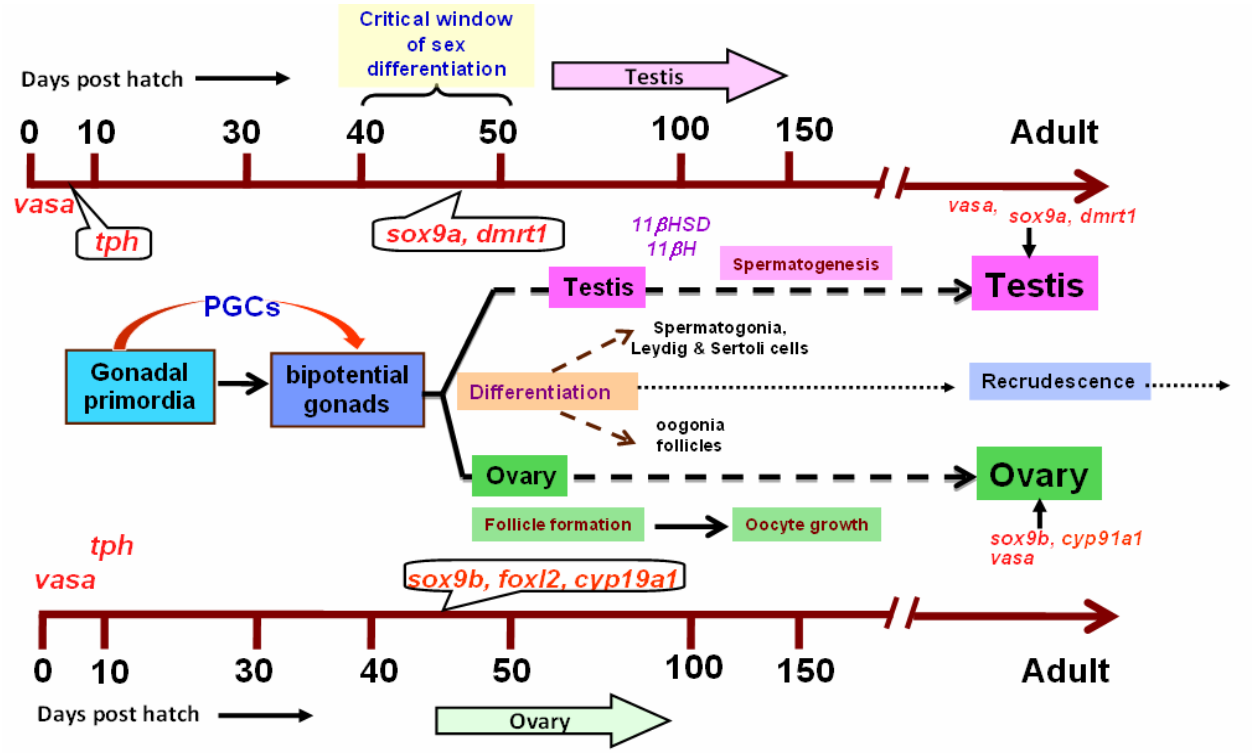

Fig. 1 Proposed molecular mechanism of gonadal sex differentiation in catfish

Research Publications

Publications from Ph.D. thesis work:

1. **Raghuveer K**, Senthilkumaran B 2010 Differential expression pattern of *sox9* duplicates during gonadal development, recrudescence and hCG-induced up regulation of *sox9* in testis of catfish, *Clarias gariepinus Reproduction* (*in press*).

2. **Raghuveer K**, Senthilkumaran B 2010 Cloning and differential expression pattern of *vasa* in the developing and recrudescing gonads in catfish, *Clarias gariepinus*. *Comparative Biochemistry and Physiology Part A* (*in press*), DOI 10.1016/j.cbpa.2010.04.017**.**

3. **Raghuveer K**, Senthilkumaran B 2009 Identification of multiple *dmrt1*s in catfish: localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment. *Journal of Molecular Endocrinology* 42: 437-448.

4. **Raghuveer K**, Sudhakumari CC, Senthilkumaran B, Wang DS, Nagahama Y, Dutta-Gupta A 2009 Differences in tryptophan hydroxylase expression in the brain of XX and XY Nile tilapia during early development. *Neuroscience Research* 65 (*Suppl. 1*) Abstract p. S219.

5. **Raghuveer K**, Rahul G, Wang DS, Bogerd J, Kirubagaran R, Rasheeda MK, Sreenivasulu G, Bhattacharya N, Tarangini S, Nagahama Y, Senthilkumaran B 2005 Effect of methyl testosterone- and ethynyl estradiol-induced sex differentiation on catfish, *Clarias gariepinus*: Expression profiles of *Dmrt1*, Cytochrome P450 aromatases and 3β-hydroxysteroid dehydrogenase. *Fish Physiology and Biochemistry* 31: 143-147.

In collaboration with Prof. B. Senthilkumaran's research team:

6. Sudhakumari CC, Senthilkumaran, B., **Raghuveer K**, Wang DS, Kobayashi T, Kagawa H, Krishnaiah Ch, Dutta-Gupta A, Nagahama Y 2010 Dimorphic

expression of tryptophan hydroxylase in the brain of XX and XY Nile Tilapia during early development. *General and Comparative Endocrinology* 166: 320-329.

7. Senthilkumaran B, Sudhakumari CC, **Raghuveer K**, Dutta-Gupta A, Nagahama Y 2009 Dimorphic Expression of GnRH, Gonadotropin and Tryptophan Hydroxylase Provides New Markers to Follow Brain Sex Development in Teleosts. *Neuroscience Research* 65 (*Suppl. 1*) Abstract p.S42**.**

8. Swapna I, Rajasekhar M, Supriya A, **Raghuveer K**, Sreenivasulu G, Rasheeda M. K, Majumdar KC, Kagawa H, Tanaka H, Dutta-Gupta A, Senthilkumaran B 2006 Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. *Comparative Biochemistry and Physiology Part A* 144: 1-10.

9. Supriya A, **Raghuveer K**, Swapna I, Rasheeda MK, Kobayashi T, Nagahama Y, Dutta-Gupta A, Majumdar KC, Senthilkumaran B 2005 Thyroid Hormone Modulation of Ovarian Recrudescence of Air-Breathing Catfish, *Clarias gariepinus*. *Fish Physiology and Biochemistry* 31: 267-270.

10. Jacob TN, Pandey JP, **Raghuveer K**, Sreenivasulu G, Dutta-Gupta A, Yoshikuni M, Jagota A, Senthilkumaran B 2005 Thyroxine-Induced Alterations in the Testis and Seminal Vesicles of Air-Breathing Catfish, *Clarias gariepinus*. *Fish Physiology and Biochemistry* 31: 271-274.

11. Rasheeda MK, Sreenivasulu G, Swapna I, **Raghuveer K**, Wang DS, Thangaraj K, Dutta-Gupta A, Senthilkumaran B 2005 Thiourea-induced alterations in the expression of some steroidogenic enzymes in the air-breathing catfish, *Clarias gariepinus Fish Physiology and Biochemistry* 31:275-279.