Involvement of brain aromatase, *FTZ-F1* and *FOXL2* in catfish ovarian development and recrudescence

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I, Parikipandla Sridevi, hereby declare that this thesis entitled "Involvement of brain aromatase, *FTZ-F1* and *FOXL2* in catfish ovarian development and recrudescence" 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 Institution for the award of any degree or diploma.

Date:

Name: Parikipandla Sridevi

Signature:

Regd. No.: 06LAPH02

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CERTIFICATE

This is to certify that this thesis entitled "Involvement of brain aromatase, *FTZ-F1* and *FOXL2* in catfish ovarian development and recrudescence" is a record of bonafide work done by Mrs. Parikipandla Sridevi, a research scholar for Ph.D. programme in the Department of Animal Sciences, University of Hyderabad under my guidance and supervision.

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

(Signature of Supervisor)

(Head of the Department)

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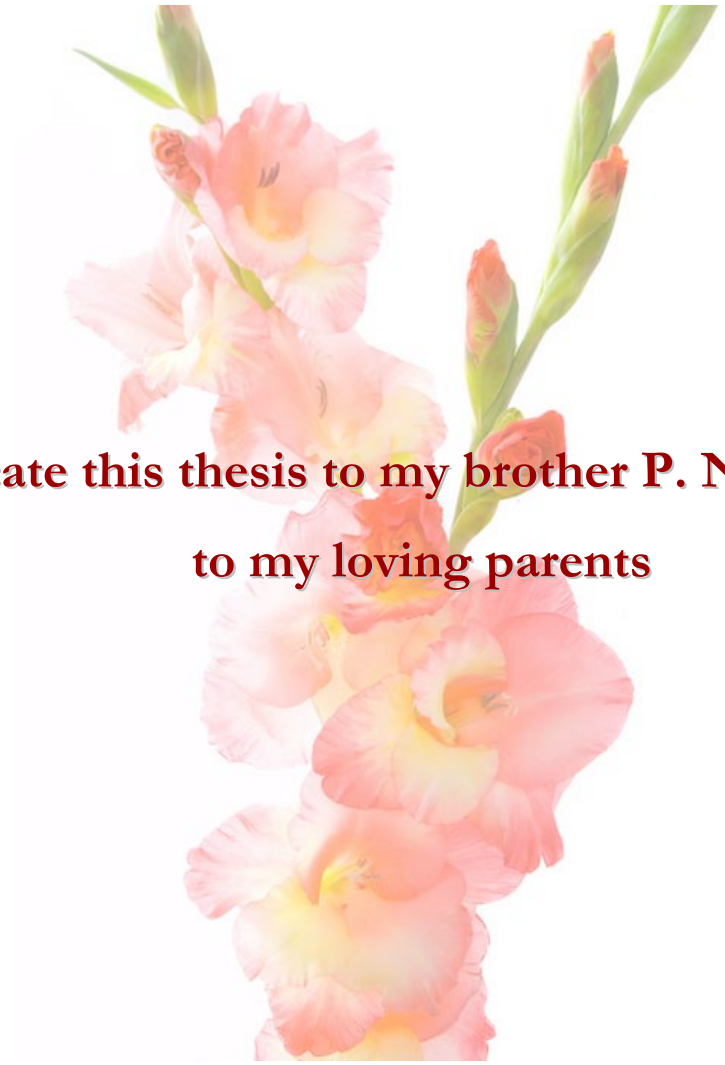

I dedicate this thesis to my brother P. Narahari and to my loving parents

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Sridevi…

Contents

General Introduction

Cytochrome P450 aromatase (*CYP19*) is the terminal enzyme in the steroidogenic pathway responsible for the conversion of androgens into estrogens. It belongs to cytochrome superfamily where cytochrome P450 enzymes (CYPs) constitute a superfamily of haem-thiolate proteins present in prokaryotes and throughout the eukaryotes. CYPs act as mono-oxygenases, with functions ranging from the synthesis and degradation of endogenous steroid hormones, vitamins and fatty acid derivatives (endobiotics) to the metabolism of foreign compounds such as drugs, environmental pollutants, and carcinogens (xenobiotics). The aromatase enzyme is part of an enzymatic complex which includes CYP19, the product of *CYP19* gene, and a NADPH-dependent cytochrome P450 reductase known as an ubiquitous flavoprotein (Simpson *et al.*, 1994). CYP19 is a microsomal enzyme localized in the smooth endoplasmatic reticulum steroidogenic cells. This enzyme is encoded by a single gene with multiple tissue specific promoters in both steroidogenic and non-steroidogenic tissues in vertebrates including mammals (Simpson *et al.*, 2002) except for pig, where multiple genes have been described (Choi *et al.*, 1996, 1997). Initially the authors have isolated two different genes encoding CYP19 which are specifically expressed in porcine periimplantation blastocysts, midpregnancy endometrium and placenta. Later, they identified three isoforms from blastocyst along with term placental and ovarian isoforms (Choi *et al.*, 1997). The CYP19 isoforms identified are expressed in the endometrium and placenta at midpregnancy, demonstrate a transition in synthesis of CYP19 isoform-specific mRNAs during placental development, and suggest the preferential involvement of the blastocyst CYP19 isoform in synthesis of estrogens that may function in feto-maternal signaling at periimplantation. Therefore, the developmental transition between isoforms may reflect functional differentiation of placental cells. Moreover, they speculate that the CYP19 products responsible for the synthesis of estrogens and perhaps other steroids, may perhaps

function in different capacities to mediate feto-maternal processes, respectively. In lower vertebrates including teleosts CYP19s play differential role during various stages of gamete development and maturation.

Interestingly, in teleosts existence of two CYP19s (ovarian form, *CYP19A1* and brain form, *CYP19A2)* is demonstrated in numerous fish species (Gelinas *et al.*, 1998; Tchoudakova and Callard, 1998; Trant *et al.*, 2001) which is a consequence of genome duplication (Simpson *et al.*, 1994) except in the eels, which belongs to the ancient group of elopomorphs (Jeng *et al.*, 2005). The *CYP19A1* gene is also called as the ‘‘gonadal aromatase” or ‘‘ovarian aromatase” (also referred as oP450arom, P450aromA, cyp19a or cyp19a1) as this gene is mainly expressed in the differentiating and adult gonads (mainly the ovary) of teleosts. *CYP19A1* gene and estrogens are pivotal for the regulation of ovarian differentiation, vitellogenesis and sex change in most teleosts (Wallace, 1985; Liu *et al.*, 2007; Nagahama and Yamashita, 2008; Kobayashi and Nagahama, 2009; Kazeto *et al.*, 2011). However, full grown immature post vitellogenic oocytes require subjugation of expression and enzymatic activity of CYP19A1 for final oocyte maturation (Nagahama and Yamashita, 2008; Senthilkumaran, 2011). Recent studies pointed out the importance of *CYP19A1* to regulate not only ovarian but also testicular differentiation in both gonochoristic and hermaphrodite fish species. The upregulation of *CYP19A1* is needed not only for triggering but also for maintaining ovarian differentiation, *CYP19A1* down-regulation is the necessary step for inducing a testicular differentiation pathway (Yamamoto, 1969; Wang *et al.*, 2010; Guiguen *et al.*, 2010), because suppressing *CYP19A1* gene expression, inhibits aromatase enzymatic activity or blocking estrogen receptivity are invariably associated with gonadal masculinization. Similarly, in the cases of temperature-induced masculinization and protogynous

sex change in hermaphrodite species, gonadal *CYP19A1* expression is turned off (Guiguen *et al.*, 2010). Despite high number of studies support the pivotal role of *CYP19A1* and estrogens, in ovarian differentiation of fish, there are still some results indicating it is as not an absolute rule.

The *CYP19A2* gene is called as the ‘‘neural aromatase” or ‘‘brain aromatase” (also referred as P450aromB, cyp19b, or cyp19a1bI) as this gene is highly expressed in the teleost brain in both males and females (Patil and Gunasekera, 2008). Recent studies have also demonstrated that the unique characteristic of sexual plasticity in teleosts (Fig. 1), is attributed to relatively much higher aromatase activity in brain of teleosts (100–1000 times higher) than in adult mammals (Pasmanik and Callard, 1985; Le page *et al.*, 2010) is due to the massive expression of *CYP19A2* gene, encoding the CYP19A2 protein (Diotel *et al.*, 2010). As suggested by these reports that neuronally derived E_2 regulates neuronal differentiation, survival and function in mammals (Toran-Allerand, 1996), this high level of *CYP19A2* gene expression is thought to be related to the exceptional capability of the teleost brain to grow indefinitely, its neuroplasticity and its neuronal regeneration capability during adulthood (Forlano *et al.*, 2001; Menuet *et al.*, 2003). Additionally, the developmental pattern is quite different from mammals where *CYP19A2* activity is highest during prenatal organization and development and declines in adulthood (Lephart, 1996). It is likely that, in fish, *CYP19A2* expression in progenitors is one of the key features enabling brain growth and brain sexualization throughout life (Peter *et al.*, 1990). Furthermore, it is known that the brain of many adult fish display sexual dimorphisms (Forlano *et al.*, 2001; Diotel *et al.*, 2010, Okubo *et al.*, 2011), though the role of *CYP19A2* expression during development in shaping these sexual dimorphisms is not well understood.

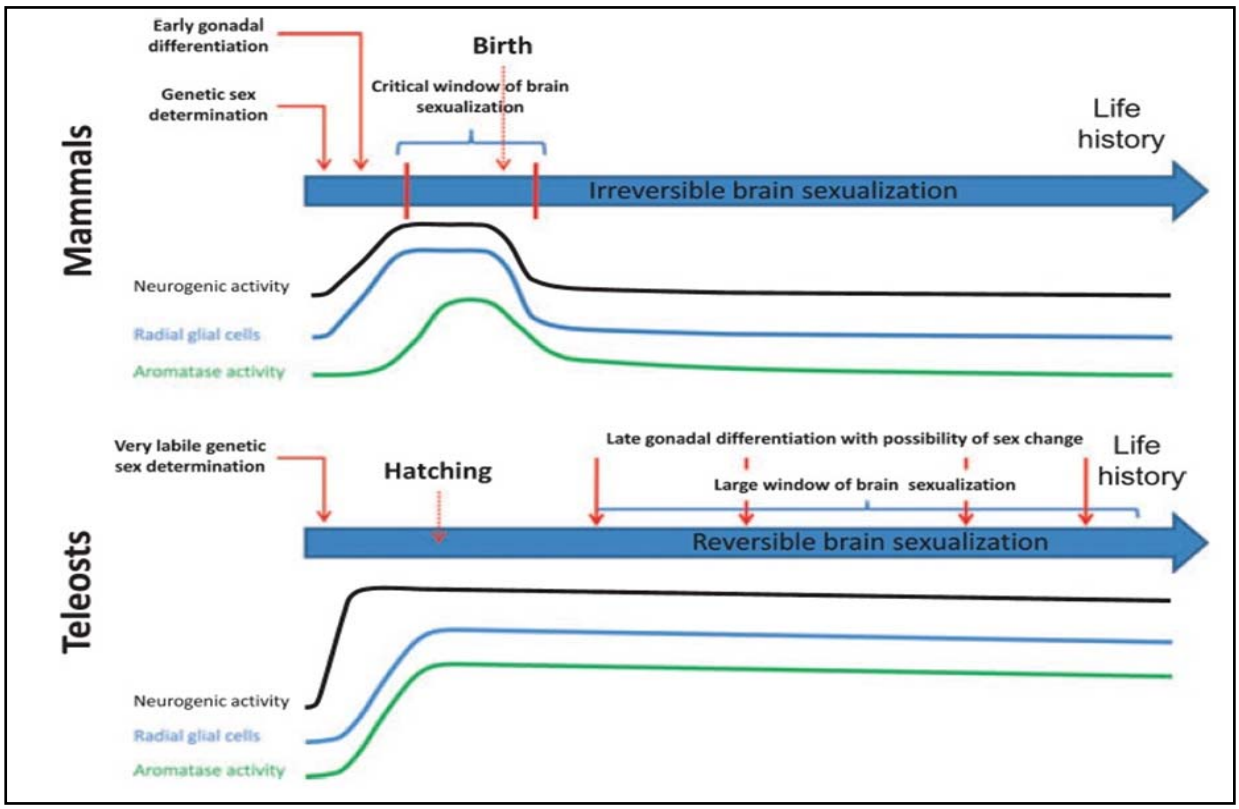

Fig.1. Schematic representation of life history in fish compared to mammals, highlighting the major differences that may affect the process of brain plasticity. (Adopted from Le Page *et al.*, 2010)

CYP19A2 expression is demonstrated as specific to brain in many teleosts, however in the Japanese medaka, (Patil and Gunasekera, 2008), rainbow trout (Blázquez *et al.*, 2008), Wrasse, (Sundaray *et al.*, 2005) and the Nile tilapia, (Kwon *et al.*, 2001; Chang *et al.*, 2005), its expression has been investigated during gonadal differentiation and was found to be expressed at low levels both in male and female gonads also. Furthermore, in the Nile tilapia, *Oreochromis niloticus* and the Japanese fugu, *Takifugu rubipres* (Sudhakumari *et al.*, 2003, 2005; Rashid *et al.*, 2007), the *CYP19A2* gene was expressed in the early developing testis and also gets induced following female-to-male androgen-induced sex change. However, the implications of this early

CYP19A2 expression in the differentiating testis are not yet known. In addition to the brain, the teleost pituitary highly expresses the *CYP19A2* gene (Gelinas *et al.*, 1998; Menuet *et al.*, 2003), whereas the mammalian pituitary is generally considered CYP19-negative except that of rat pituitary (Callard *et al.*, 1983). This further suggests that *CYP19A2* gene may have a role in the pituitary gland of teleosts and other vertebrates.

Structurally, like other vertebrate CYP19 genes and teleost *CYP19A1*, *CYP19A2* also exhibits a series of evolutionary well conserved domains, including the I-helix region, the Ozol's peptide region, the aromatase-specific region and the heme-binding region. Moreover, these three putative functional domains identified in mammalian *CYP19* forms, were significantly more identical (97, 83 and 100% respectively) than the rest of the predicted CYP19A2 protein in teleosts (Trant *et al.*, 2001). The anatomical basis of *CYP19A2* in teleosts was first documented in the plainfin midshipman, where the highest mRNA and protein signals were mainly detected in the ventricular surface of the telencephalon, preoptic area and hypothalamus (Forlano *et al.*, 2001). Based on the radial morphology of the CYP19A2-positive cells, and the fact that it co-localized with glial fibrillary acid protein and vimentin (both glial specific markers), *CYP19A2* was reported to be almost exclusively expressed in radial glial cells (Forlano *et al.*, 2001). Such cells are observed in abundance in the pallial and subpallial regions, in the preoptic area and the mediobasal hypothalamus. A similar distribution pattern was also described in the rainbow trout, zebrafish, pejerrey, and bluehead wrasse (Menuet *et al.*, 2003, 2005; Strobl-Mazzulla *et al.*, 2005). Additionally, *CYP19A2* expressing radial glial cells are also observed in the thalamus, the periventricular layer of the torus semicircularis, the optic tectum and around the fourth ventricle (Forlano *et al.*, 2001; Menuet *et al.*, 2005; Strobl-Mazzulla *et al.*, 2005; Diotel *et al.*, 2010).

Thus, radial glial cells, expressing *CYP19A2*, sustain the high neurogenic activity of adult fish. This cell proliferation is not restricted to the telencephalon, but is observed in all forebrain regions (Pellegrini *et al.*, 2007).

It has also been suggested that the expression of *CYP19A2* influences the brain-pituitary-gonadal axis of reproductive physiology, especially with reference to steroid feedback onto gonadotropin and/or gonadotropin-releasing hormone (GnRH) secretion (Khan *et al.*, 1999) however, little attention has been received to address this hypothesis. Earlier sex steroids along with estradiol 17β (E_2) levels were measured in plasma and ovaries of the catfish, *Clarias batrachus*, mainly over the course of their annual reproductive cycle (Singh and Singh, 1987; Joy *et al.*, 2000) and also in *Clarias gariepinus* (Rasheeda *et al.*, 2010). Seasonal fluctuations in *CYP19A1* expression and activity levels are well documented in female sea bass and the Nile tilapia where they have attributed these fluctuations are accountable for follicular growth and vitellogenesis (González and Piferrer, 2003; Chang *et al.*, 2005). But this was not analysed earlier for *CYP19A2*.

Ovarian differentiation and the processes of follicle development, oocyte maturation and ovulation are complex events, requiring the coordinated action of regulatory molecules (Fig. 2). Similar to other vertebrates, hormones from the hypothalamo-hypophyseal-gonadal axis play important roles in regulating follicle development (Ankley *et al.*, 2004). Special emphasis is placed on how follicle development and oocyte maturation in adult females is regulated by gonadotropins, ovarian steroids and growth factors produced by the ovary (Nagahama, 1994; Bhattacharya *et al.*, 2007). Under the influence of GnRH, the pituitary secretes Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) which act upon the gonads, stimulating their growth, production of eggs or sperm and synthesis of gonadal hormones

(Holland *et al.*, 2001). Feedback systems between the gonads, hypophysis and hypothalamus regulate expression and release of GnRH, gonadotropins (FSH and LH) and sex steroids.

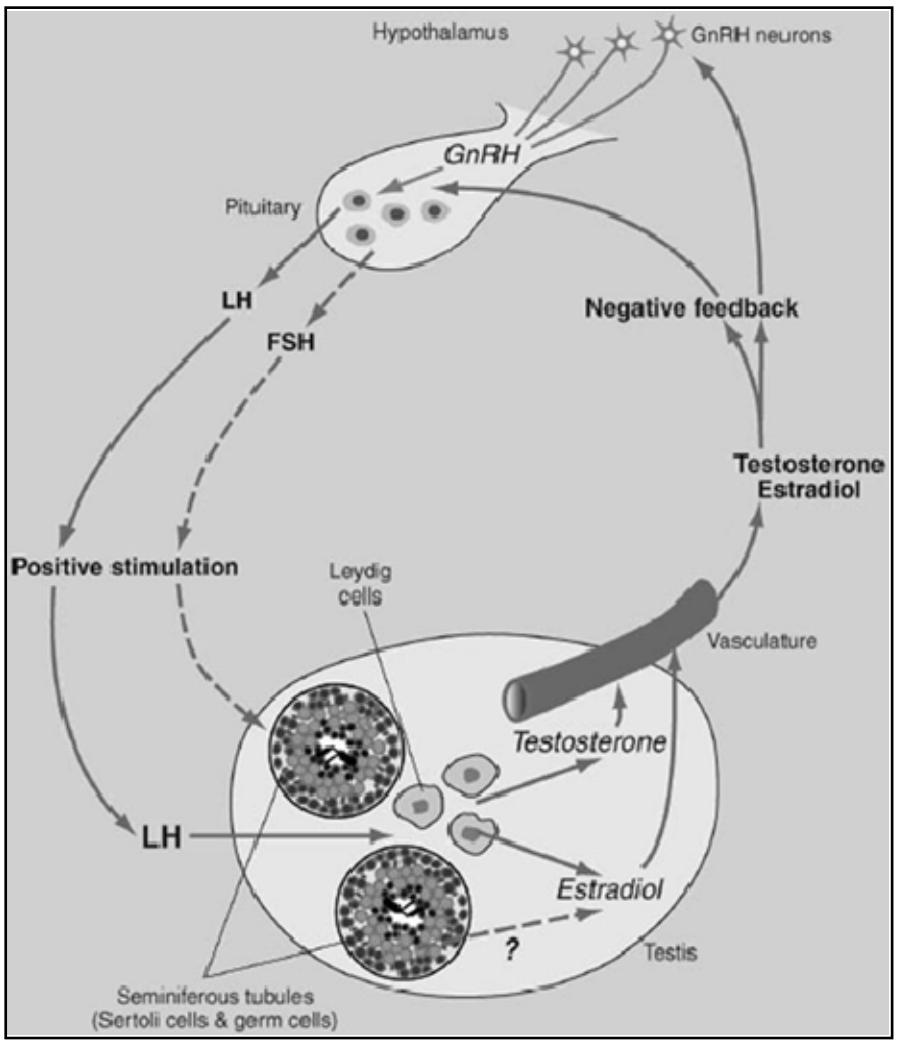

Fig.2. Overview of the teleost hypothalamo-hypophyseal-gonadal axis. (Adopted from Ankley *et al.*, 2004)

Direct innervation of the hypophysis by the neurons originating from the hypothalamus precludes the requirement of hypothalamo-hypophyseal-portal system in teleosts (Peter *et al.*, 1990). In fishes, the factors that are involved in regulating gonadotropin 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.*, 1990; Joy *et al.*, 1998; Goos *et al.*, 1999; Senthilkumaran *et al.*, 2001). Pituitary LH induces receptor-mediated changes in gonadal enzymes like 11β-hydroxylase and *CYP19A1* (Kagawa *et al.*, 1982). In this regard, a two-cell type model has been proposed (Nagahama, 1994). The outer thecal cell layer under the influence of gonadotropin produces the androgen substrate (testosterone) which diffuses into the granulosa cell layer where the *CYP19A1* is localized for the conversion of E_2 (Nagahama, 1994). Similarly during oogenesis, plasma FSH levels induced follicular E_2 production, which in turn, stimulated hepatic vitellogenin synthesis (Wallace, 1985; Specker and Sullivan, 1994) followed by a rise in plasma LH levels, subsequently gonadotropin binding to its receptor on granulosa cells (Oba *et al.*, 1999) and stimulates a sequence of events including shift in steroidogenesis, acquisition of oocyte maturation competence, production of maturation-inducing hormone (MIH), MIH dependent resumption of meiosis and cytoplasmic maturation (Goswami and Sundararaj, 1974; Kanamori and Nagahama, 1988; Haider, 2003; Matsuyama *et al.*, 2005; Nagahama and Yamashita, 2008; Senthilkumaran, 2011) has been demonstrated. Although MIH is generally considered as mediator of LH-induced meiotic resumption, other factors like activins (Ge, 2000) are also known to mediate or modulate this process have been shown to induce final oocyte maturation. However in catfish, single form of gonadotropin has been purified, which showed seasonal variation and the interactions between fish gonadotropin and their receptors, appear to be less discriminatory (Bogerd, 2005; Kirubagaran *et al.*, 2005). We also analysed the responsiveness of catfish *CYP19A2* gene and enzyme activity after induction with gonadotropin (hCG) to correlate with *CYP19A1* expression in earlier findings from amago salmon where

gonadotropins potentiated oocyte growth and maturation (Kagawa *et al.*, 1982; Young, 1986). Additionally, earlier report from the North African catfish have demonstrated the presence of LH receptor in ovary and brain and *in vitro* studies showed positive response of LH receptor by catfish LH, salmon LH, human LH, hCG, and human FSH by Vischer and Bogerd (2003) which made possible the use of non-homologous gonadotropin, i.e., hCG to study the modulation.

The *CYP19A2* promoter region has also been investigated in fish and it contains many putative sites for transcription factors, mostly shared both in the *CYP19A1* and *CYP19A2* genes, with the notable exception that while *CYP19A2* contains sites for putative neurotrophic factors and estrogen responsive elements (ERE). It is also reported that ERα is probably needed for programming of the hypophysis that involves sex-dependent expression of neuronal CYP19 (Roselli and Klosterman, 1998). Furthermore, our study attempts to describe the 5' flanking region of *CYP19A2* gene and putative *cis*-acting elements therein. It is theorized that the isolation of *CYP19* genes and subsequent analysis of their 5' flanking regions for putative regulatory elements will aid towards the elucidation of the physiological mechanisms controlling sex differentiation process. In the context of gonadal sex differentiation, several binding motifs were identified in *CYP19* promoter sequences earlier. Among them, Ad4 binding protein/steroidogenic factor 1 (NR5A1/Ad4BP/SF-1), liver receptor homologue 1 (NR5A2/LRH-1), fushi tarazu factor 1 (FTZ-F1), cAMP responsive elements (CRE), GATA binding proteins, forkhead domain transcription factor L2 (FOXL2), Wilms' tumor 1 protein (WT1) and half sites for estrogen responsive elements (ERE) and estrogen receptor α (ERα) were majorly identified in different teleosts. All these factors are actually known to be important players in the sex differentiation cascade of vertebrates (Brennan and Capel, 2004). But among these putative

transcription factors potentially acting as cis-elements only a few of them have been experimentally demonstrated to directly regulate fish *CYP19*s. Reports exist on a plausible role of ERE and Ad4BP/SF-1 in regulating the spatial expression of CYP19 in the brain and ovary, respectively. In mammals, the Ad4BP/SF-1 is a key regulator of endocrine function and sex determination (Sadovsky *et al.*, 1995). The synergistic expression of *Ad4BP/SF-1* and *CYP19A1* in tilapia ovary during vitellogenesis has been reported by Yoshiura *et al.* (2003). Several genes belonging to NR5A family homologous to *Ad4BP/SF-1* have been linked to the process of gonadal differentiation in zebrafish, for example, the *Drosophila* fushi tarazu factor 1 (*FTZ-F1*) and Liver Receptor Homologue 1 (*LRH-1*) (reviewed by von Hofsten and Olsson, 2005). FTZ-F1 has also been reported to potentially regulate *CYP19A1* in medaka (Watanabe *et al.*, 1999)**.** *FTZ-F1* genes in teleosts are of central interest as they are involved in regulating interrenal development and thereby steroid biosynthesis, as well as that they show expression patterns congruent with reproductive tissue differentiation and function. Kuo *et al.* (2005) have put forward that *FTZ-F1* genes are strongly linked to steroid biosynthesis as the regulatory region of *CYP19* contains the binding sites for *FTZ-F1* genes in zebrafish. Thus, in teleosts, *FTZ-F1* could play an important role in the initiation of puberty via regulation of steroidogenic enzyme gene expression.

FOXL2 is one of the very few ovarian differentiation genes known in vertebrates (Baron *et al.*, 2004) which has been shown to interact with ligand binding domain of Ad4BP/SF-1 and bind to promoter of *CYP19A1* and hence transcriptionally regulating it in the Nile tilapia (Wang *et al.*, 2007). Interestingly, while it is well known that *FOXL2* upregulates *CYP19A1* expression, ultimately leading to ovarian differentiation (Wang *et al.*, 2007), recent data in mice have

elegantly demonstrated that inducible deletion of *FOXL2* in adult ovarian follicles causes instant upregulation of testis-specific genes including Sox9, the critical SRY target gene (Uhlenhaut *et al.*, 2009). *FOXL2* has been reported to be related to *CYP19A1* and ovarian development in rainbow trout and medaka (Baron *et al.*, 2004; Nakamoto *et al.*, 2006). The results on *FOXL2*, and *CYP19A1* were closely related to teleost sex differentiation, and the gonadotropin subunits were possibly related to ovarian differentiation and oocyte development (Swanson *et al.*, 1991; Liu *et al.*, 2007). Though binding elements for FTZ-F1 and FOXL2 in the *CYP19A2* promoter region have been reported in teleosts, their regulatory role with reference to *CYP19A2* has not yet been elucidated.

Teleosts 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). Further, their sex can be manipulated or completely reversed by exogenous sex steroid treatments around the critical period of sex determination/differentiation (Nagahama, 2005; Kobayashi *et al.*, 2009; Raghuveer and Senthilkumaran, 2009). Estrogens have long been regarded as important hormones for ovarian differentiation in non-eutherian vertebrates. Administration of estrogens can reverse phenotypic males to female has been demonstrated in teleosts. The effects of estrogen treatment on fish sex differentiation have been investigated since the late fifties, however, physiological and molecular studies on the role of estrogens in fish have initially been developed mainly in relation to vitellogenesis only. A number of consensus transcriptional regulatory elements were identified in the 5'flanking regions of *CYP19A1* and *CYP19A2* genes (Kazeto *et al.*, 2001; Yoshiura *et al.*, 2003; Chang *et*

al., 2005) that are responsive to estradiol analogues, which suggests that *CYP19* genes would make excellent transcriptional targets for endocrine disruption. As mentioned before, the *CYP19's* has a proximal promoter of a well-characterized ERE, and hence can be sensitive to estrogens and the mutation of which abolishes the response to estrogens (Menuet *et al.*, 2005; Diotel *et al.*, 2010; Le Page *et al.*, 2008). Kazeto and Trant, (2005) demonstrated that the addition of ethynyl estradiol to zebrafish fry up-regulates *CYP19A2* while the gonad isoform remains unaffected, suggesting specific transcriptional regulation for the brain isoform via ERE. Studies on the effect of estradiol analogues like diethylstilbestrol (a non steroidal analogue, DES) and ethynyl estradiol (steroidal analogue, EE_2) on the expression of both forms of *CYP19s* and their transcription factors in different age group fish would be interesting if correlated to ovarian development.

In depth research work related to involvement of CYP19s in mammalian sex differentiation has been reported along with few teleosts where are mostly of the work restricted to daily or fortnight breeders like zebrafish, medaka and tilapia. In this regard, annual breeders that undergo seasonal pattern of gonadal 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. In order to study the expression pattern and to understand the role of transcription factors in regulating the steroidogenic enzyme genes during sex differentiation and gonadal recrudescence we chose to work on the North African air-breathing catfish, *Clarias gariepinus* (Fig. 3). It is a gonochoristic annual breeder undergoing seasonal pattern of reproductive cycle (Fig. 4). Major reasons for the

Fig.3. Photograph of the North African air-breathing catfish, *Clarias gariepinus*. Classification: Eukaryota; Chordata; Vertebrata; Teleostii; Siluriforms; *Clarias*.

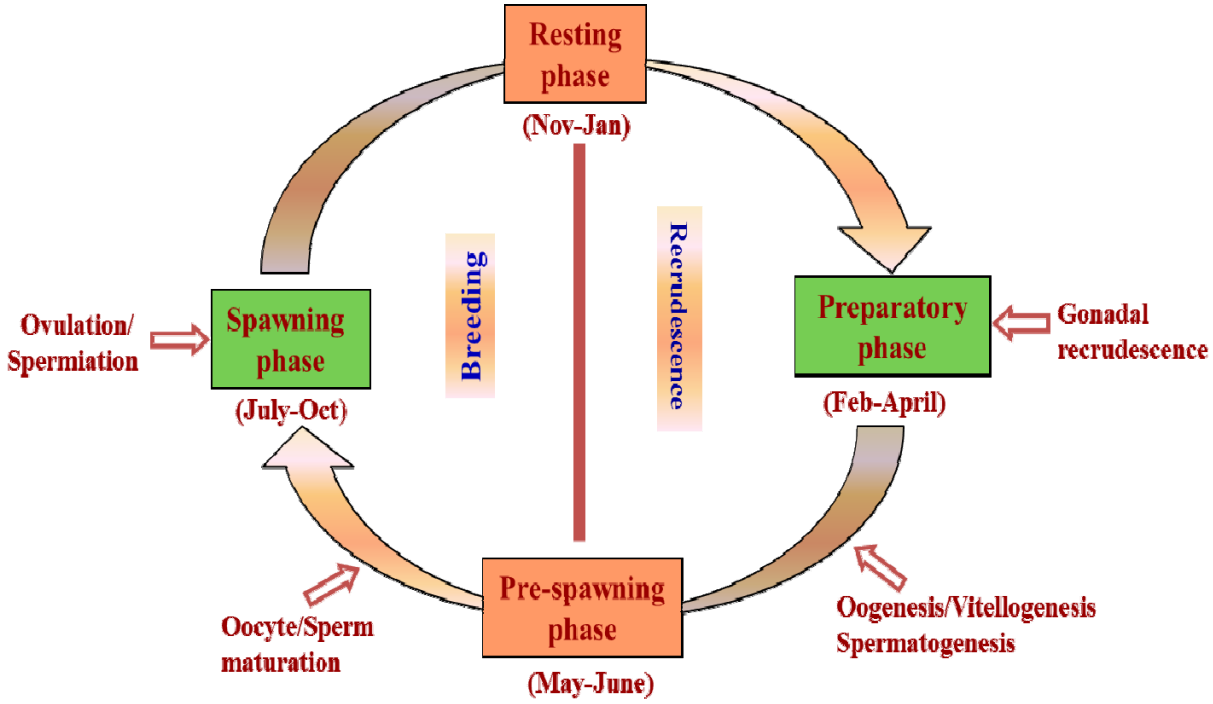

Fig.4. Reproductive cycle of *C. gariepinus*: Is under the influence of catfish gonadotropin-releasing hormone-gonadotropin-sex steroids.

choice of this animal model include synchronized reproductive cycle and fast growth (Fig. 5). As we have established *in vitro* fertilization, breeding and rearing in our laboratory, developmental studies are also possible. Moreover, *in vivo* studies can be done without sacrificing animals when it comes to collection of oocytes (Sreenivasulu and Senthilkumaran, 2009).

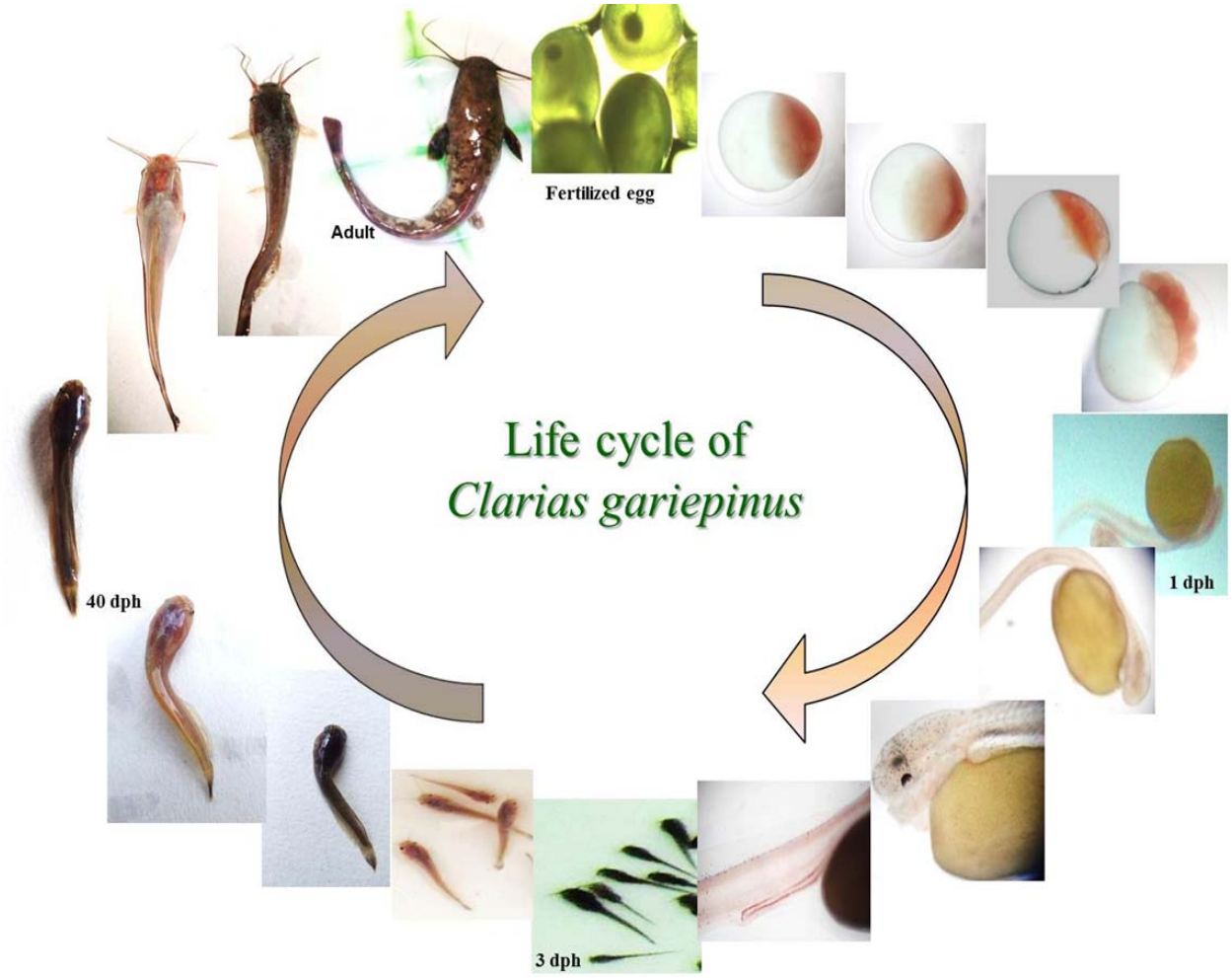

Fig.5. Life cycle of *C. gariepinus* from fertilized egg stage to an adult. These photographs have been taken in our aquaculture facility after *in vitro* fertilization and breeding.

In the backdrop of this existing state of knowledge, we designed our studies in order to understand the role of *CYP19A2* in brain sex differentiation and ovarian development by

simultaneous analysis the expression profile and aromatase enzyme activity in brain during ovarian cycle. The potential transcription factors, *FTZ-F1* and *FOXL2* were also studied for their expression pattern during development and recrudescence and after *in vivo* hCG induction in the brain of catfish, *C. gariepinus*. In addition, the transcriptional regulation of *CYP19A2* by these transcription factors was analyzed in the present study. The effect of estradiol analogues (DES and EE_2) on CYP19 expression and activity was also studied to understand the potential influence of estrogens on ovarian development.

Objectives of the present study

- Cloning, expression and enzyme activity analysis of brain aromatase in the air-breathing catfish, *Clarias gariepinus,* during ovarian development, recrudescence and after *in vivo* hCG induction

- Cloning, expression and ontogeny of brain *FTZ-F1* in the catfish, *Clarias gariepinus,* during ovarian development, recrudescence and after *in vivo* hCG induction

- Cloning and differential expression of *FOXL2* during ovarian development and recrudescence in the catfish, *Clarias gariepinus*

- FTZ-F1 and FOXL2 synergistically up-regulate catfish brain aromatase gene transcription by specific binding to the promoter motifs

- Effects of estradiol analogues, diethylstilbestrol and ethynyl estradiol on the expression of aromatases and their transcription factors in the brain and ovary of catfish, *Clarias gariepinus,* during ovarian differentiation

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Chapter I

Cloning, expression and enzyme activity analysis of brain aromatase in the air-breathing catfish, *Clarias gariepinus,* during ovarian development, recrudescence and after *in vivo* hCG induction

Abstract

In the present study, we analyzed the importance of brain form of aromatase (*CYP19A2)* during ovarian development and recrudescence of the North African/air-breathing catfish, *Clarias gariepinus*. We cloned *CYP19A2* (1786 bp) which showed only 47% homology with *CYP19A1* of the catfish. Characterization of encoded protein in non-steroidogenic COS-7 cells could efficiently catalyze the aromatization reaction by producing estradiol-17β (E_2) from testosterone. Tissue distribution pattern revealed preferential expression of *CYP19A2* in brain. Quantitative real-time PCR analysis of *CYP19A2* in brain revealed high levels of transcripts in the prespawning phase of ovarian cycle. Ontogeny studies displayed sexual dimorphism, with an early expression of *CYP19A2* in brain. Phase-dependent rise in the expression and enzyme activity of aromatase in brain after hCG treatment revealed the stimulatory role of gonadotropin during preparatory and prespawning phases. Lack of influence of hCG treatment during spawning phase endorsed it further. A good correlation of expression and enzyme activity suggests a plausible role of *CYP19A2* during ovarian differentiation and ovarian cycle either directly or indirectly through hypothalamo-hypophyseal-gonadal axis.

Introduction

Cytochrome P450 aromatase (P450arom) is the endoplasmic reticular enzyme catalyzing the production of estrogens from androgens. P450 arom via production of estradiol-17β (E_2) plays a potent role in preserving the continuity of life in diverse vertebrates including fishes by sustaining important physiological process, reproduction. Its role in oogenesis, sexual behavior, sex change in hermaphrodites and temperature sensitive fishes (Yamamoto, 1969; Donaldson, 1996; Chang *et al.*, 2005) is well characterized. In addition, changes in the expression and/or

activity of P450arom along with variations in serum E_2 levels have often been demonstrated during sex change and ovarian differentiation in different fish species (Tanaka *et al.*, 1992; Kwon *et al.*, 2001; Blazquez and Piferrer, 2004; Goto-Kazeto *et al.*, 2004; Barney *et al.*, 2008; Guiguen *et al.*, 2010). Not only in fishes but also in chicken, aromatase vis-à-vis E_2 is required for ovarian development or differentiation (Smith and Sinclair, 2004). This enzyme is encoded by a single gene with multiple tissue specific promoters in both steroidogenic and non-steroidogenic tissues in vertebrates including mammals (Simpson *et al.*, 2002). On the contrary, in teleosts existence of two P450arom (ovarian form, *CYP19A1* and brain form, *CYP19A2*) is demonstrated in numerous fish species (Gelinas *et al.*, 1998; Tchoudakova and Callard, 1998; Trant *et al.*, 2001) which is a consequence of genome duplication (Simpson *et al.*, 1994). These two isoforms differ in their sequences, with not more than 60% homology between them (Trant *et al.*, 2001). In addition, both forms differ in their enzyme kinetics and also show preferential expression in brain and gonad during development and reproduction in male and female fish (Zhao *et al.*, 2001; Kobayashi *et al.*, 2004; Sudhakumari *et al.*, 2005). In their physiological roles, while *CYP19A2* is implicated for neuroplasticity and neurogenesis (Forlano *et al.*, 2001), *CYP19A1* is involved in ovarian differentiation (Kwon *et al.*, 2001; Chang *et al.*, 2005; Sudhakumari *et al.*, 2005; Matsuoka *et al.*, 2006; Blazquez *et al.*, 2008; Esterhuyse *et al.*, 2008) and gametogenesis (Ijiri *et al.*, 2003; Kobayashi *et al.*, 2004). Reports exist from fishes belonging to varied reproductive strata on *CYP19A1*'s specific role during sex determination/differentiation (Nagahama, 2005; Ijiri *et al.*, 2008) and regulation of vitellogenesis during reproductive cycle (Nagahama *et al.*, 1994; Chang *et al.*, 1997; Nakamura *et al.*, 2005), but the role of *CYP19A2* and its involvement in brain sex differentiation is still unclear. There is evidence of seasonal variation in transcript levels of *CYP19A2* in channel catfish brain (Kazeto

and Trant, 2005), which is relayed via gonadotropins to ovary. However, it will be intriguing to study the role of *CYP19A2* during gametogenesis with reference to gonadotropins. Interestingly, catfish *CYP19A2* expression was found to be brain-specific (Kazeto and Trant, 2005), while in the Nile tilapia, *CYP19A1* transcript was exclusively detected in the ovary (Sudhakumari *et al.*, 2003, 2005; Chang *et al.*, 2005) which poses another diverse situation worthwhile to probe its specificity. Therefore extensive information is required on plausible role of *CYP19A2* during ovarian differentiation, which could be achieved by studying its spatiotemporal expression and enzyme activity during reproductive cycle and gonadal differentiation. Such study will assist in garnering data if *CYP19A2* displayed any disparity in its expression pattern vis-à-vis activity during gametogenesis. To accomplish this, we initially cloned cDNA of *CYP19A2* from the brain of catfish and studied its expression pattern and enzyme activity during gametogenesis and ovarian cycle in brain. We also checked the change in expression pattern and enzyme activity in brain after *in vivo* hCG induction during ovarian recrudescence, using qRT-PCR and radiometric enzyme assay, respectively.

Materials and Methods

Animals

Male and female juveniles of *Clarias gariepinus* belonging to different age groups were obtained from our laboratory aquaculture facility by breeding and rearing. The hatchlings were kept in the glass water tanks supplied with circulating filtered water under natural photoperiod and ambient temperature. They were fed with live tubeworms *ad libitum* till they grew to fingerling size (~5-6mm). Later on, they were given chopped goat liver or pelleted fish food till adult. Maintenance of adult fish and its annual reproductive cycle were described earlier (Raghuveer and

Senthilkumaran, 2009). In brief, the various phases of female reproductive (annual/seasonal) cycle, namely, preparatory, prespawning, spawning, resting were closely followed by catfishes (adult) maintained more than a year in outdoor tanks under natural conditions. Brain from various age group fish as well as from adult at different phases were collected by sacrificing them (decapitation) after immersing in mild ice-cold water. The tissues were stored at -80ºC before being assayed for various parameters.

Total RNA isolation and 1st strand cDNA synthesis

Total RNA from the brain of adult and juvenile catfish was isolated using Sigma TRI-reagent (Sigma, St. Louis, MO, USA). The quality and concentration of total RNA was assessed by using NanoDrop (ND-1000, NanoDrop technologies, USA) spectrophotometer and checked in formaldehyde agarose gel. Total RNA from adult tissue was reverse transcribed to obtain first strand cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA) and oligodT_{18} primers following the manufacturer's instructions. The efficiency of the transcription was checked by performing a PCR for *β-actin,* a constitutively expressed gene.

Molecular cloning of catfish *CYP19A2*

A set of degenerate primers were designed from conserved region by aligning the existing sequences of teleost *CYP19A2* using Lasergene software, (release 3.05; DNASTAR, Madison, WI, USA) which yielded partial cDNA fragment of 242 bp from brain which was then cloned in pGEMT-easy vector (Promega, Madison, WI, USA) and sequence was determined. The identity of amplified partial cDNA was analyzed by NCBI-BLAST.

Construction of cDNA libraries and screening

Brain cDNA library of catfish was constructed as per the method described earlier (Senthilkumaran *et al.*, 2002). Briefly, total RNA from brain of catfish was prepared using Sigma TRI-reagent. Using 1.5mg of total RNA, mRNA was prepared with oligotex-mRNA kit (Qiagen, GmbH, Germany). Using 5µg of mRNA, cDNA synthesis was carried out using a Stratagene cDNA synthesis kit (Stratagene, Cedar Creek, TX, USA). Ends of cDNA were blunted and ligated with *EcoR*I adaptors using T4 DNA ligase and phosphorylated by polynucleotide kinase. Then the cDNA was size fractionated using Sepharose CL-4B columns (Amersham, Buckinghamshire, England). Fractionated cDNAs were ligated with UNI-ZAP-XR vector and packaged into lambda phage heads using Giga pack II–gold packaging extract kit (Stratagene). Screening of the brain cDNA library for *CYP19A2* was performed using RT-PCR amplified partial cDNA fragment as probe which was radiolabelled with ^{32}P-dCTP using random primer labeling kit (Perkin Elmer, Boston, MA, USA). After three rounds of screening, positive clones were isolated by single clone excision. The pBluescript phagemids obtained after excision were sequenced bi-directionally using ABI prism capillary 316 DNA sequencer and the sequence identity was analyzed by NCBI-BLAST.

Functional characterization *CYP19A2* of catfish expressed in COS-7 cells

Functional characterization of *CYP19A2* in COS-7 cells was performed following the method (Chang *et al.*, 2005) described earlier. In brief, about 3x10^5 COS-7 cells were plated on 6-cm tissue culture plate holding 4ml of DMEM with or without (during transfection) 10% (v/v) fetal calf serum and grown at 37ºC in 5% CO_2 until confluent. After confirming the sequence integrity of the *CYP19A2* ORF in pCDNA3.1$^+$ TOPOV5-His mammalian expression vector (Invitrogen),

it was transfected in COS-7 cells using Tfx20 (Promega) reagent. Mock pcDNA3.1 (ORF insert locked in reverse direction) was used as a negative control. After 24 h incubation, 30nM of testosterone was added as substrate to the cells. Subsequently after 12 h, the culture medium was separated from cells by centrifugation at 1000Xg, followed by extraction twice with diethyl ether and evaporation in vacuum centrifuge. The steroids were reconstituted in 100µl EIA buffer supplied in E_2 EIA kit (Cayman). The E_2 produced in the culture medium was measured using a enzyme linked immunoassay (EIA) kit for E_2 (Cayman) according to the manufacturer's protocol. A recovery of 93-95% was obtained by the extraction procedure described in E_2 EIA kit. Cross-reactivity of the E_2 antisera to estradiol-3-glucoronide was (14%), estrone (12%), estradiol-17-glucoronide (10%), estriol (0.3%), 5α-dihydrotestosterone (0.06%), testosterone (<0.01%). The minimal detection threshold was 20 pg/ml for E_2. After measurements, the conversion rates were calculated and the values of cross-reactivity were subtracted. Intra- and inter assay coefficient of variations were (n=5; mean ± SEM) 1.38 ± 0.12% and 1.65 ± 0.05%, for E_2 produced from *CYP19A2* transfected plate. Results were expressed as mean ± SEM of three replicates each from three independent analyses. Data analysis was carried out using one-way ANOVA followed by Dunnett's test. Significance was accepted at $P< 0.05$.

Quantitative real time PCR (qRT-PCR) for *CYP19A2* during reproductive cycle of catfish

Expression of *CYP19A2* was analyzed by qRT-PCR. To study the expression pattern during different phases, total RNA (5µg) was obtained by dissecting the brain from five fish (n=5).Gene-specific primers were designed for *CYP19A2* in intron-exon boundaries by aligning the existing sequences. The forward primer located in intron-exon boundary of exon I and II and reverse primer located in intron-exon boundary of exon II and III. Absence of genomic DNA contamination in the RNA was confirmed by using non-reverse transcribed samples as templates.

In addition, absence of DNA in total RNA was ensured by treating with DNAseI before proceeding for 1st strand cDNA synthesis. Primers for *β-actin* used as the reference gene were designed from *β-actin* cDNA obtained from catfish which is listed in Table. 1. Primer specificity for each primer pair was confirmed by cloning and sequencing the amplicon followed by dissociation curve analysis. Real-time PCR was performed on an ABI Prism® 7500 fast thermal cycler (Applied Biosystems, Foster, CA,USA) using SYBR Green 1 (Applied Biosystems). Each sample was run in triplicate in a final volume of 25 µl containing 0.3 µl of cDNA, 10 pmol of each primer, and 12.5 µl of Power SYBR® Green PCR master mix. During PCR, fluorescence accumulation resulting from DNA amplification was analyzed using the sequence detector software (Applied Biosystems). Comparative C_T method was used to quantify the target genes abundance. RQ Manager 1.2 (Applied Biosystems) was used to compile data from all plates and compare expression levels. Transcript abundance of both the genes were normalized to that of *β-actin* and reported as fold change in abundance relative to the values obtained in preparatory phase using $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen, (2001).

Changes in aromatase activity in brain at different phases of female reproductive cycle

The aromatase activity in brain of female catfish collected at different phases of ovarian cycle was assayed as per the method described (Tsai *et al.*, 2000) with minor modifications. Briefly, microsomes from the above mentioned tissues were prepared by homogenizing 2g of tissue in 3ml of potassium phosphate buffer pH 7.4 (KPO_4 buffer) followed by centrifugation at 9000Xg for 20 min at 4ºC to clear debris, and a final spin at 105,000Xg for 1 hour at 4ºC. The microsomal pellet was then rinsed with buffer and resuspended in 500µl of KPO_4 buffer/0.1mM EDTA/20% v/v glycerol. Aromatase activity was measured by incubating 300µg of microsomal

protein and 0.6µM of 1β-[^{3}H] androstenedione in 500µl of 100mM KPO_4 (pH 7.4), 1mM EDTA, 1mM β-$NADP^+$, 5mM glucose-6-phosphate and 10units of glucose-6-phosphate dehydrogenase at 37°C for 1 hour in a water shaker. The reaction was stopped by adding iced 10% trichloroacetic acid containing 20mg charcoal/ml. After centrifugation at 1000Xg for 10 min at 4ºC, the supernatant was collected in scintillation vials containing 3ml of cocktail-T (SRL chemicals, Mumbai, Maharashtra, India). The radioactivity was measured using Wallac 1409-liquid scintillation counter. Data analysis was carried out using one-way ANOVA followed by Tukey's test. The entire procedure was repeated thrice and the results were expressed as means ± SEM of three replicates for each brain sample collected individually from five fish (n=5). Significance was accepted at $P < 0.05$.

Table. 1. List of primers used for analysis of *CYP19A2*

S. No.	Primer	Sequence 5' to 3'	Purpose
1.	DF1	TGGWYKGGDCATHGGBACDGC	To amplify partial cDNA fragment
2.	DR1	GGVCCDGTBRVGCTTTRG	To amplify partial cDNA fragment
3.	RT Fw	GGTCTGGGGATTGGGACAGC	qRT-PCR
4.	RT Rv	CACAGCAGATGACTTGCTTAG	qRT-PCR
5.	Sp Fw	CCAGGTCCATACTGGTTACTG	RT-PCR
6.	Sp Rv	CACAGCAGATGACTTGCTTAG	RT-PCR
7.	β-actin-Fw	ACCGAAGTCCATCACAATACCAGT	qRT-PCR
8.	β-actin-Rv	GAGCTGCGTGTTGCCCCTGAG	qRT-PCR

qRT-PCR for *CYP19A2* and aromatase activity in brain of developing catfish

Expression pattern of *CYP19A2* was assessed in developing male and female catfish by qRT-PCR. The brain and gonadal tissues were dissected out using fine forceps under a stereozoom microscope (Leica, Wetzlar, Germany) from developing juvenile catfish, after morphological (also histological) identification of testis and ovary at 45, 60, 75, 100 and 150 days post hatch (dph), under sterile conditions. From histological observation it was evident that the critical period of sex differentiation was around 35-50 dph when the gonads get morphologically distinguishable in air-breathing catfish (Raghuveer and Senthilkumaran, 2009). Hence, we performed the ontogeny study from 45 dph, which permitted us to unambiguously identify and isolate the ovary and testis from the catfish juveniles for expression and enzyme activity analyses on aromatase. For ontogeny studies (45, 60, 75, 100 and 150 dph), the brain and gonadal tissues from 20-25 juveniles were dissected and pooled to have 5 biological samples (brain/ovary/testis tissue) from 4-5 juvenile fish constitute one sample, n=5) for total RNA preparation (2.5µg). For aromatase activity analysis 10-15 (brains) were pooled to have 5 biological. Pooling of brains from 2-3 juvenile fish constitute one biological sample (n=5) as per their age. The tissue samples were then immediately snap-frozen in liquid N_2 and kept at -80ºC for subsequent analysis.

Semi-quantitative RT-PCR and qRT-PCR analysis of tissue distribution pattern of *CYP19A2*

Total RNA (5µg) was isolated from different tissues and reverse transcribed to first-strand cDNA using Superscript III (Invitrogen) reverse transcriptase. PCR reaction using specific (Sp) primers designed for *CYP19A2* given in Table. 1. The PCR was carried out at 94°C for 45 sec, 60°C for 30sec, and 72° for 1 min for 30 cycles using a dual-block thermal cycler ABI 9700 (Applied Biosystems). The tissue distribution pattern was reconfirmed using qRT-PCR as per the method

described above using primer sets used for seasonal / reproductive cycle expression analysis of *CYP19A2*.

hCG-induced *in vivo* study of expression and activity of *CYP19A2*

Fish were collected during different phases i.e., February (early preparatory), end of May (early prespawning), September (late spawning) and injected with hCG (1000 IU/kg body weight; Pubergen, Uni-Sankyo, Hyderabad, India), intraperitoneally. Brain tissue from individual fish at every 4 h up to 12 h was collected by sacrificing the fish after exposing it to ice-cold water. The expression and activity analyses of *CYP19* following hCG induction were performed as elucidated above. Present experiment was conducted using different batches of female catfish (n=5) for each time point (4/5 time points using 20/25 fish).

Immunocytochemisty

Immunocytochemistry was carried out using heterologous primary antibody (antibody was raised against tilapia aromatase) on brain samples collected from 250 dph fish. Avidin-biotin peroxidase method was followed for cellular localization of *CYP19* gene as described by (Swapna *et al.* 2006). In short, 5µm thick ovarian sections were cut, deparaffinized and blocked using 10% normal goat serum for 1 hour at room temperature. Then primary antibody was added (heterologus antibody from Nile tilapia aromatase, 1:750 times dilution was standardized for this study) and sections were incubated at 4ºC for 24 h in humid chamber. Subsequently, they were incubated with HRP labeled secondary antibody (Bangalore Genei, India) washed with 0.1M phosphate buffer saline pH 7.4 and developed using commercially supplied 3' 3' diaminobenzidine for 3-10 min. Images were taken with a motic microscope fitted with motic camera.

Statistical Analysis

All the data were expressed as mean ± SEM. Significance among group was tested by ANOVA followed by Student's-Newman-Keuls' test. Difference among groups were considered significant at $P<0.05$.

Results

Molecular cloning of *CYP19A2* from catfish brain

A set of degenerate primers yielded a partial cDNA of 242 bp from catfish brain (Fig. 1) whose sequence identity was confirmed by NCBI-BLAST. Full length cDNA of *CYP19A2* clone was obtained from brain cDNA library (6 identical clones) using radiolabelled partial cDNA as probe. The *CYP19A2* cDNA was 1786 bp in length with 1494 bp ORF, 180 bp 5' UTR, 112 bp 3'UTR and AATAA as polyadenylation signal present 53 bp before the poly-A tail (Fig. 2). The *CYP19A2* cDNA encoded a protein containing 498 amino acids. ClustalW multiple alignment revealed the presence of conserved signature domains which includes the heme binding region, the I-helix region and the aromatase specific sequence in both isoforms plus a membrane spanning region at the N-terminal side typical of the brain form among teleosts (Fig. 3). *CYP19A2* of catfish specifically displayed high homology in the signature domain regions with aromatases cloned from other teleosts. The nucleotide sequence *CYP19A2* was deposited in GenBank (Accession number: GU220076). Phylogenetic analysis by neighbour-joining method exhibited 88-72% homology with other teleost brain aromatase and only 47% homology with *CYP19A1* of catfish (Fig. 4).

Functional characterization in COS-7 cells

A flow chart depicting the methodology for functional characterization of *CYP19A2* is provided in Figure 5A. The *CYP19A2* ORF of 1.5 kb was cloned into the mammalian cell expression vector pCDNA3.1 (Fig. 5B). The transiently expressed ORF of *CYP19A2* in COS-7 cells was able to transform the substrate testosterone to E_2 demonstrating that the inserted cDNA in mammalian expression vector was indeed functional possessing the property to aromatize C_{19} androgens to C_{18} estrogens. The catalytic efficiency was expressed as percentage conversion of testosterone to E_2. Mock transfected cells of *CYP19A2* (obtained by reverse orientation of the ORF in the expression vector) showed no significant production of E_2 from testosterone. The percentage conversion of the substrate testosterone to E_2 by *CYP19A2* was 38% (Fig. 5C).

Phase-dependent (reproductive cycle) expression and enzyme activity of *CYP19A2* in brain

CYP19A2 exhibited maximum transcript levels in the prespawning phase when compared to preparatory, spawning and regressed phases (Fig. 6). A flow chart depicting the methodology of measurement of aromatase activity is provided in Figure 7A. The aromatase activity in brain tissue corroborated well with the *CYP19A2* transcripts during different phases of ovarian cycle (Fig.7B).

Ontogeny of *CYP19A2* expression and aromatase activity in brain

The qRT-PCR analysis at 45, 60, 75,100 and 150 dph in juvenile catfish revealed early onset of *CYP19A2* transcript in female brain from 45 dph (Fig. 8A) with levels gradually increasing till 75 dph and then more or less similar levels were observed after 75 dph. Transcript of *CYP19A2* in male brain was detected only from 100 dph. On the other hand, no expression of *CYP19A2*

was detected in the ovary however faint expression was spotted in testis from 60 dph. The enzyme activity analysis demonstrated elevation in aromatase activity in an age-dependent manner and the aromatase activity was high in female brain (Fig. 8B) when compared to male brain during early development in catfish.

Tissue distribution of *CYP19A2*

Using semi-quantitative RT-PCR analysis, expression of *CYP19A2* was detected only in brain (Fig. 9A) and qRT-PCR analysis further endorses the expression pattern of *CYP19A2* (Fig. 9B).

Expression and activity of *CYP19A2* in brain of female catfish during preparatory, prespawning and spawning phases after *in vivo* induction using hCG

In vivo hCG treatment during the preparatory phase resulted in significant increase of *CYP19A2* expression and aromatase activity in the brain of preparatory phase catfish from 8 to 24h (Fig. 10A and B) while in the prespawning phase it demonstrated a peak in transcript level at 8h However, the enzyme activity analysis in the prespawning phase demonstrated considerably higher activity in at 8h corroborating with the transcript levels (Fig. 11A and B). There was no significant change in the transcript and enzyme activity after hCG induction in the spawning phase (Fig. 12A and B).

Immunocytochemisty

Immunocytochemical localization of aromatase in the cross section of 250 dph brain showed positive immune reactivity in pre-optic area and hypothalamus (Fig. 13A, B and C). The sagittal section of juvenile catfish brain showed immunoreactivity in hypothalamus-mid brain area (Fig. 13D, E and F).

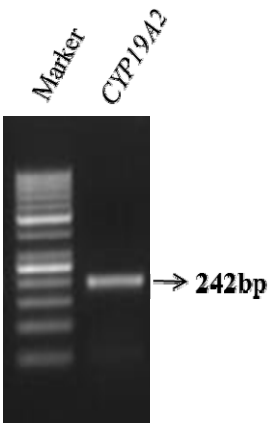

Fig.1. Amplification of partial *CYP19A2* cDNA fragment.

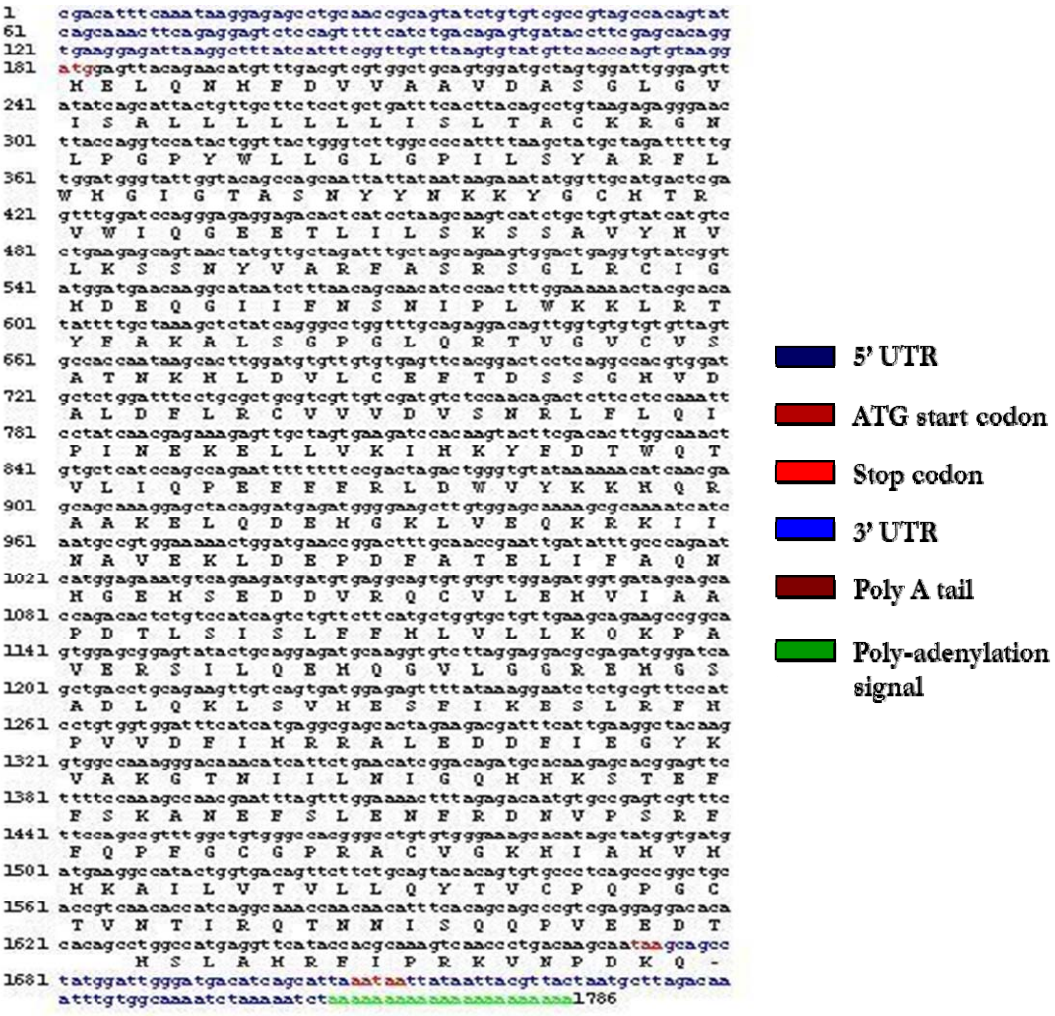

Fig.2. Nucleotide and deduced amino acid sequence of catfish full-length *CYP19A2* cDNA.

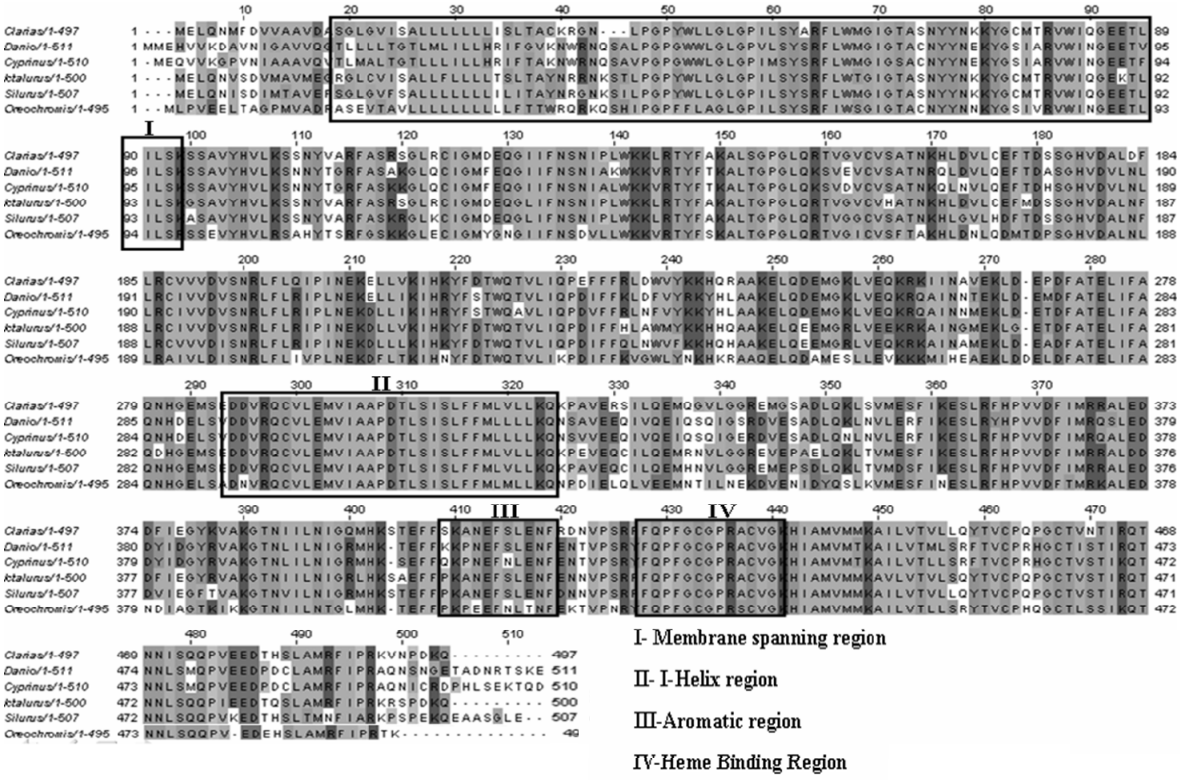

Fig.3. ClustalW multiple alignment of catfish *CYP19A2* with other vertebrate counterparts. GenBank accession numbers of *CYP19A2* sequences are :- *Ictalurus punctatus*: AF417239; *Oreochromis niloticus*: AF472621; *Silurus meridonalis*: AY325907; *Danio rerio*: AF226619; *Cyprinus carpio*: EU375456 and *Clarias gariepinus*: GU220076.

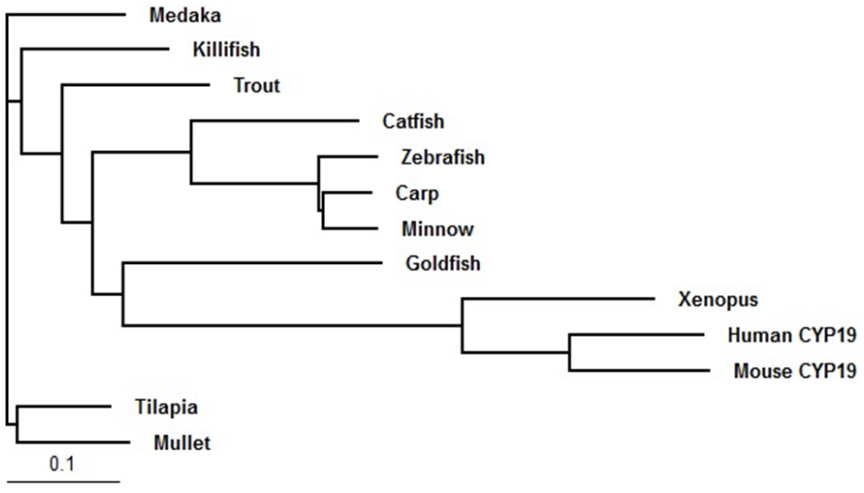

Fig.4. Phylogenetic tree of catfish *CYP19A2*. Phylogenetic analysis was done with ClustalX

software 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 tree was generated using the TreeView software package version 1.4. GenBank accession numbers of the sequences used in phylogenetic tree are as follows *CYP19A2* accession numbers: African catfish: GU220076; Zebrafish: AF226619; Medaka: AY319970; Tilapia: AF472621; Trout: AJ311938; Carp: EU375456; Killifish: AY494837; Minnow: AB190291; Goldfish: AB009336; Mullet: AY859423; Human CYP19: NM000103; Mouse CYP19: NP031836; Xenopus CYP19: FJ644565.

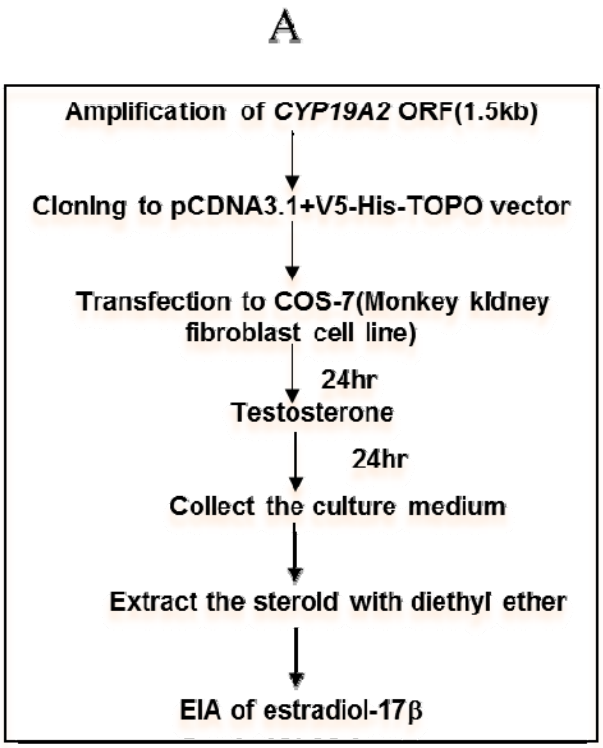

Fig.5. A) Flow chart of methodology used for functional characterization of *CYP19A2*.

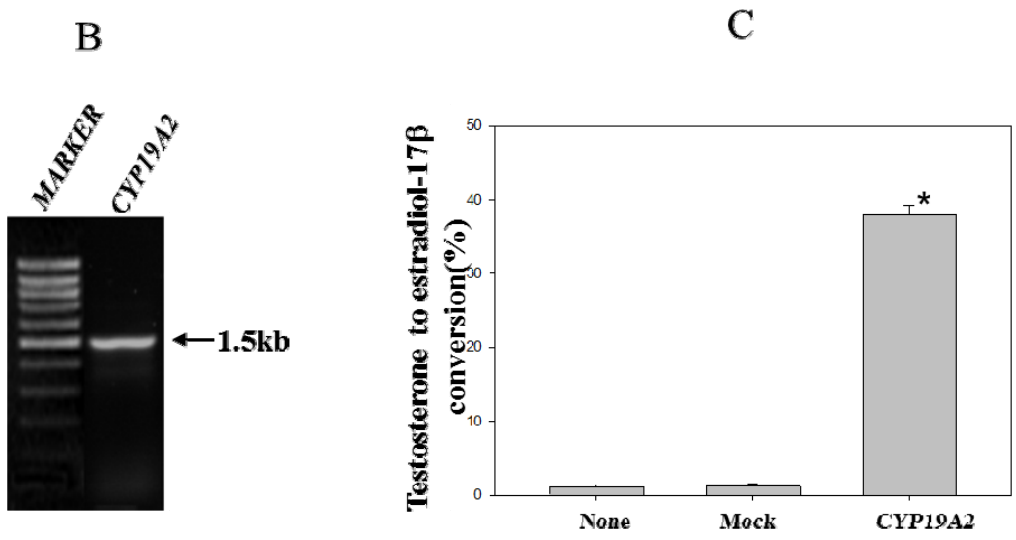

Fig.5. Functional characterization of *CYP19A2*. B) Amplified PCR product of *CYP19A2* ORF cDNA. B) Histogram showing % conversion of testosterone to estradiol-17β by recombinant CYP19A2 protein. * indicates the significance at P<0.05.

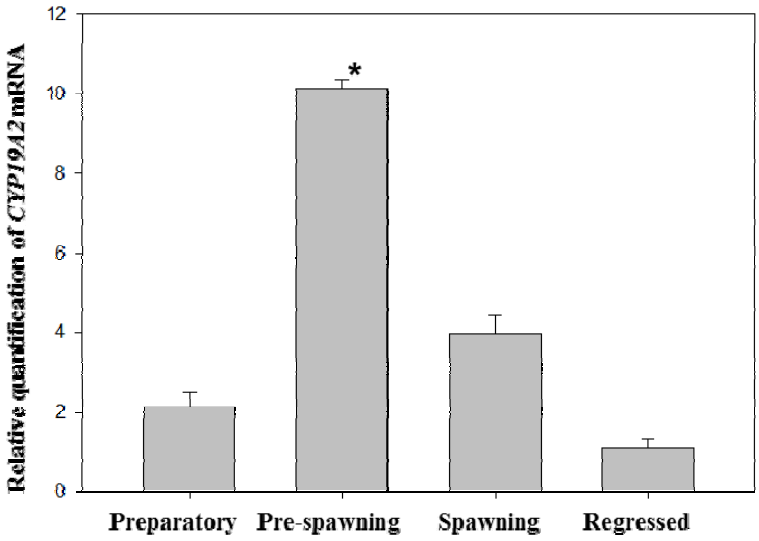

Fig.6. Changes in transcript levels of *CYP19A2* in different phases of ovarian cycle from female brain. * indicates the significance at P<0.05.

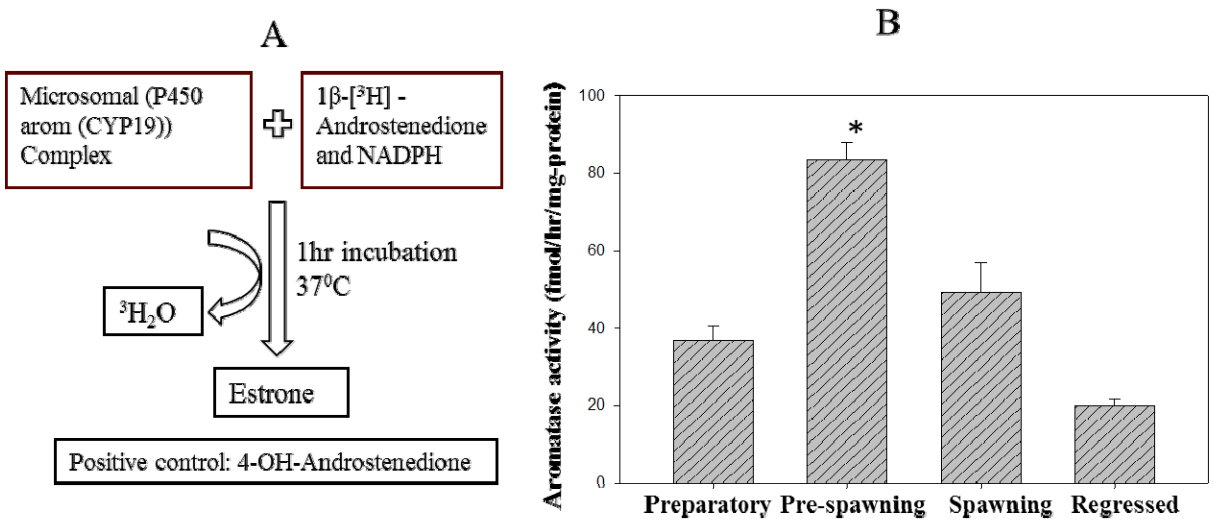

Fig.7. Aromatase activity in brain A) Flow chart of methodology used to measure the aromatase (CYP19) activity in brain B) Changes in aromatase activity in female brain during different phases of ovarian cycle in catfish. * indicates the significance at P<0.05.

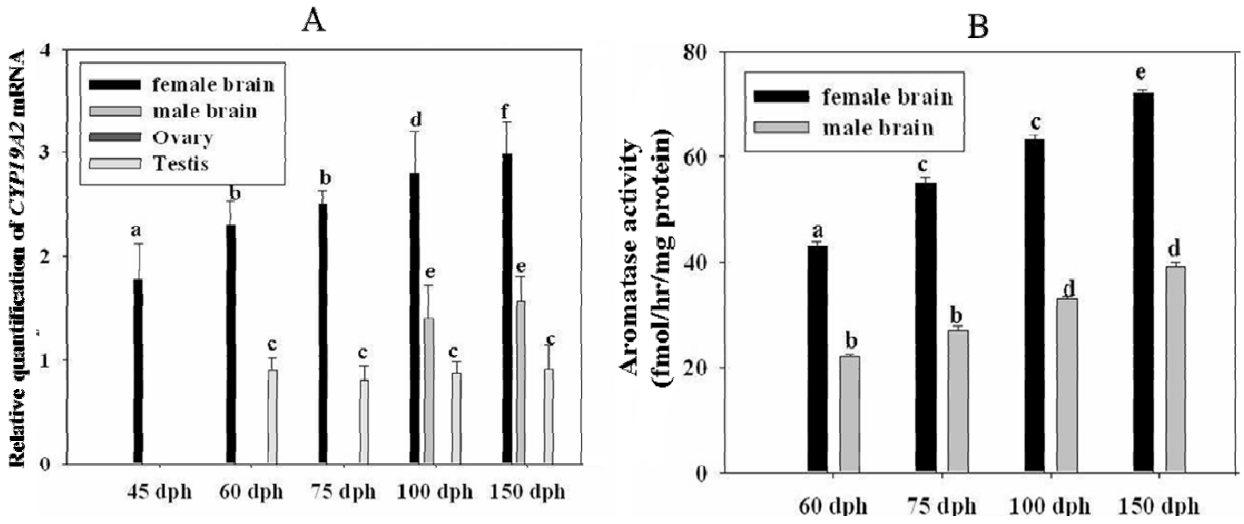

Fig.8. Changes in transcript levels of *CYP19A2* and aromatase activity during development. A) temporal expression pattern of *CYP19A2* in juvenile catfish at 45, 60, 75, 100 and 150 dph in gonads and brain of male and female catfish, B) aromatase enzyme activity at 60, 75, 100 and 150 dph in male and female brain tissue. Different alphabets indicate groups that are not significantly different P<0.05.

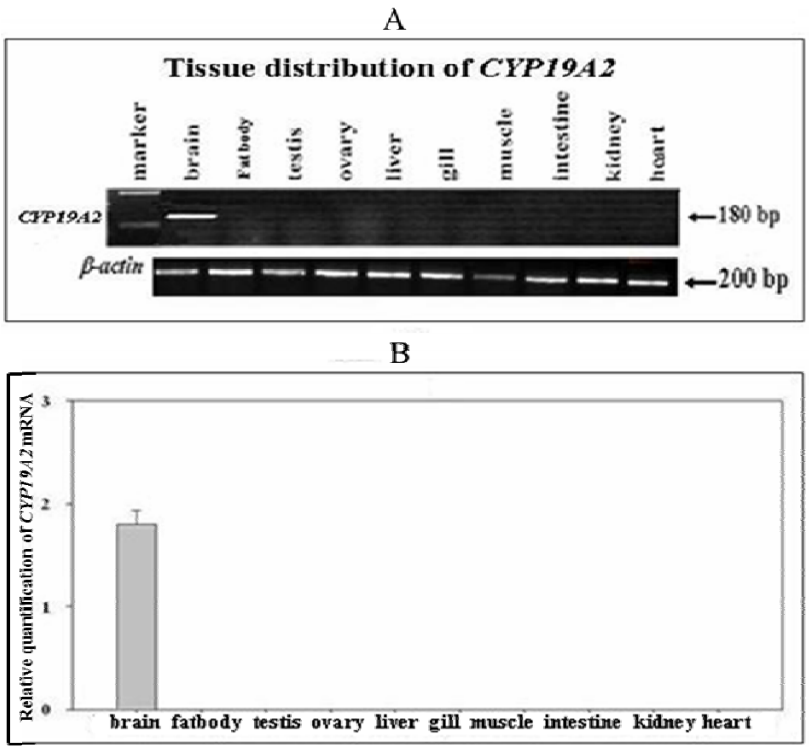

Fig.9. Tissue distribution pattern of *CYP19A2*. A) Semi-quantitative RT-PCR analysis of *CYP19A2* expression B) qRT-PCR analysis of *CYP19A2* expression in different tissues of adult female catfish.

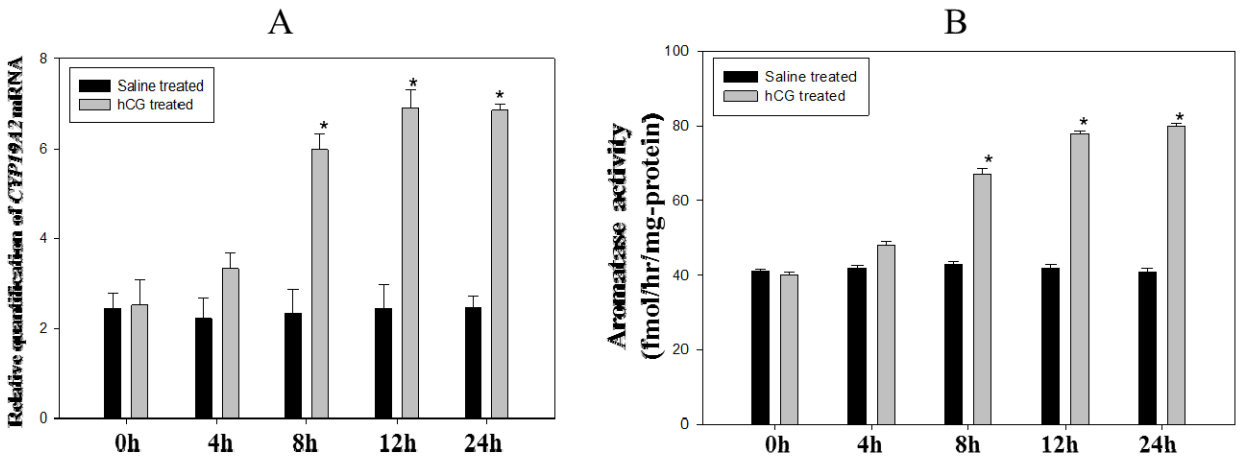

Fig.10. Changes in expression pattern of *CYP19A2* after *in vivo* hCG-induction in brain of preparatory phase of catfish A) qRT-PCR analysis of *CYP19A2* B) enzyme activity of aromatase, compared to control.* indicates the significance at $P<0.05$.

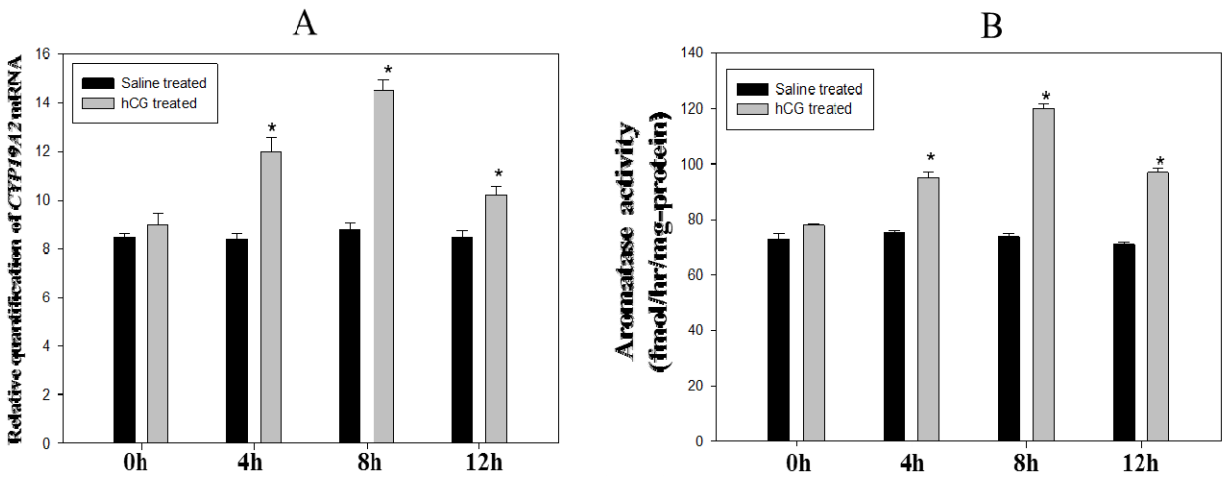

Fig.11. Changes in expression pattern of *CYP19A2* after *in vivo* hCG-induction in brain of prespawning phase of catfish A) qRT-PCR analysis of *CYP19A2* B) enzyme activity of aromatase, compared to control.* indicates the significance at P<0.05.

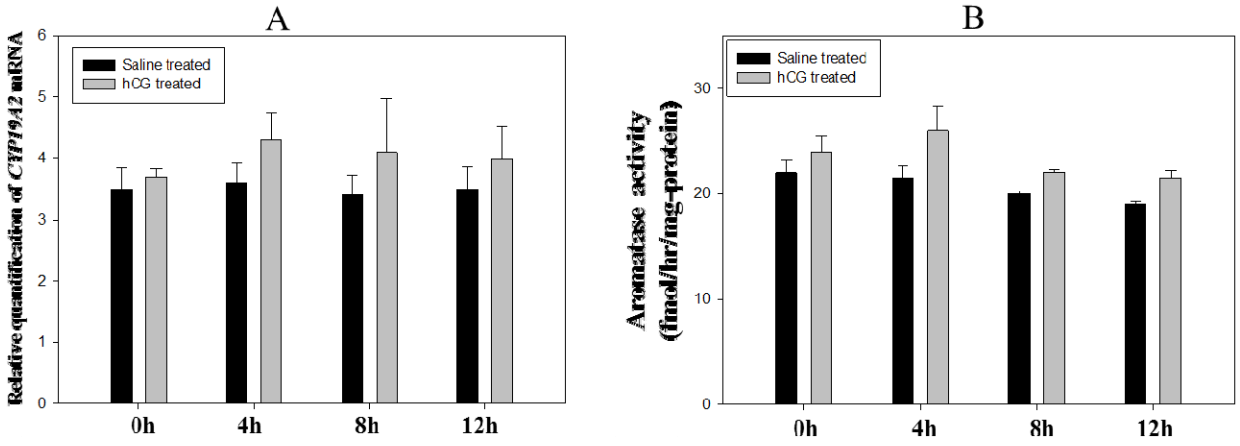

Fig.12. Changes in expression pattern of *CYP19A2* after *in vivo* hCG-induction in brain of spawning phase of catfish A) qRT-PCR analysis of *CYP19A2* B) enzyme activity of aromatase, compared to control.* indicates the significance at P<0.05.

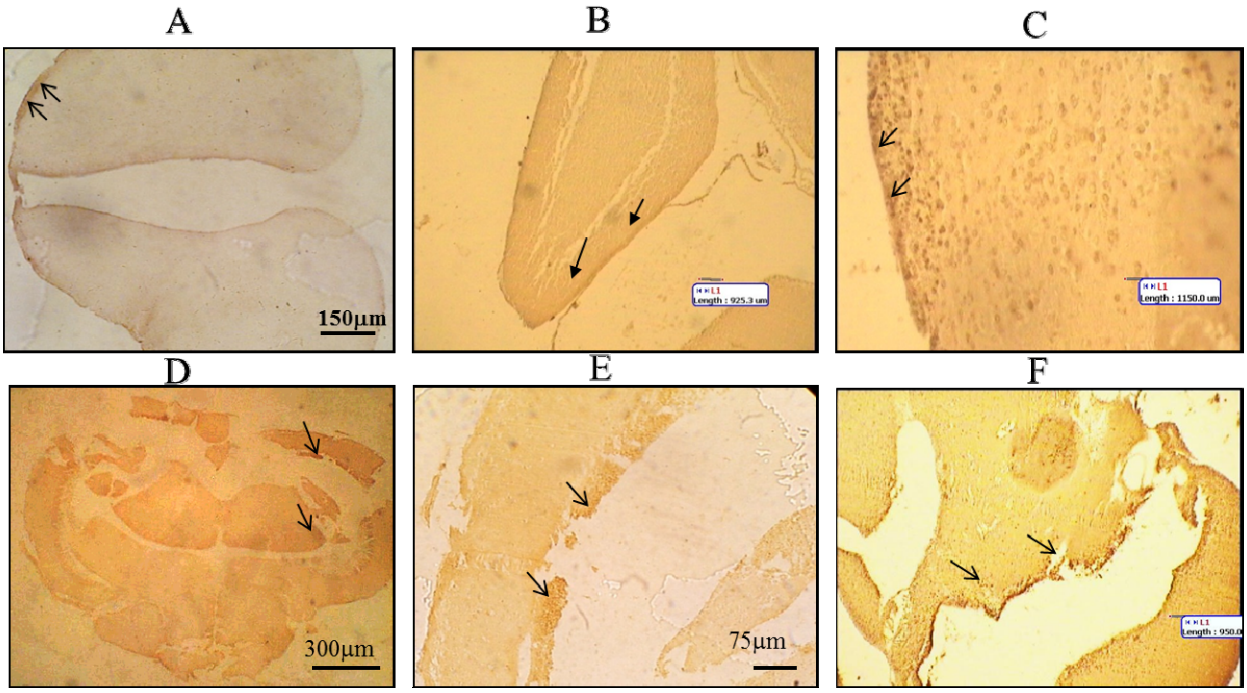

Fig.13. Immunocytochemical localization of aromatase in brain of catfish A), B), C) cross section of 250 dph female brain through pre-optic area-hypothalamus and D), E), F) Sagittal section of brain through hypothalamus-mid brain area of 250 dph catfish. Arrow marks indicate immunoreactivity.

Discussion

Various approaches have been attempted to validate the significance of *CYP19A2* during development and ovarian differentiation in the brain of teleosts. There are also increasing evidences from different fish species supporting the involvement of the brain form of aromatase, *CYP19A2* in neuronal differentiation, synaptogenesis and sexual behavior (see Diotel *et al.*, 2010). In the present study, we focused on elucidating the contribution of the *CYP19A2* during ovarian differentiation and gametogenesis. This was accomplished by initially analyzing the transcript and/or activity of *CYP19A2* in brain, ovary and testis of juvenile catfish from 45 dph up till the catfish approaching puberty (150 dph), followed by monitoring, the transcript levels

(by qRT-PCR), the aromatase activity in the brain in relation to the pattern of serum E_2 levels during different phases. Lastly, we also tried to study the responsiveness of *CYP19A2* transcript levels and enzyme activity after hCG (non-homologous gonadotropin) induction at different phases of ovarian cycle. Hence, the present work, virtually tried to deal with various parameters that contributed in the maintenance of female reproductive cycle (mRNA, terminal enzyme (aromatase) activity and its modulation by gonadotropins) leading to the production of E_2 to demonstrate the possible contribution of *CYP19A2,* either directly or indirectly in ovarian differentiation and growth.

Cloning, phylogenetic analysis and functional characterization of *CYP19A2*

Iniatially we cloned the full length cDNA of *CYP19A2* from catfish brain. When compared to reports from many teleosts like goldfish (Tchoudakova and Callard, 1998), zebrafish (Kishida and Callard, 2001) and channel catfish (Trant, 1994; Kazeto and Trant, 2005), and from the results obtained earlier in our laboratory on cloning of *CYP19A1* in air-breathing catfish (Rasheeda *et al.*, 2010), it was evident that the transcript length of brain form (*CYP19A2*: 1786 bp) is shorter than the ovarian form (*CYP19A1*: 1941 bp) although the protein encoded by the brain form followed the usual norm of being 18 amino acids lesser than the ovarian form. Phylogenetic analysis revealed that these two paralogous genes shares 47% homology, indicating that they have evolved separately after genome duplication, albeit preserving a greater identity in their functional domains thus reinforcing the essential role of these regions. The ovarian form exhibits a separate branch within the siluriforms clade in the distance tree, similarly the brain form also shares greater identity with brain form of siluriforms. Transiently expressed *CYP19A2* protein in non-steroidogenic mammalian COS-7 cells catalyzed the aromatization of testosterone to E_2 indicating that the cloned cDNA indeed codes for aromatase and the efficiency of the brain

form *CYP19A2* was lower than reported *CYP19A1* efficiency which is in accordance with aromatases cloned from the Nile tilapia (Chang *et al.*, 2005).

Phase-dependent (seasonal / reproductive cycle) expression and enzyme activity in brain

Expression of *CYP19A2* correlated well with the activity and the plasma E_2 levels in, *C. gariepinus*. The transcript levels and enzyme activity in brain exhibited seasonal variation peaking at prespawning phase, a phase when the ovaries were filled mostly with vitellogenic and early post vitellogenic oocytes, in the synchronous spawning catfish, followed by a steep decline in the transcript and activity of aromatase as the oocyte ensued maturation. Similar seasonal pattern of *CYP19A2* transcript levels was reported in adult killifish and channel catfish with mRNA levels peaking prior to the spawning phase (Gelinas *et al.*, 1998; Kazeto and Trant, 2005). We substantiate our findings of seasonal pattern of *CYP19A2* with the previous report (Kazeto and Trant, 2005) from channel catfish that demonstrated the significance of *CYP19A2* in regulating the gonadotropins levels directly or indirectly by local production of E_2 in brain, thus implying a plausible role of *CYP19A2* in the brain-pituitary-gonadal axis. Further there are also evidence supporting the low aromatase activity observed in catfish brain when compared to ovary, from few other fish species viz., *Chasmichthys dolichognathus* (Agohaze), *Leucopsarion petersii* (Shiro-yu), yellowfin goby, rainbow trout and coho salmon (Sasaki and Asahina, 2006). Alternatively high catalytic activity of fish brain compared to ovarian tissue is also demonstrated in goldfish (Pasmanik and Callard, 1988; Zhao *et al.*, 2001).

Ontogeny of *CYP19A2*

The relative change in *CYP19A2* transcript levels were studied after the phenotypic sex differentiation in *C. gariepinus* beginning at 45 dph followed by enzymatic activity at 60 dph.

This aromatase activity at 60 dph in male brain is due to the presence of *CYP19A1* in brain (Rasheeda *et al.* 2010), though transcripts of *CYP19A2* were not observed in 60 dph male brain. Present study revealed dimorphic expression pattern of *CYP19A2* from 45 dph with early expression in female juveniles suggesting a crucial role of estrogen in the maintenance of ovarian differentiation and development. Furthermore, detection of late onset of *CYP19A2* expression in male brain warrants the non-requirement of estrogens during early testicular differentiation, reinforcing the validity of the gynoinductor hypothesis of E_2 (Yamamoto, 1969). These above findings in catfish are in accordance with data documented in juvenile sea bass (Blazquez and Piferrer, 2004) which exhibited higher *CYP19A2* expression in female brain compared to male brain at 20 days post fertilization suggesting *CYP19A2* role in sea bass sex differentiation and also concurrent with reports from zebrafish and the Atlantic halibut where high expression of *CYP19A2* prior to and after gonadal differentiation clearly indicated the role of *CYP19A2* as a key determinant of phenotypic sex in fish (Trant *et al.*, 2001; Matsuoka *et al.*, 2006). However, Kallivretaki *et al.* (2007) have ruled out the possibility of *CYP19A2*'s role during sex differentiation by detecting similar pattern of *CYP19A2* expression and localization in zebrafish brain from both the sexes, concomitant to the findings in carp (Barney *et al.*, 2008). Nevertheless, in zebrafish embryos *CYP19A2* expression occurred sooner and reached higher levels compared to *CYP19A1*, and was E_2 inducible (Sawyer *et al.*, 2006). The sexual dimorphic expression pattern displayed by catfish juveniles similar to roach (Lange *et al.*, 2008) indicates *CYP19A2* may contribute to ovarian differentiation and sexual development. The contribution of the *CYP19A1* might be greater than the *CYP19A2*. The significance of faint expression of *CYP19A2* during catfish testicular differentiation which is in agreement with reports from the

Nile tilapia and the Japanese medaka (Sudhakumari *et al.*, 2003, 2005; Chang *et al.*, 2005; Patil and Gunasekera, 2008) is yet to be resolved.

Tissue distribution pattern of *CYP19A2*

Similar to channel catfish (Trant, 1994; Kazeto and Trant, 2005), in the catfish, *C. gariepinus* *CYP19A2* is expressed only in brain and Rasheeda *et al.* (2010) also showed predominant expression of *CYP19A1* in ovary, brain and many other steroidogenic and non steroidogenic tissues, contradictory to the reports from goldfish, the Nile tilapia, zebrafish and southern catfish where *CYP19A1* was exclusively found in ovary while *CYP19A2* was detected both in ovary and brain (Tchoudakova and Callard, 1998; Trant *et al.*, 2001, Chiang *et al.*, 2001; Kwon *et al.*, 2001; Sudhakumari *et al.*, 2003: 2005; Chang *et al.*, 2005; Zhihao *et al.*, 2007). On the other hand, the catfish brain form was confined to the adult brain nevertheless ontogeny study detected faint testicular expression of *CYP19A2* in testes from 60 dah. However, unlike the Nile tilapia (Sudhakumari *et al.*, 2003, 2005; Chang *et al.*, 2005) *CYP19A2* transcript was detected exclusively in brain as that of channel catfish (Kazeto and Trant, 2005). Discrepancy among fishes in the spatial expression of the two isoforms in steroidogenic and non-steroidogenic tissues may be explained as a consequence of species specific gene specialization that befits their mode of reproduction, sexual and courtship behavior and their habitat, however to implicate a biological role *CYP19A2* one has to measure the protein in those tissues independently.

Phase-dependent (seasonal / reproductive cycle) expression and activity of *CYP19A2* after *in vivo* hCG treatment

Earlier report from the North African catfish has demonstrated the presence of LH receptor in ovary and brain. Further *in vitro* study showed positive response of LH receptor by catfish LH,

salmon LH, human LH, hCG, and human FSH (Vischer and Bogerd, 2003) which made possible the use of heterogenous gonadotropin i.e. hCG to study the modulation, if any, on the transcript and activity level of *CYP19A2* at different reproductive phases of adult female catfish. Our findings revealed a positive response in the activity and transcript levels of aromatase after hCG induction in the preparatory and prespawning phases in brain while no response was seen in the spawning phase. Increase in the aromatase activity in the isolated ovarian follicles after the treatment of gonadotropins or agents that raise the intracellular cAMP levels has been demonstrated in several fish species (Nagahama *et al.*, 1991; Kagawa *et al.*, 2003; Senthilkumaran *et al.*, 2004). However, gonadotropins had no influence on aromatase in the oocytes that were undergoing final oocyte maturation (Yoshiura *et al.*, 2003; Senthilkumaran *et al.*, 2004; Sreenivasulu and Senthilkumaran, 2009; Senthilkumaran, 2011). Direct effects, if any, on *CYP19A2* needs to be ascertained based on the reports on *de novo* synthesis of aromatizable androgen in teleost brain (see Diotel *et al.*, 2010) and the neuroestrogen thus synthesized might govern, the gonadotropin secretion by pituitary, leading to folliculogenesis and ovarian recrudescence. In the present study, administration of hCG did not induce *CYP19A2* or selectively *CYP19A2* during spawning phase when levels of E_2 are very low (Joy *et al.*, 1998; Rasheeda *et al.*, 2010). This perhaps indicates that gondadotropin regulates gonadal recrudescence primarily via *CYP19A1* and *CYP19A2* might contribute secondarily via E_2 production either from ovary or neuroestrogens. In accordance to the results on transcripts, the aromatase activity in brain and ovary was also inducible by hCG treatment during preparatory and prespawning phases but not in spawning. This may further substantiate the effect of gonadotropin (hCG) on *CYP19A2*. Judging from the response of *CYP19A2* after gonadotropin treatment, it may be interesting to understand its regulation in catfish.

Conclusion

In summary, full-length cDNA of *CYP19A2* was cloned from brain of catfish which exhibited high homology with other siluriforms. Purified recombinant protein of *CYP19A2* catalyzed the aromatization of testosterone to E_2. Present study demonstrated the stage-dependent expression and activity of *CYP19A2* in brain during ovarian cycle. Based on our results, we propose that *CYP19A2* might also play an important role in ovarian differentiation and development, either directly or indirectly. Interestingly, the changes in expression of *CYP19A2* after hCG treatment in brain during different phases of reproductive cycle shows its responsiveness to gonadotropins.

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Chapter II

Cloning, expression and ontogeny of brain *FTZ-F1* in the catfish, *Clarias gariepinus,* during ovarian development, recrudescence and after *in vivo* hCG induction

Abstract

The fushi tarazu factor-1 (FTZ-F1), encodes an orphan nuclear receptor belonging to the nuclear receptor family 5A (NR5A). NR5A family members namely, adrenal 4-binding protein or steroidogenic factor-1 (Ad4BP/SF-1), Liver receptor homologue 1 (LRH-1) etc., play a pivotal role in the regulation of aromatases. Present study aimed to understand the possible role of this transcription factor in regulating the brain form of aromatase (*CYP19A2*) during development, recrudescence and after hCG induction in an annually breeding teleost model, *Clarias gariepinus*. To accomplish this, we cloned the full length cDNA of *FTZ-F1* from the brain of catfish and its developmental expression was analyzed. Its transcripts are evident during early stages of development and showed maximum levels during gonadal differentiation period. The tissue distribution pattern both at transcript and protein level revealed its expression was prominent in brain along with liver, kidney and testis. The expression pattern of *FTZ-F1* during reproductive cycle and after *in vivo* hCG induction analogous to that of *CYP19A2,* (Chapter I) indicating its direct involvement in recrudescence. Based on our previous results of Chapter I and the present data it is plausible to implicate the plausible role for *FTZ-F1* in the regulation of *CYP19A2* in teleosts.

Introduction

Fushi tarazu factor 1 (FTZ-F1) is an orphan nuclear receptor that was initially identified as an activator of *fushi tarazu*, a pair-ruled homeobox gene involved in segmentation in *Drosophila* (Ueda and Hirose, 1990; Lavorgna *et al.*, 1991). Since then numerous *FTZ-F1* homologues have been recognized in several species. Presently, FTZ-F1 constitutes a distinct subfamily (NR5A) in the superfamily of nuclear receptors (Kuo *et al.*, 2005). It includes two major subgroups of

related genes with separate function and expression patterns in higher vertebrates. The NR5A1 subgroup contains the *FTZ-F1* homologue, which is also designated as the steroidogenic factor-1 (*SF-1*) or adrenal 4-binding protein (*Ad4BP*; Honda *et al.*, 1993) or *Ad4BP/SF-1* (Yoshiura *et al.*, 2003). In vertebrates, the biosynthesis of steroid hormone is regulated by the tissue-specific expression of cytochrome P450 enzymes (Miller, 1998) and analysis of the promoter motifs of steroid hydroxylase genes revealed the presence of elements specific for a transcription regulator, FTZ-F1 (Ohno *et al.*, 1994; Lala *et al.*, 1992). *Ad4BP/SF-1*, a homologue of *FTZ-F1,* has been identified as an important regulator of steroidogenesis due to its regulatory influence on several cytochrome P450 (CYP) enzymes involved in steroidogenic pathways (Hammer and Ingraham, 1999). Hence, this group of transcription factors has been shown to be critical for normal physiological entrainment of hypothalamo-hypophyseal-gonadal axis in reproduction and sexual differentiation of gonads in vertebrates (Ikeda *et al.*, 1993). The NR5A2 subgroup contains the liver receptor homologue 1 (*LRH-1*) or α-feto protein transcription factor (FTF), which regulates the expression of the α-feto protein (Galarneau *et al.*, 1996) and is involved in cholesterol metabolism (Nitta *et al.*, 1999) of mammals.

In non-mammalian vertebrates, role of FTZ-F1 has not been fully understood. Teleost *FTZ-F1* has been put forward as a likely candidate upstream regulator of several genes involved in steroidogenesis, and it has been suggested that teleost *FTZ-F1* may be involved in feminization of developing testis in response to estrogen exposure (Govoroun *et al.*, 2001). The expression patterns of teleost *FTZ-F1* homologues partly correlate to mammalian patterns (Lin *et al.*, 2000; von Hofsten *et al.*, 2001), but their roles in reproduction are yet to be elucidated in detail, although few reports are available in salmonids (Higa *et al.*, 2001; von Hofsten *et al.*, 2002).

In several teleosts, *FTZ-F1* and its homologues are reported to be involved in the regulation of either form of aromatases. The expression of *FTZ-F1* has not yet been described in teleost brain and studies regarding protein expression patterns are limited. This prompted us to clone the full length cDNA of *FTZ-F1* from the brain of air-breathing catfish, *Clarias gariepinus* and analyze its expression pattern in brain during reproductive cycle and after *in vivo* treatment of human chorionic gonadotropin (hCG). Western blot analysis was performed to understand its expression pattern at protein level using purified FTZ-F1 antibody raised against an antigenic peptide designed from deduced amino acid sequence of catfish FTZ-F1. Our interesting observation of specific expression of *FTZ-F1* specifically in brain but not in ovary like that of *CYP19A2* (shown in Chapter I), opens up a new avenue of research to unravel the regulatory role of former over the later.

Materials and methods

Animals and sampling

Breeding and rearing of catfish (*C. gariepinus*) at different age groups was already described in Chapter I. The catfish hatchlings were collected at different time points: 0, 5, 10, 20, 30, 50, 75, 100 and 200 days post hatch (dph) were sacrificed by briefly dipping them in mild ice-cold water as per the animal ethical conditions and brains were dissected using a fine forceps under stereo zoom microscope (Leica, Germany) except for 0 day where in whole body was used and since, it was not possible to isolate brain from 10 and 20 dph larva, we used whole head for total RNA isolation. Five biological samples (n=5) were used for each time point of ontogeny study. To perform seasonal cycle studies, adult fishes were collected in the months of February, May, September and December corresponding to preparatory, prespawning, spawning and regressed

phases, respectively. All the tissue samples collected were snap frozen in liquid nitrogen and stored briefly at -80 °C until use.

Molecular cloning of *FTZ-F1* from brain

Total RNA from brain of catfish was isolated and first strand cDNA was synthesized as described in Chapter I. Based on the alignment of known *FTZ-F1* sequences using Lasergene software (release 3.05; DNASTAR, Madison, WI, USA), a set of degenerate primers were designed and synthesized. Using degenerate primers (Table. 1) a partial cDNA fragment was amplified from brain. The amplicon obtained was cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and sequence was determined. In order to obtain full length sequence of *FTZ-F1* cDNA, RNA-ligase mediated RACE system (Invitrogen, Carlsbad, CA, USA) was used. RACE primers provided in the kit and gene specific primers were designed from partial fragment of *FTZ-F1* as per the manufacturer's protocol (Table. 1). The RACE products obtained were cloned into pGEM-T easy vector, subsequently sequenced and their identity was analyzed by NCBI-BLAST.

Polyclonal antibody generation and peptide affinity purification

Based on the deduced amino acid sequence of FTZ-F1, an antigenic peptide, H_2N-KAEH**P**DPYSGSPDS-COOH was synthesized and conjugated to keyhole limpet hemocyanin carrier protein commercially (USV Limited, Mumbai, India). This peptide was dissolved in PBS (pH 7.4) and used for antibody generation. Three-months old New Zealand white rabbits used for antibody raising were housed and handled as per the guidelines of Institutional Animal Ethics Committee and CPCSEA. Prior to injection, the lateral ear vein was bled to collect pre-immune serum. The animals were injected subcutaneously with 200 µg of antigenic peptide emulsified

with Freund's complete adjuvant followed by two booster injections (100 µg) of antigen emulsified with Freund's incomplete adjuvant. The serum was collected and the presence of the antibody was confirmed by dot blot method (Talbot *et al.*, 1984). All the rabbits in the present study were used following the guidelines of Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals and also after obtaining prior permission. The antibody raised was further affinity purified using CNBr-activated Sepharose™ 4B (Amersham Biosciences). Briefly, the FTZ-F1 antigenic peptide was coupled to the beads. The serum was passed through the column for binding. The bound antibody was eluted with 200 mM glycine (pH 2.8). The IgG fraction was checked for the presence of heavy and light chains.

Table. 1. List of primers used for analysis of *FTZ-F1*.

S.N0	Primer	Sequence 5' to 3'	Purpose
1.	DFw	GCWCTVCGTGATKGCRGACYAGASKCTG	To amplify partial cDNA fragment
2.	DRv	GAKAGYCCAGWCCCGASTCAGAMAYGMCG	To amplify partial cDNA fragment
3.	5' P	GTCCCCGCACACAGGACAGAGCTC	RACE
4.	5' N	GCAGGCCGTAGTGATACCCGGACAC	RACE
5.	3' P	GAGCGCCCAGGCCGAGGAGTACCTG	RACE
6.	3' N	GCTGCATGCCAAGAGAGCCACCACC	RACE
7.	RTFw	GGAGGAGCTCTGTCCTGTGTGCG	RT-PCR & qRT-PCR
8.	RTRv	GCTGCGTCTTATCGATGCCGCAG	RT-PCR & qRT-PCR
9.	β-actin Fw	ACCGAATGCCATCACAATACCAGT	RT-PCR & qRT-PCR
10.	β-actin Rv	GAGCTGCGTGTTGCCCCTGAG	RT-PCR & qRT-PCR

qRT-PCR analysis of *FTZ-F1*

Total RNA was isolated from various tissues, different phases of reproductive cycle, ontogeny and various time points after hCG induction. The standardization and methodology used for qRT-PCR were mentioned in Chapter I. The primers for real time PCR were designed from the conserved region of DNA binding domain as the *FTZ-F1* gene (Table. 1) by considering the intron-exon boundaries. Primers for endogenous control *β-actin* cDNA were designed from *β-actin* sequence obtained from catfish (Table. 1).

In vivo hCG induction

For *in vivo* hCG induction studies, catfish were injected with 1000 IU/kg body weight hCG (Pubergen, Uni-Sankyo Pvt. Ltd., India) intraperitoneally. Brain was collected at 0, 4, 8, 12 and 24 h time points. Controls were treated with physiological saline. Experiments were performed (n=5) with different batches of female fish during preparatory (March) and prespawning (May) phases. Dosages of hCG were chosen based on our earlier report (Sreenivasulu and Senthilkumaran, 2009).

SDS-PAGE and Western blot analysis

Different tissues from adult prespawning phase catfish were collected and snap frozen in liquid N_2 and homogenized in protein extraction buffer [10 mM Tris-Cl (pH 7.4), 0.1% Triton X-100, 1 mM PMSF, 1 mM EDTA and 1 mM DTT] followed by centrifugation at 500Xg for 15 min to remove debris. The aliquots of the supernatant were used immediately to avoid the loss of activity. SDS-PAGE (12%) and western blot analysis was carried out respectively.

Immunocytochemistry

Immunocytochemistry was done as per the method described previously by Swapna *et al.* (2006). In brief, brain tissue from catfish was fixed in Bouin's fixative (saturated picric acid: formaldehyde: glacial acetic acid, 15: 5: 1) for 1 h and embedded in paraplast (Sigma) after dehydration with graded alcohol and xylene. Paraplast sections at 3-5 µm sections were prepared using a rotary microtome (Leitz). Sections were then deparaffinized and hydrated, and later blocked with 10% normal goat serum for 1 h at room temperature. Purified anti-FTZ-F1 polyclonal antibody (1:500) was applied to the sections and incubated for 1 h at room temperature in a humid chamber. Following incubation with primary antibody, sections were incubated with either HRP (Bangalore Genei) labeled secondary antibody, washed with PBS and developed using 3' 3' diaminobenzidine (DAB) for 3-10 min. Images of sections developed with DAB were taken with Olympus CX41 light microscope fitted with a digital camera.

Statistical analysis

All the data were expressed as mean ± SEM. Significance among groups was tested by ANOVA followed by Student's-Newman-Keuls' test using Sigmastat (version 11) software. Differences among groups were considered significant at $P<0.05$.

Results

Cloning of *FTZ-F1* from brain of catfish

A partial cDNA of 243 bp was obtained using the set of degenerate primers (DFw and DRv) (Fig. 1A). The sequence identity of the fragment was confirmed by NCBI-BLAST. Full length *FTZ-F1* cDNA obtained by using RACE was 2355 bp long with 319 bp of 5' untranslated region

(UTR) (Fig. 1B), 626 bp 3' UTR (Fig. 1C) and polyadenylation signal ATTAAA which is 24 bp upstream of poly-A tail. The open reading frame (ORF) is 1410 bp long (Fig. 1D), encoding 469 amino acid residues. The full length nucleotide sequence along with the deduced amino acid sequence was depicted in Figure 2. ClustalW alignment revealed the presence of a signature ligand binding domain, DNA binding domain and AF-2 regions, which are conserved for all transcription factors belonging to nuclear receptor superfamily and are well preserved among teleosts (Fig. 3). Phylogenetic analysis revealed that catfish *FTZ-F1* showed high homology with NR5A1 group genes of most teleosts and other known vertebrate counter parts (Fig. 4).

Western blot analysis

The flow chart showing methodology of production of polyclonal antibody against rabbit and the peptide affinity purification methodology by using Sepharose 4B beads (Amersham) were provided in Figures 5 and 6, respectively. The purification profile of FTZ-F1 antibody (IgG fraction) heavy and light chain is shown in Fig. 7A. The elution profile of affinity chromatography of the antibody raised against the synthetic peptide was depicted in Figure 7B. The antibody generated against this peptide detected a ~45 kDa protein from the brain protein homogenate, which matched with predicted size of FTZ-F1 protein of teleosts including catfish (Fig. 8A). Synthetic peptide pre-incubated with the purified fraction of antibody was used as a negative control to determine its specificity against FTZ-F1 protein (Fig. 8B).

Tissue distribution of *FTZ-F1*

The tissue distribution pattern of *FTZ-F1* was analyzed by semi-quantitative RT-PCR (Fig. 9A) and also by qRT-PCR showed highest expression in brain (Fig. 9B). Low levels of expression

were also seen in liver, kidney and testis. The tissue distribution of FTZ-F1 protein correlated well with that of the transcript levels when probed with the FTZ-F1 antibody (Fig. 9C).

Ontogenic expression of *FTZ-F1* in brain of catfish

The expression of *FTZ-F1* monitored from 0 dph to adult stage at different time intervals in brain by qRT-PCR (Fig. 10) which revealed differential expression pattern in males and females. The expression gradually increased from 0 dph to 40 dph and peaked maximum by 50 dph. Thereafter, expression of *FTZ-F1* gradually decreased from 50 to 200 dph, and elevated again at 300 dph and in adult female brain which was significant. In male brains, its expression decreased drastically from 50 to 300 dph and an increase in the adult male brain was observed.

Expression of *FTZ-F1* during reproductive cycle

FTZ-F1 expression was analyzed using qRT-PCR in different phases of reproductive cycle in brain (Fig. 11). The *FTZ-F1* showed highest expression during prespawning phase when compared to preparatory and spawning phases. The expression was found to be low in the regressed phase.

Expression of *FTZ-F1* after hCG treatment, *in vivo*

Brain collected at 0, 4, 8, 12 and 24 h after the hCG induction *in vivo* showed a gradual increase from 0 to 8 h that was persistent till 12 h during preparatory phase (Fig. 12A) whereas in prespawning phase the expression was found to be highest at 4 h and decreased gradually at later time points when compared to saline treated controls (Fig. 12B).

Localization of FTZ-F1 in brain of catfish

Immunocytochemical localization of FTZ-F1 was evident in cross section of 20 dph fish head region (Fig. 13A, B) and in the cross section of brain through pre-optic area–hypothalamus in 150 dph fish (Fig. 13C, D). Sagittal section of brain through hypothalamus-mid brain area in also adult catfish indicated positive immunoreactivity (Fig. E, F).

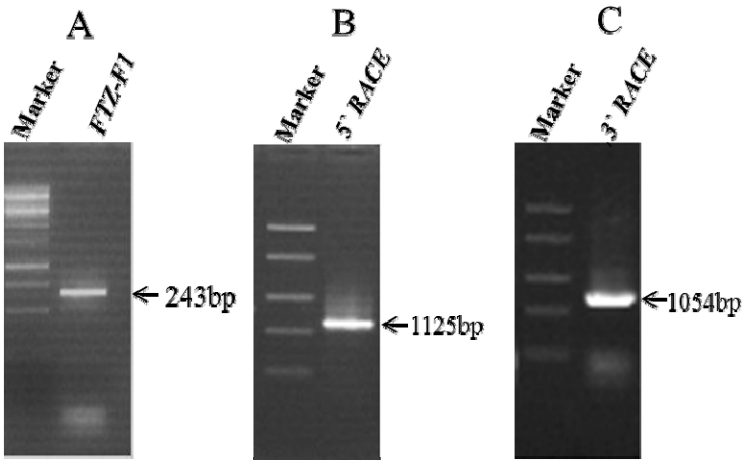

Fig.1. Amplification products of *FTZ-F1* A) partial *FTZ-F1* cDNA fragment from catfish brain. B), C) 5' and 3' RACE products to obtain full-length cDNA of *FTZ-F1*.

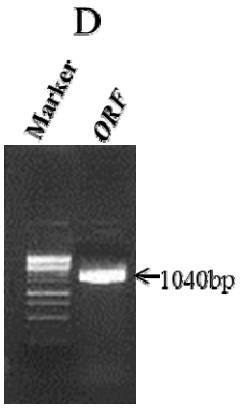

Fig.1. D) Amplification product of *FTZ-F1* ORF region.

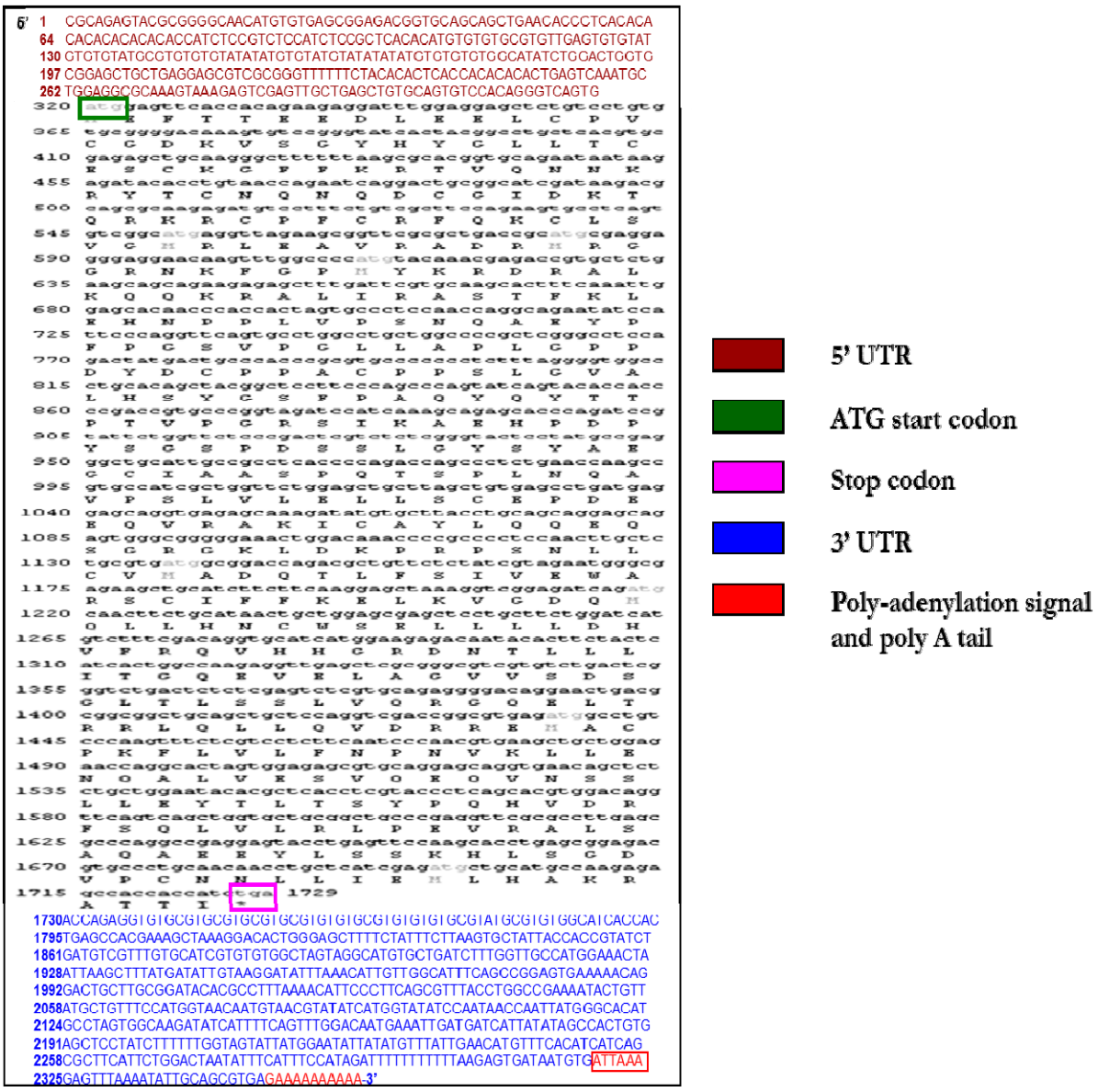

Fig.2. Nucleotide and deduced amino acid sequence of catfish full-length *FTZ-F1* cDNA.

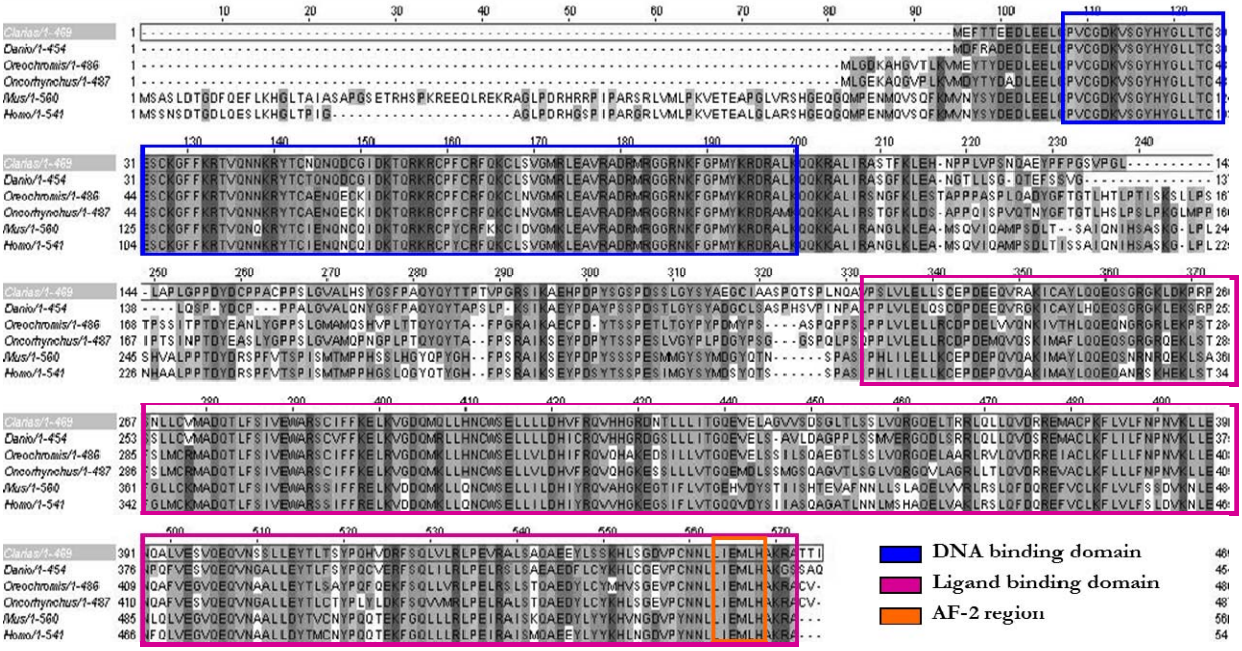

Fig.3. ClustalW multiple alignment of catfish FTZ-F1 with other vertebrate counterparts. GenBank accession numbers of FTZ-F1 sequences are:- *Oreochromis niloticus*: AB060814; *Oncorhynchus mykiss*: AY879314; *Danio rerio*: NM131463; *Mus musculus*: AF511594 and *Homo sapiens*: BC118571.

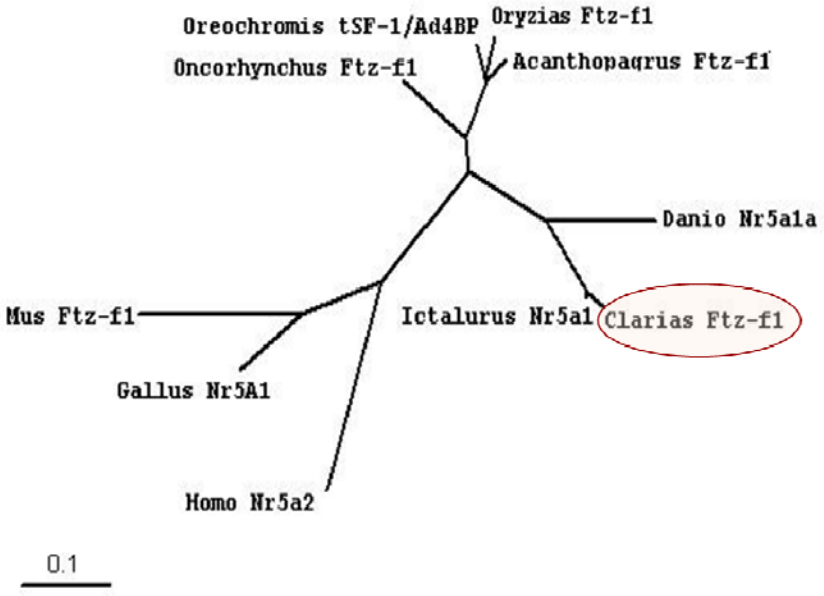

Fig.4. Phylogenetic tree of catfish FTZ-F1. Phylogenetic analysis was done with ClustalX

software 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 tree was generated using the TreeView software package version 1.4. GenBank accession numbers of the sequences used in phylogenetic tree are as follows: *Ictalurus punctatus*: DQ000612; *Oreochromis niloticus*: AB060814; *Oryzias latipes*: AB016834; *Oncorhynchus mykiss*: AY879314; *Danio rerio*: NM131463; *Acanthopagrus schlegelii*: AY491379; *Gallus gallus*: NM205077; *Mus musculus*; AF511594 and *Homo sapiens*; BC118571.

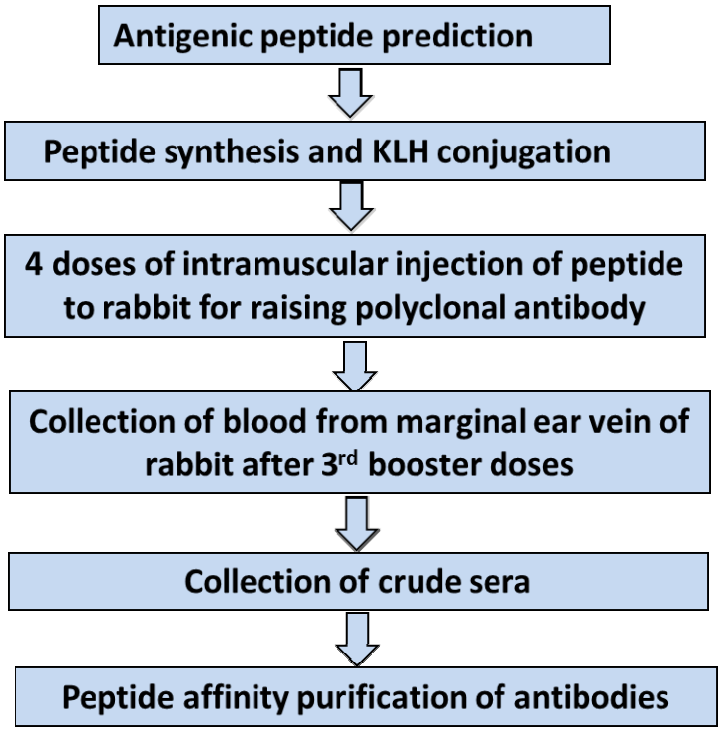

Fig.5. Flow chart of methodology used for raising polyclonal antibody in rabbit against different proteins using antigenic peptide.

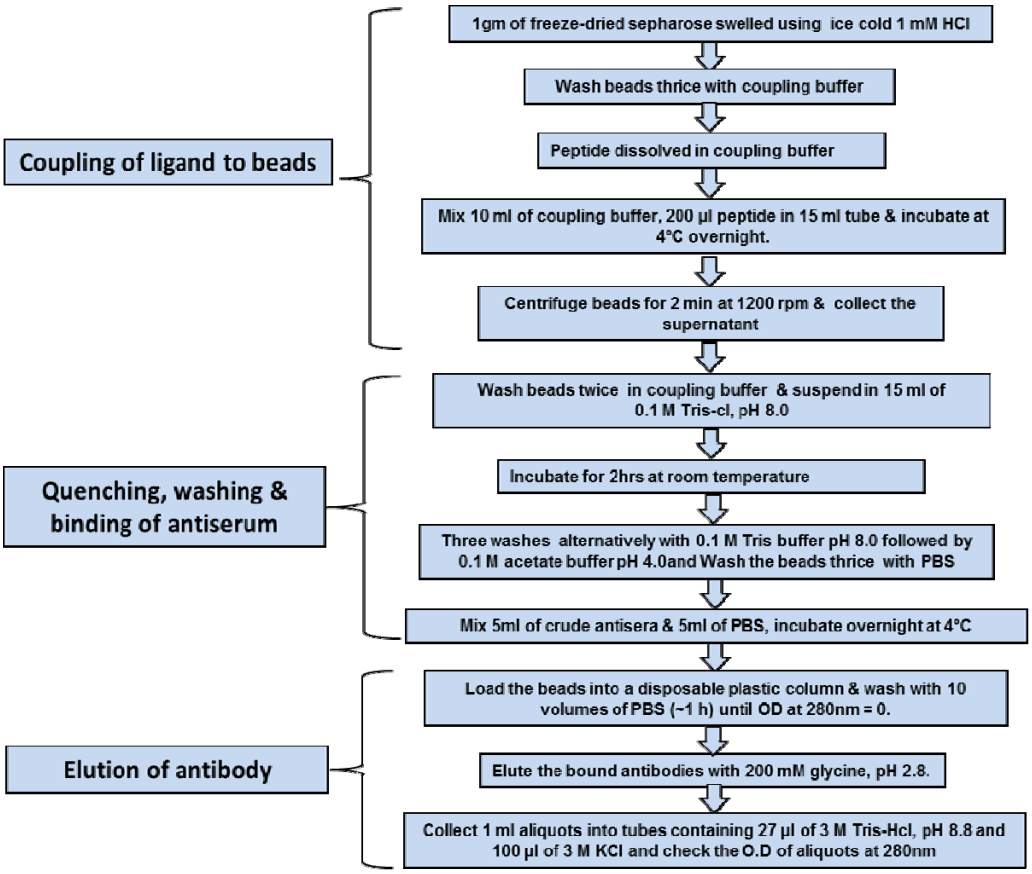

Fig.6. Flow chart of methodology used for peptide affinity purification of antibodies using Sepharose 4B beads raised against antigenic peptide.

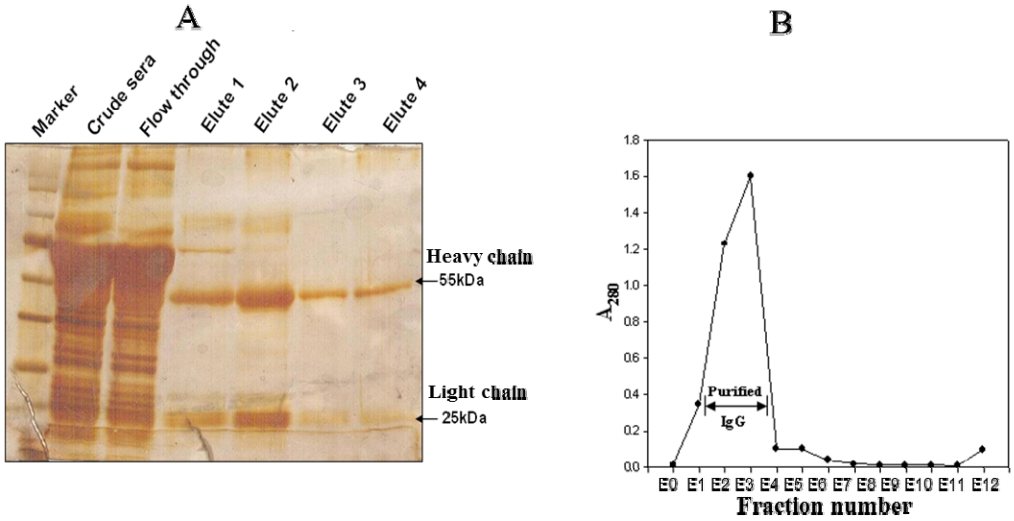

Fig.7. Peptide affinity purification profile of FTZ-F1 antibody. A) Silver stained gel showing

IgG fraction of FTZ-F1 antibody. Bands detected corresponding to ~55kDa (heavy chain) and ~25kDa (light chain). B) Affinity chromatography elution profile of FTZ-F1 antibody.

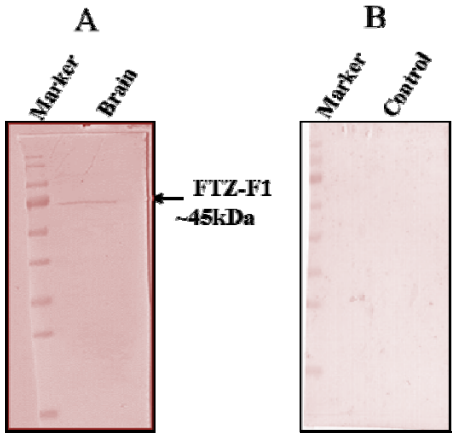

Fig.8. Western blot analysis of brain homogenate with FTZ-F1 antibody A) Western blot showing band (~45kDA) corresponding to FTZ-F1 protein. B) No signal in control (peptide pre absorbed with FTZ-F1 antibody).

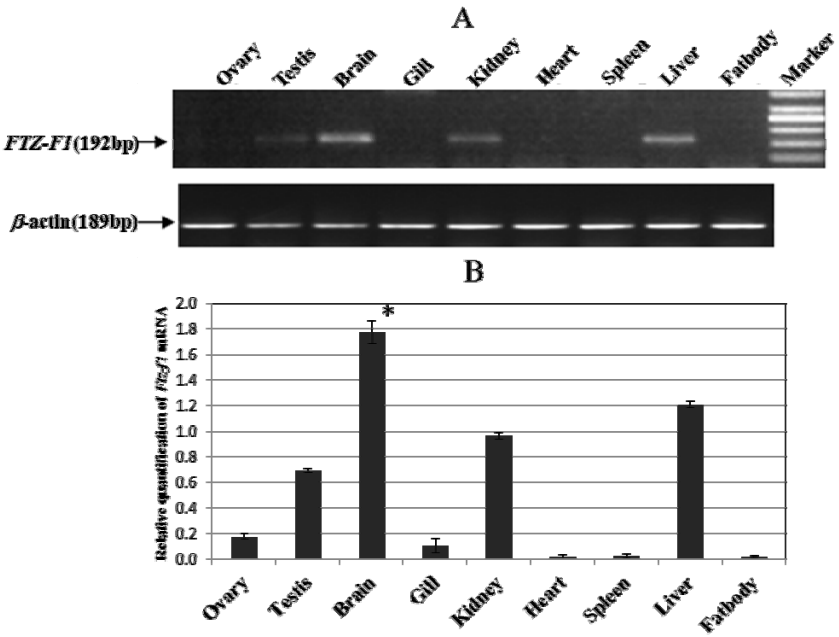

Fig.9. Tissue distribution pattern of *FTZ-F1*. A) Semi-quantitative RT-PCR analysis of *FTZ-F1* expression in different adult female catfish tissues. B) qRT-PCR analysis of *FTZ-F1* expression in different tissues of adult female catfish. * indicates the significance at $P < 0.05$.

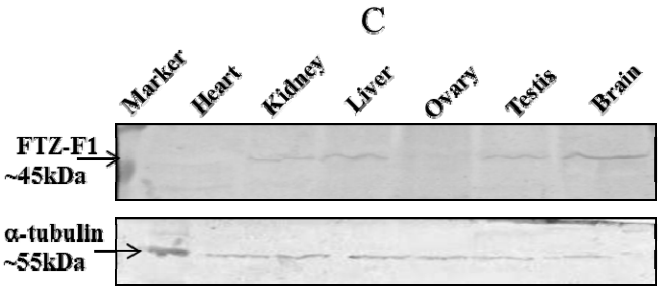

Fig.9. C) Western blot analysis of tissue distribution pattern of FTZ-F1. Western blot using FTZ-F1 antibody in various tissues (upper panel) and western blot using α-tubulin antibody (~55kDa) (lower panel) used as equal loading control.

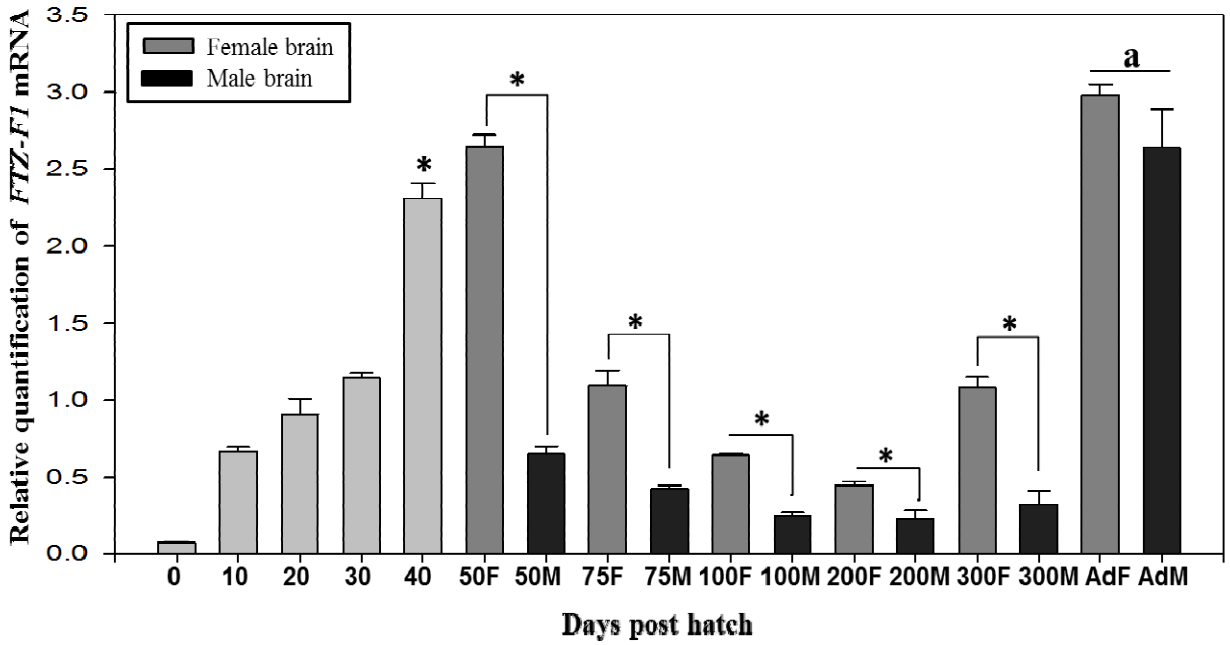

Fig.10. Changes in FTZ-F1 mRNA levels during ontogeny in male and female brains of catfish. * indicates the significance at $P < 0.05$, [a] denotes the significance at $P<0.05$ from 50/75 - 300 dph to adult for respective sex.

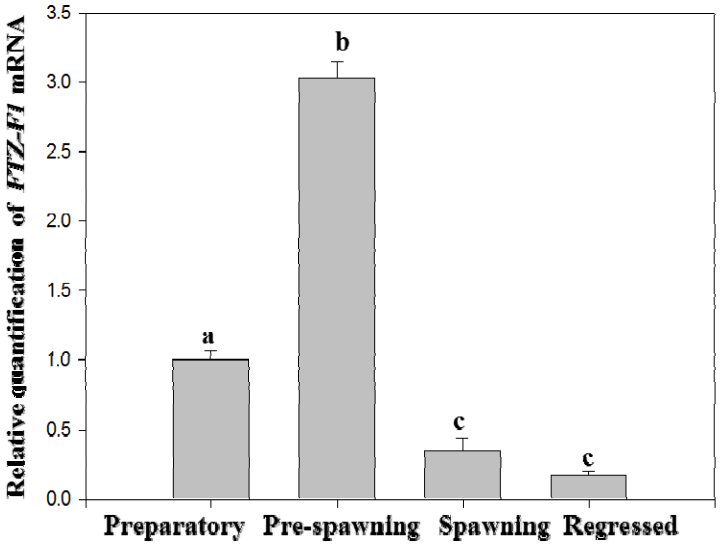

Fig.11. Changes in *FTZ-F1* mRNA levels in brain during different phases of ovarian cycle of catfish. Different alphabets indicate groups that are significantly different P< 0.05.

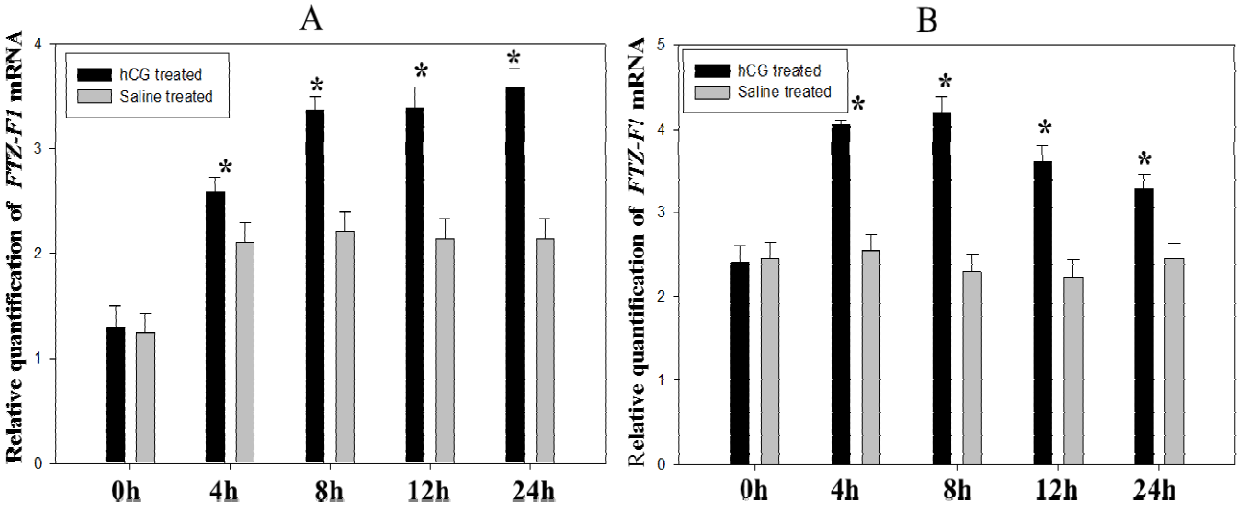

Fig.12. Changes in *FTZ-F1* mRNA levels in brain at different time points after *in vivo* hCG treatment A) Preparatory and B) Pre-spawning phase of ovarian cycle compared to saline treated controls. * indicates the significance at P < 0.05.

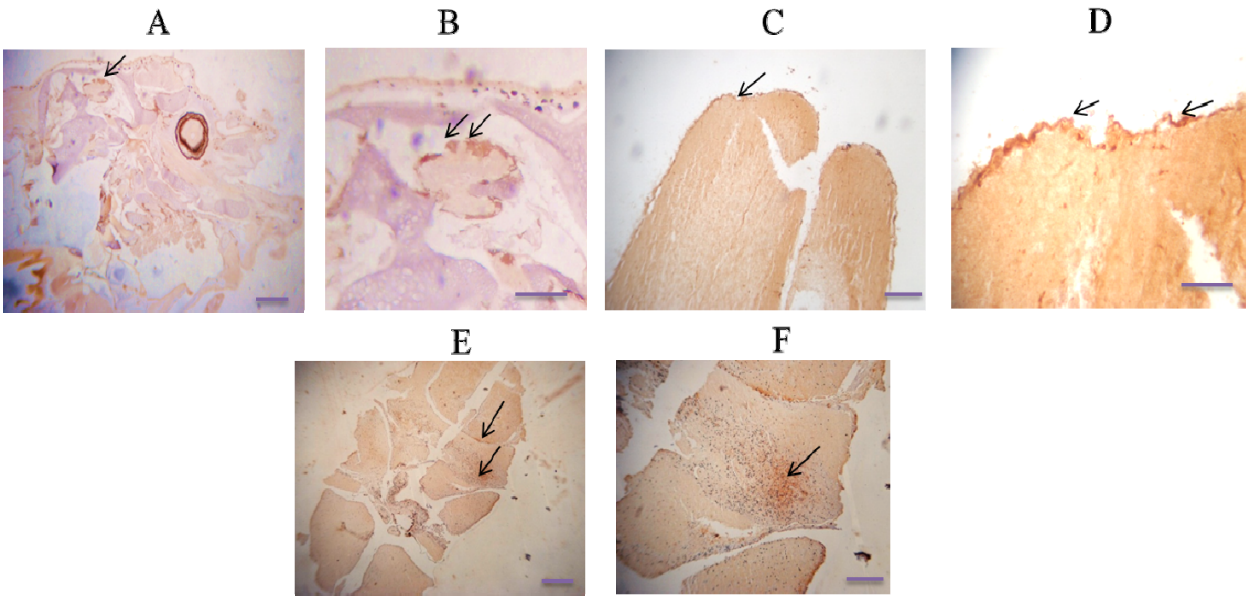

Fig.13. Immunocytochemical localization of FTZ-F1 A), B) Cross section of 20 dph fish head region C), D) Cross section of brain through pre-optic area-hypothalamus in 150 dph fish E), F) Sagittal section of brain through hypothalamus-mid brain area in adult catfish. Arrow marks indicate immunoreactivity. Scale bar indicates 50 µM.

Discussion

In the present study, we isolated the *FTZ-F1* cDNA from the brain of catfish using RACE strategy after obtaining a partial cDNA fragment using degenerate primers. Multiple amino acid (deduced) sequence alignment indicated that the catfish *FTZ-F1* had the typical structural features of nuclear receptor of FTZ-F1 subfamily. There are three conserved regions in the FTZ-F1 related proteins, which are the putative DNA-binding and ligand-binding/ dimerization domains (Wang *et al.*, 1989; Honda *et al.*, 1993). Despite the number of studies on vertebrate *FTZ-F1* genes during recent years, there exists a discrepancy in the nomenclature and functions of these genes. The primary structure of *FTZ-F1* gene was earlier reported in silkworm (Sun *et al.*, 1994), *Xenopus laevis* (Ellinger-Ziegelbauer *et al.*, 1994), mouse (Lala *et al.*, 1992), rat

(Nomura *et al.*, 1995; Galarneau *et al.*, 1996), bovine (Honda *et al.*, 1993), human (Oba *et al.*, 1996), zebrafish (Liu *et al.*, 1997), chicken (Kudo and Sutou, 1997), and rainbow trout (Ito *et al.*, 1998). More recently, *FTZ-F1* homologs have been isolated from other vertebrates and other phyla where four isoforms of *FTZ-F1* were reported in zebrafish (Liu *et al.*, 1997) and two isoforms of *FTZ-F1* were identified in chicken (Kudo and Sutou, 1997), shrimp (Chan and Chan, 1999), frog (Nakajima *et al.*, 2000) and arctic char (von Hofsten *et al.*, 2002, 2003). We obtained a single form of *FTZ-F1* similar to most other teleosts like rainbow trout (Ito *et al.*, 1998), medaka (Watanabe *et al.*, 1999) and orange spotted grouper (Zhang *et al.*, 2004) where only one form is reported. Though the sequence analysis of the catfish *FTZ-F1* cDNA by NCBI-BLAST tool showed identity with those of the *NR5A1* group members (*Ad4BP/SF-1*) than those of NR5A2 members (*LRH-1/FTF*) of the *FTZ-F1* family, the clustalW alignment and phylogenetic tree generated using the amino acid sequence by the neighbor-joining method did not classify the catfish *FTZ-F1* homologue into either the Ad4BP/SF-1 or the LRH-1/FTF group. Hence we designate the catfish *FTZ-F1* clone as *FTZ-F1/NR5A1*.

The spatial expression pattern revealed its presence predominantly in brain along with lower levels in liver and kidney specifies its distribution similar to *LRH-1* in teleosts (von Hofsten *et al.*, 2003; Matsuyama *et al.*, 2007). Similar expression pattern was also reported earlier in zebrafish where zFF1a expressed in the different brain regions (von Hofsten *et al.*, 2001) and zFF1b was restricted to the pancreas and diencephalon region of brain (Chai and Chan, 2000). This was also analogous to orange spotted grouper where *FTZ-F1* expression was evident in the hypothalamus, pituitary and forebrain which was related to function of *SF-1* similar to mammals where expression of *SF-1* is essential for the development of gonadotrops of pituitary (Parker and Schimmer, 1997).

Developmental expression of *FTZ-F1* in brain has also been observed in other species (Chai and Chan, 2000; von Hofsten *et al.*, 2001, 2003; Liu *et al.*, 2004) although the functions and expression patterns are diverse between different species. In this study, we observed an early expression *FTZ-F1* in brain which reached a maximum during 50 dph where 35-50 dph is critical window of sex differentiation in catfish (Raghuveer and Senthilkumaran, 2009). A sexually dimorphic expression pattern of *FTZ-F1* was also observed in male and female brains of developing catfish. Comparable expression pattern was observed for *CYP19A2* of catfish (Chapter I) which is indicative of involvement of *FTZ-F1* in differentiation and recrudescence through brain-pituitary-gonadal axis along with *CYP19A2*. The reports from zebrafish where expression of both zFF1a and b increased during development and peaked close to hatch in brain (von Hofsten *et al.*, 2001) further endorse our results.

We demonstrated the expression of *FTZ-F1* in brain in different phases of reproductive cycle of catfish where its predominant expression during prespawning phase was observed similar to brain form of aromatase supporting its role in the regulation of *CYP19A2* thus indirectly involved in sex differentiation and recrudescence. We also observed increased transcript levels of *FTZ-F1* in the brain of catfish in response to hCG treatment during recrudescence whose pattern is similar to that of *CYP19A2*. Localization of FTZ-F1 in hypothalamic preoptic area-hypothalamus of brain of catfish corroborates well with localization data on CYP19A2 (Chapter I).

Expression of *CYP19A1* (ovarian form) has also been shown to be regulated by *FTZ-F1* in medaka (Watanabe *et al.*, 1999) and in rainbow trout (Kanda *et al.*, 2006). It was earlier reported that these nuclear receptor family members can bind similar response element (i.e.) half sites related to hexamer AGGTCA on the promoter CYPs and can activate the transcription (Honkakoski and Negishi, 2000). Report in medaka by Matsuyama *et al.* (2007), showed that

LRH-1 potentially activates the promoter of *CYP19A2* in medaka. In tilapia, it has been demonstrated that *FOXL2* (a member of the forkhead/HNF-3-related gene family of transcription factors) interacts through its fork head domain with the ligand-binding domain of *Ad4BP/SF-1* (*NR5A1*) to form a hetero-dimer which eventually regulates *CYP19A1* transcription (Wang *et al.*, 2007). Taken together, by considering the expression pattern and morphology, the catfish FTZ-F1 might have a potential role in transcriptional regulation of *CYP19A1/A2* as that of LRH-1 or Ad4BP/SF-1 and might be activated by FOXL2 which can bind to its DNA binding domain as that of *Ad4BP/SF-1*.

Conclusion

In conclusion, we isolated and cloned *FTZ-F1* cDNA from brain of catfish. Based on the deduced amino acid sequence homology and the expression of the cDNA, it can be considered a potential nuclear receptor belonging to the FTZ-F1 subfamily. *FTZ-F1* has been implicated in the sexual differentiation of many vertebrates. However, reports in mammals suggest that NR5A1 members proposed to serve as a critical regulator of *CYP19A1/A2* gene expression and in teleosts, it has been shown that FTZ-F1 homologues regulate both forms of aromatases individually or synergizes with FOXL2. Our results on spatio-temporal expression patterns and also during reproductive cycle of FTZ-F1 in brain corroborates well with the similar study on catfish regarding expression patterns of *CYP19A2* (Chapter I). Taken together, our results indicate a decisive role for *FTZ-F1* in sexual differentiation process through regulation of *CYP19A2*.

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Chapter III

Cloning and differential expression of *FOXL2* during ovarian development and recrudescence in the catfish, *Clarias gariepinus*

Abstract

FOXL2 is a member of the forkhead/HNF-3-related gene family of transcription factors which provides tissue-specific gene regulation. It is known to regulate *CYP19A1* that plays a crucial role in ovarian differentiation. To understand the role of *FOXL2* in ovarian development and recrudescence, we cloned the full-length cDNA of *FOXL2* and studied its spatio-temporal expression both at transcript and protein levels in the air-breathing catfish, *C. gariepinus*. Based on its deduced amino acid sequence, an antigenic peptide conjugated with a carrier protein was synthesized and used for raising antibody that cross-reacted specifically with FOXL2. Tissue distribution pattern of *FOXL2* revealed its presence prominently in ovary and female brain. Highest expression of *FOXL2* was observed in prespawning phase both in ovary and brain during reproductive cycle indicating an important role for this correlate in ovarian recrudescence. Treatment of hCG, *in vitro* and *in vivo*, induced the expression of ovarian *FOXL2* during preparatory and prespawning phases. Similar type of enhanced expression was evident in the brain after hCG-induction during the prespawning phase. The ontogeny of *FOXL2* showed a sexual dimorphic expression pattern in gonads and brain. Based on our previous studies, the expression pattern of *FOXL2* was found to be synchronous not only with that of *CYP19A1* but also with *CYP19A2*. Taken together, present study substantiates the role of *FOXL2* in the regulation of CYP19s in teleosts and also designates *FOXL2* as a potential marker for ovarian differentiation in catfish.

Introduction

A well-synchronized interaction between the transcription factors and hormones, more specifically, sex steroids, is essential for mechanism(s) that lead to gonadal differentiation. In most teleosts, the proportion of androgens and estrogens is very important for sexual/gonadal differentiation which is restricted by cytochrome P450 aromatase availability. It regulates the amount of estrogen, primarily estradiol-17β. The levels of estradiol are kept within the traversable range for ovarian differentiation by the aromatase (Trant *et al.*, 2001; Fenske and Segner, 2004). In fish, two forms of cytochrome P450 aromatases are known, namely, *CYP19A1* and *CYP19A2* (Kwon *et al.*, 2001; Liu *et al.*, 2007). The ovarian aromatase, *CYP19A1* plays a predominant role in ovarian differentiation while *CYP19A2* is specifically expressed in the brain which is indirectly involved in gonadal differentiation probably through the hypothalamo-hypophyseal-gonadal axis (Chang *et al.*, 2005; Sawyer *et al.*, 2006; Rasheeda *et al.*, 2010).

The expression of *CYP19A1* is regulated by several factors such as *Ad4BP/SF-1*, *FOXL2* and anti-mullerian hormone (Wang *et al.*, 2007; Yamaguchi *et al.*, 2007). *FOXL2* is a member of the forkhead/HNF-3-related gene family of transcription factors that is involved in ovarian differentiation and many other developmental processes including cellular differentiation (Carlsson and Mahlapuu, 2002). *FOXL2* interacts through its fork head domain with the ligand-binding domain of *Ad4BP/SF-1* to form a hetero-dimer which eventually enhances the *Ad4BP/SF-1* mediated *CYP19A1* transcription (Nakamoto *et al.*, 2006; Wang *et al.*, 2007). Recently, it was shown that *FOXL2* suppresses the testicular differentiation mainly through repression of *Sox9* regulatory element that

promotes testis-specific expression of *Sox9* leading to gonadal sex reversal in mice (Uhlenhaut *et al.*, 2009). The expression of *FOXL2* has been analyzed both in mammals (Crisponi *et al.*, 2001; Cocquet *et al.*, 2002) and non-mammalian vertebrates (Loffler *et al.*, 2003). The existing data denotes the ovary specific expression of *FOXL2* not only in mouse embryos, but also in chick, turtle and fish around the time of female sex differentiation (Loffler *et al.*, 2003; Wang *et al.*, 2004; Liu *et al.*, 2007). These findings suggest a conserved role of *FOXL2* in vertebrate ovarian development. *FOXL2* was localized in stroma and interstitial cells of the gonads of normal XX and sex-reversed XY tilapia wherein *CYP19A1* and *Ad4BP/SF-1* were normally found before the occurrence of morphological sex differentiation (Wang *et al.*, 2007). Our earlier studies in the catfish demonstrated elevated expression and activity of both *CYP19A1* and *CYP19A2* during prespawning phase and after hCG induction (Rasheeda *et al.*, 2010). It is plausible that *FOXL2* may contribute for changes in both forms of aromatase expression and activity during ovarian cycle and ontogeny. Though extensive study has been carried out on aromatases, the role of *FOXL2* during ovarian development and recrudescence is not well established. In this context, it would be intriguing to explore the spatio-temporal expression pattern of *FOXL2* during reproductive cycle and gonadal differentiation. This analysis might further provide enough evidences for the significance of *FOXL2* in the regulation of aromatases.

To accomplish this, we report full length cloning of *FOXL2* from *Clarias gariepinus* that was employed to characterize the expression at molecular level and also to design a specific peptide used for generation of an antibody specifically immunoreactive to *FOXL2* protein. Further, we studied its expression during gonadogenesis, ovarian cycle

and after *in vivo* hCG induction at different phases of seasonal cycle in ovary and female brain.

Materials and methods

Catfish breeding and sampling

C. gariepinus were bred and reared in the aquaculture facility as per the details described previously (Chapter I). Hatchlings at different age groups, i.e., 0 (less than 23 hours post hatch), 10, 20, 30, 40, 50, 100 and 200 days post hatch (dph) were sacrificed by briefly dipping them in mild ice-cold water as per the animal ethical procedures. From 50 dph onwards, sex was identified based on morphology and histology of gonadal sections as the gender was clearly evident from this age group. Brain and mesonephric gonadal complex (MGC) were isolated using fine sterile forceps under a stereozoom dissection microscope from 30, 40 and 50 dph juvenile fish. It was difficult to isolate MGC complex from 5, 10 and 20 dph larva, and hence we used trunk region by removing most of the muscular and intestinal part for total RNA isolation. Such a strategy has been utilized for a similar type of ontogenic study in catfish (Raghuveer and Senthilkumaran, 2010). Five biological samples (n=5) were used for each time point for ontogeny study. To perform seasonal cycle studies, adult fishes were collected in the months of February, May, September and December corresponding to preparatory, prespawning, spawning and regressed phases, respectively. All the tissue samples collected were snap frozen in liquid N_2 and stored briefly at -80 °C until use.

Molecular cloning of *FOXL2* from ovary

Total RNA from ovary of catfish was isolated and the first strand cDNA was synthesized, as per the method described in Chapter I. Based on the alignment of known *FOXL2* sequences using Lasergene software (release 3.05; DNASTAR, Madison, WI, USA), a set of degenerate primers were designed and synthesized (Table. 1) and a partial cDNA fragment was amplified from ovary. The amplicon obtained was cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and sequence was determined. In order to obtain full length sequence of *FOXL2* cDNA, RNA-ligase mediated RACE system (Invitrogen, Carlsbad, CA, USA) was used. RACE primers provided in the kit and gene specific primers were designed from partial fragment of *FOXL2* as per the manufacturer's protocol (Table. 1). The RACE products obtained were cloned into pGEM-T easy vector, subsequently sequenced and their identity was analyzed by NCBI-BLAST.

Polyclonal antibody generation and peptide affinity purification

Based on the deduced amino acid sequence of FOXL2, an antigenic peptide, H_2N-DTASSAEKEQTKSD-COOH was synthesized and conjugated to keyhole limpet hemocyanin carrier protein commercially (USV Limited, Mumbai, India). The methodology of antibody raising and purification are explained in Chapter II.

qRT-PCR analysis of *FOXL2* for tissue distribution, reproductive cycle and ontogeny

Total RNA was isolated from various tissues for tissue distribution studies, brain and gonadal tissues during different phases of reproductive cycle, ontogeny and various time points after hCG induction. The standardization and methodology followed for qRT-PCR

were mentioned in Chapter I. The primers for real time PCR were designed from the conserved region of DNA binding domain as the *FOXL2* gene (Table. 1). Primers for endogenous control *β-actin* were designed from *β-actin* cDNA obtained from catfish (Table. 1).

Table.1. List of primers used for analysis of *FOXL2.*

S.N0	Primer	Sequence 5' to 3'	Purpose
1.	DFw	TGYGAGGAYATGTTYGAGAAGGG	To amplify partial cDNA fragment
2.	DRv	CCCARTAHGARCARTGCATCAT	To amplify partial cDNA fragment
3.	5' P	GCGGGGAGAGGTAACCGTAACTGTC	RACE
4.	5' N	GCCGTCGTCGATAGTTTCCCTTCT	RACE
5.	3' P	CACCATCAGCAGCTCAGTCCGGCC	RACE
6.	3' N	GCACCGGGCTTCAGTTCGCGCGTT	RACE
7.	RTFw	CATGGCTATACGCGACAGCTC	RT-PCR & qRT-PCR
8.	RTRv	CCAGTAGTTCCCCTTCCTCTC	RT-PCR & qRT-PCR
9.	β-actin Fw	ACCGAATGCCATCACAATACCAGT	RT-PCR & qRT-PCR
10.	β-actin Rv	GAGCTGCGTGTTGCCCCTGAG	RT-PCR & qRT-PCR

In vitro and *in vivo* hCG induction

For *in vitro* studies, catfish were sacrificed by decapitation and oocytes with centrally located germinal vesicles were collected. About two hundred oocytes were then incubated (separately for each time point) in triplicates in oocyte incubation medium (Senthilkumaran and Joy, 2001) with 100 IU/ml of hCG (Pubergen, Uni-Sankyo Pvt. Ltd., India). Ovarian follicles were collected at different time points viz., 0, 2, 4, 8 and 12

h and then used for further analysis. For *in vivo* hCG induction studies, fish were injected with hCG (1000 IU/kg body weight) intraperitoneally. Brain and oocytes were collected at the time points 0, 4, 8, 12 and 24 h. Controls were treated with physiological saline. Both *in vitro* and *in vivo* studies are performed (n=5) with different batches of female fish during preparatory (March), prespawning (May) and spawning (October) phases. Dosages of hCG are chosen based on earlier report (Sreenivasulu and Senthilkumaran, 2009).

SDS-PAGE and Western blot analysis

The ovarian tissue was snap frozen in liquid nitrogen and homogenized in protein extraction buffer [10 mMTris-Cl (pH 7.4), 0.1% Triton X-100, 1 mM PMSF, 1 mM EDTA and 1 mM DTT] followed by centrifugation at 12,000rpm for 15 min to remove debris. The aliquots of the supernatant were used immediately to avoid the loss of activity. SDS-PAGE (12%) and western blot analysis was carried out respectively.

Immunocytochemistry

Immunocytochemistry was carried out in ovarian sections collected from 100 dph and adult catfish using purified FOXL2 antibody. Avidin-biotin peroxidase method was followed for cellular localization of FOXL2 protein in paraplast sections as described by Swapna *et al.* (2006) and also in Chapter I. Photomicrograph images were taken with Olympus CX41 light microscope fitted with a digital camera. For immunofluorescence, adult ovary sectioned with cryostat was used. Following incubation with primary antibody, sections were incubated with tetramethylrhodamine isothiocyanate (TRITC; Thermo Scientific) labeled secondary antibody, washed with PBS and nuclear counter

staining was done with diamidino-2-phenylindole (DAPI; Thermo Scientific). Images of phase-contrast and immunofluorescence pictures were taken using a laser-confocal microscope (Leica).

Statistical analysis

All the data were expressed as mean ± SEM. Significance among groups was tested by ANOVA followed by Student's-Newman-Keuls' test using Sigmastat (version 11) software. Differences among groups were considered significant at $P<0.05$.

Results

Cloning of *FOXL2* from ovary of catfish

A partial cDNA of 485 bp was obtained using the set of degenerate primers (F1 and R1) (Fig. 1A). The sequence identity of the fragment was confirmed by NCBI-BLAST. The full length *FOXL2* cDNA obtained by using RACE was 1058 bp long with 34 bp of 5' untranslated region (UTR) (Fig. 1B), 118 bp 3' UTR (Fig. 1C) and polyadenylation signal AATAAA which is 14 bp upstream of poly-A tail. The open reading frame (ORF) is 906 bp long (Fig. 1D), encoding 301 amino acid residues. The nucleotide and deduced amino acid sequence of catfish full-length *FOXL2* cDNA along with all the characteristics of a full length cDNA are shown in Fig. 2. ClustalW alignment revealed the presence of a signature DNA binding domain which is conserved for all winged helix transcription factors and is well preserved among teleosts (Fig. 3). Phylogenetic analysis revealed that catfish *FOXL2* showed high homology with zebra fish and other known

vertebrate counter parts (Fig. 4). The nucleotide sequence of *FOXL2* was deposited in GenBank and the accession no is HQ680981.

Western blot analysis

The purification profile of IgG heavy and light chains is shown in Fig. 5A. The elution profile of affinity chromatography of the antibody raised against the synthetic peptide is depicted in Fig. 5B. The antibody generated against this peptide detected a ~34 kDa protein from the ovarian protein homogenate, which matched with predicted size of FOXL2 protein of teleosts including catfish (Fig. 6A). Synthetic peptide pre-incubated with the purified fraction of antibody was used as a negative control to determine its specificity against FOXL2 protein (Fig. 6B).

Tissue distribution of *FOXL2*

The tissue distribution pattern of *FOXL2* was analyzed by semi-quantitative RT-PCR (Fig. 7A) and also by qRT-PCR which shows its expression predominantly in ovary, brain and pituitary (Fig. 7B). Low levels of expression were also seen in gills and kidney. The tissue distribution of FOXL2 protein correlated with that of the transcripts level when probed with the FOXL2 antibody (Fig. 7C).

Ontogenic expression of *FOXL2*

The expression of *FOXL2* monitored from 0 dph to adult stage at different time intervals both in gonads and brain by semi-quantitative RT-PCR (Fig. 8A and 9A) and also by qRT-PCR (Fig. 8B and 9B) which revealed differential expression pattern in males and females. The expression in gonads gradually increased from 0 to 40 dph and reached

maximum between 40 to 50 dph in females. Thereafter, expression of *FOXL2* decreased from 50 to 200 dph and elevated again at 300 dph and adult ovary which was significant. In male gonads, its expression decreased drastically from 50 dph to adult stage. In brain, *FOXL2* expressed in a similar pattern to that of gonads till 50 dph female brain and an increasing trend was observed from 100 to 300 dph which peaked in adult. Interestingly, a very low or undetectable expression of *FOXL2* was observed in 50 and 100 dph male brain but later it expressed in lower levels in adult male brain.

Expression of FOXL2 during the reproductive cycle

FOXL2 expression was analyzed using semi-quantitative RT-PCR in different phases of reproductive cycle both in brain and ovary (Fig. 10A and B). The *FOXL2* showed highest expression during prespawning phase both in brain and ovary when compared to preparatory and spawning phases. The expression was lowest in regressed phase. In order to confirm these results, qRT-PCR was performed (Fig. 10C). A similar pattern of *FOXL2* expression was observed in ovary, at protein level too (Fig. 10D). Further, FOXL2 protein was not detected in regressed phase.

Expression during hCG-induced oocyte maturation, *in vitro* and *in vivo*

Oocytes collected at 0, 2, 4, 8 and 12 h after the hCG induction both i*n vitro* and *in vivo* showed a gradual increase from 0 to 8 h that was persistent till 12 h during preparatory phase (Fig. 11A and 12A) whereas in prespawning phase the expression was found to be highest at 2 h and decreased at later time points when compared to saline treated controls (Fig. 11B and 12B). The expression of *FOXL2* displayed no significant difference when induced in spawning phase (Fig. 11C and 12C). A similar pattern to that of ovary was

observed in the brain collected at 0, 4, 8, 12 h after *in vivo* hCG treatment in prespawning phase where there was a surge in the expression of *FOXL2* by 4 h and it was maintained at later time points too (Fig.13A). These results were further confirmed by qRT-PCR (Fig. 13B) as well.

Localization of FOXL2 in ovary of catfish

Immunolocalization analysis in ovary of 100 dph catfish confirmed its intense immunoreactivity (IR) in the follicular layer of the pre-vitellogenic oocytes which also extended to the cytoplasm (Fig.14A and B). No immunoreactivity was observed with peptide preabsorbed with FOXL2 antibody which is used as the negative control (Fig. 14C). Immunofluorescence analysis in adult catfish ovarian sections revealed FOXL2 IR mainly in the follicular layer, extended towards cytoplasm as similar to DAB stained sections (Fig. 15A). Figure 16B shows the nuclear counter staining of oocytes with DAPI which shows fluorescence in the follicular layer of the oocyte. Merge of phase contrast and confocal images clearly revealed the localization of FOXL2 in catfish ovary.

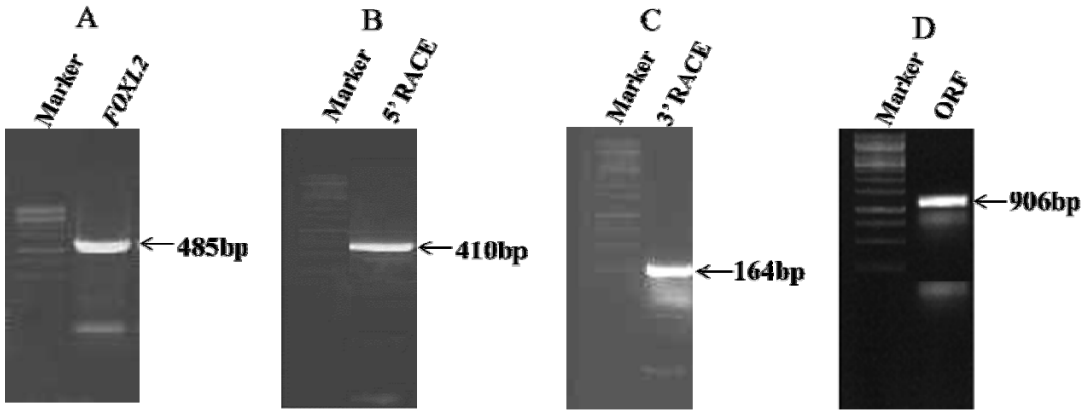

Fig.1. Amplification products of *FOXL2* (A) partial *FOXL2* cDNA fragment from catfish ovary B), C) 5' and 3' RACE products using gene specific primers to obtain full-length cDNA of *FOXL2* and D) ORF of *FOXL2*.

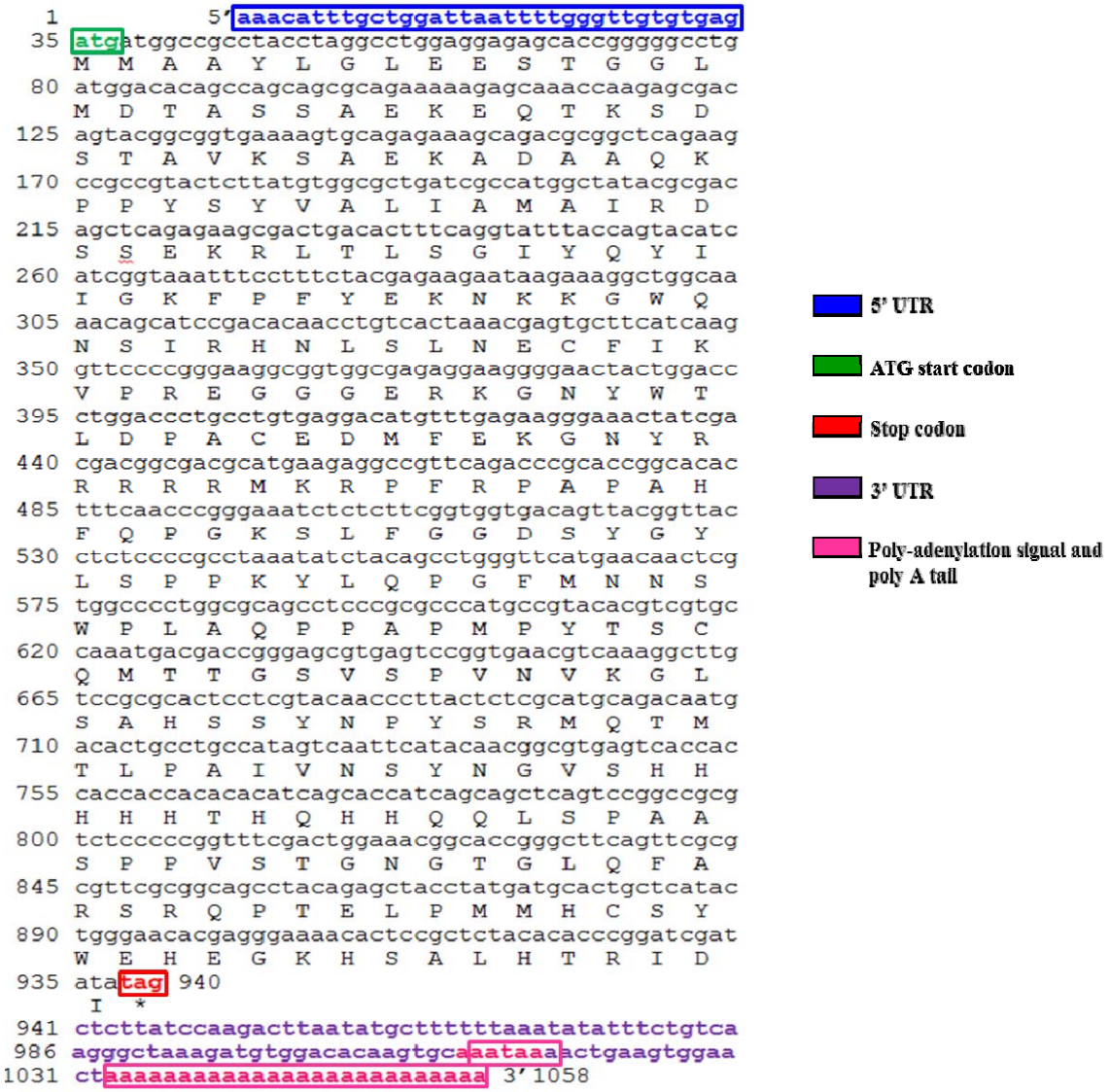

Fig.2. Nucleotide and deduced amino acid sequence of catfish full-length *FOXL2* cDNA.

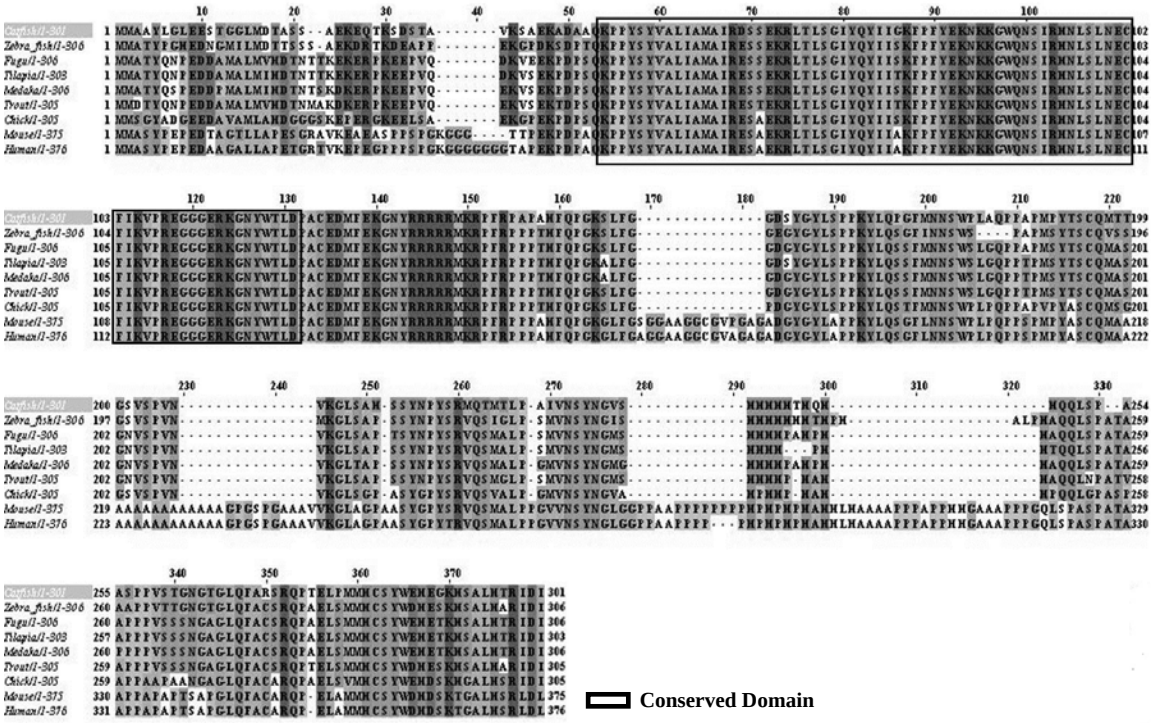

Fig.3. ClustalW multiple alignment of catfish FOXL2 with other vertebrate counterparts. GenBank accession Numbers of *FOXL2* sequences are :- Fugu; CAAB01001061, Trout; CA354643, Tilapia; AY554172, Medaka; AB252055, Catfish; HQ680981, Zebrafish; AL929010, Chick; NP001012630, Mouse; AF522275 and Human; AF301906.

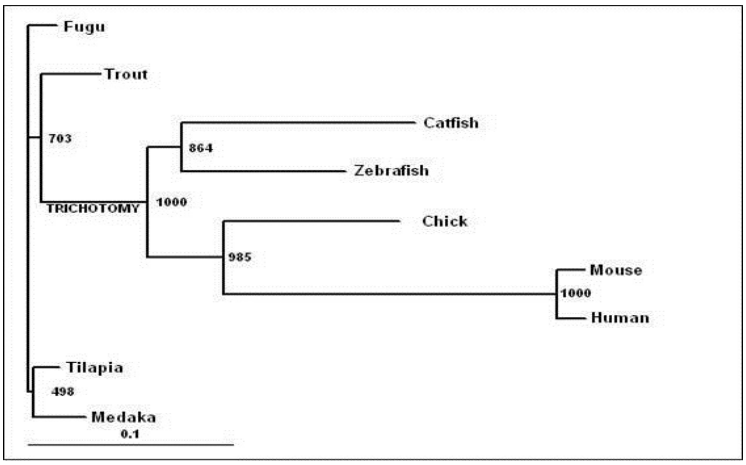

Fig.4. Phylogenetic tree of catfish *FOXL2*. Phylogenetic analysis was done with ClustalX using the Neighbor-Joining (NJ) method and a bootstrap analysis with 1000 replicates

was used to assess the strength of nodes in the tree (http://www.ddbj.nig.ac.jp). Phylogenetic tree was generated using the TreeView software package version 1.4. GenBank accession numbers of the sequences used in phylogenetic tree are as follows :- Fugu; CAAB01001061: Trout; CA354643: Tilapia; AY554172: Medaka; AB252055: Catfish; HQ680981: Zebrafish; AL929010: Chick; NP001012630: Mouse; AF522275 and Human; AF301906.

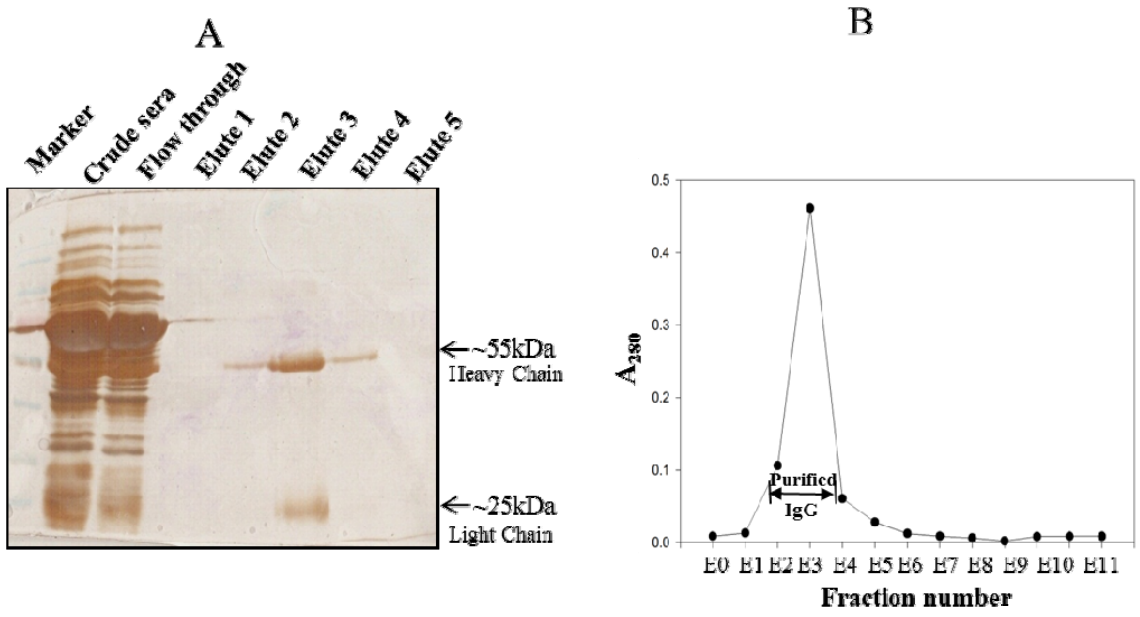

Fig.5. Peptide affinity purification profile of FOXL2 antibody. A) Silver stained gel showing IgG fraction of FOXL2 antibody. Bands detected corresponding to ~55kDa (heavy chain) and ~25kDa (light chain). B) Affinity chromatography elution profile of FOXL2 antibody

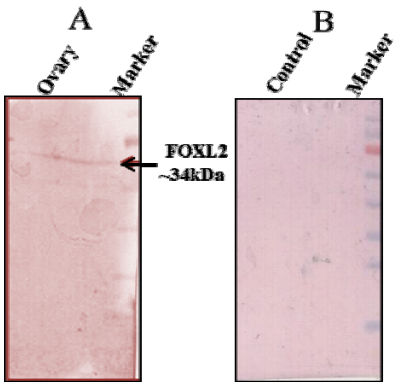

Fig.6. Western blot analysis of ovary homogenate with FOXL2 antibody A) Western blot showing band (~45kDA) corresponding to FOXL2 protein. B) No signal in control (peptide pre absorbed with FOXL2 antibody).

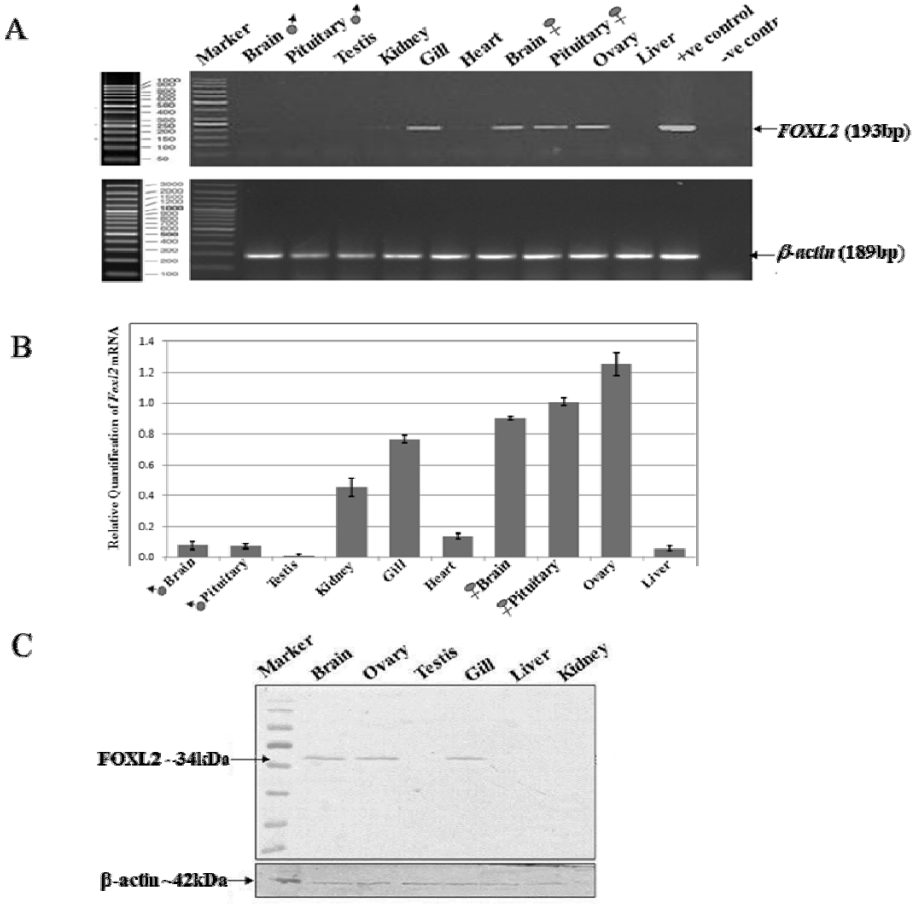

Fig.7. Tissue distribution pattern of *FOXL2* in different female tissues A) Semi-quantitative RT-PCR analysis of tissue distribution pattern of *FOXL2*. B) qRT-PCR

analysis of *FOXL2* expression in different tissues of adult female catfish. C) Western blot analysis (upper panel) of FOXL2 protein (~34kDa) expression in various tissues. Western blot using β-actin (~42kDa) antibody (lower panel) in different tissue samples used as equal loading control.

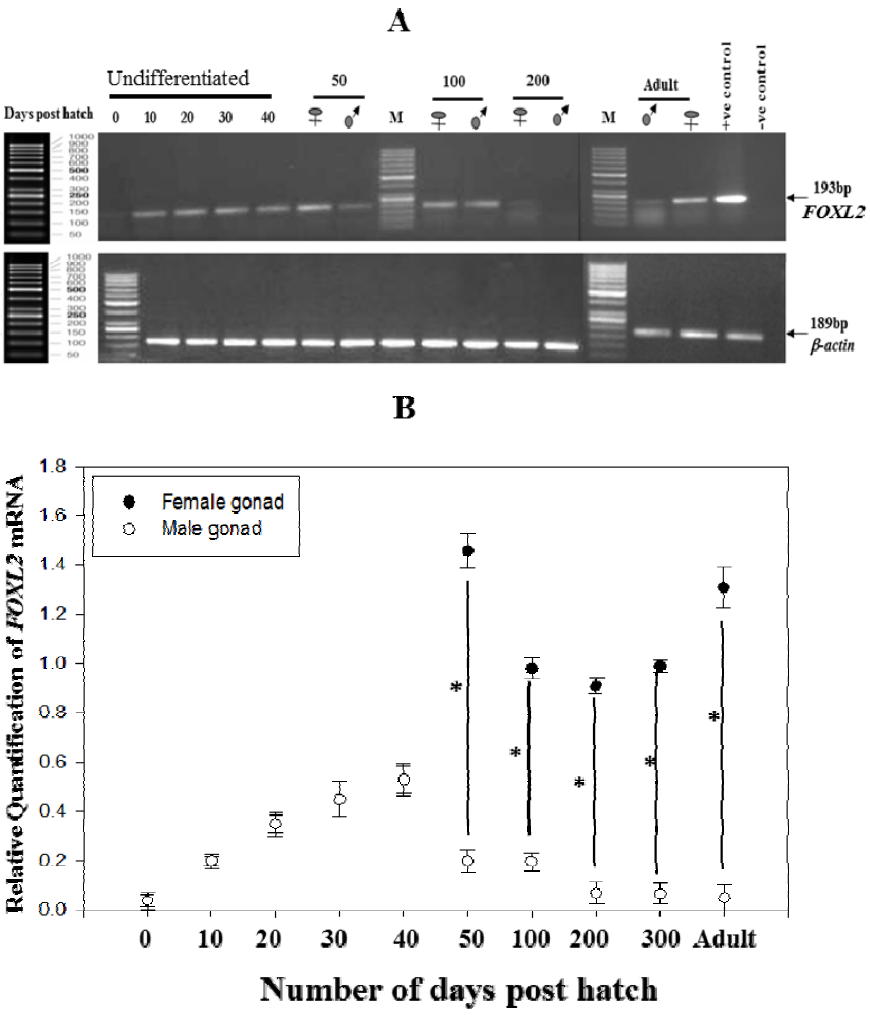

Fig.8. Ontogeny expression of *FOXL2* in gonads of different age group catfish. A) Semi-quantitative RT-PCR analysis *FOXL2* ontogeny expression. B) Changes in *FOXL2* mRNA levels during ontogeny in different stages of embryonic and gonadal development using qRT-PCR. * indicates the significance at $P < 0.05$.

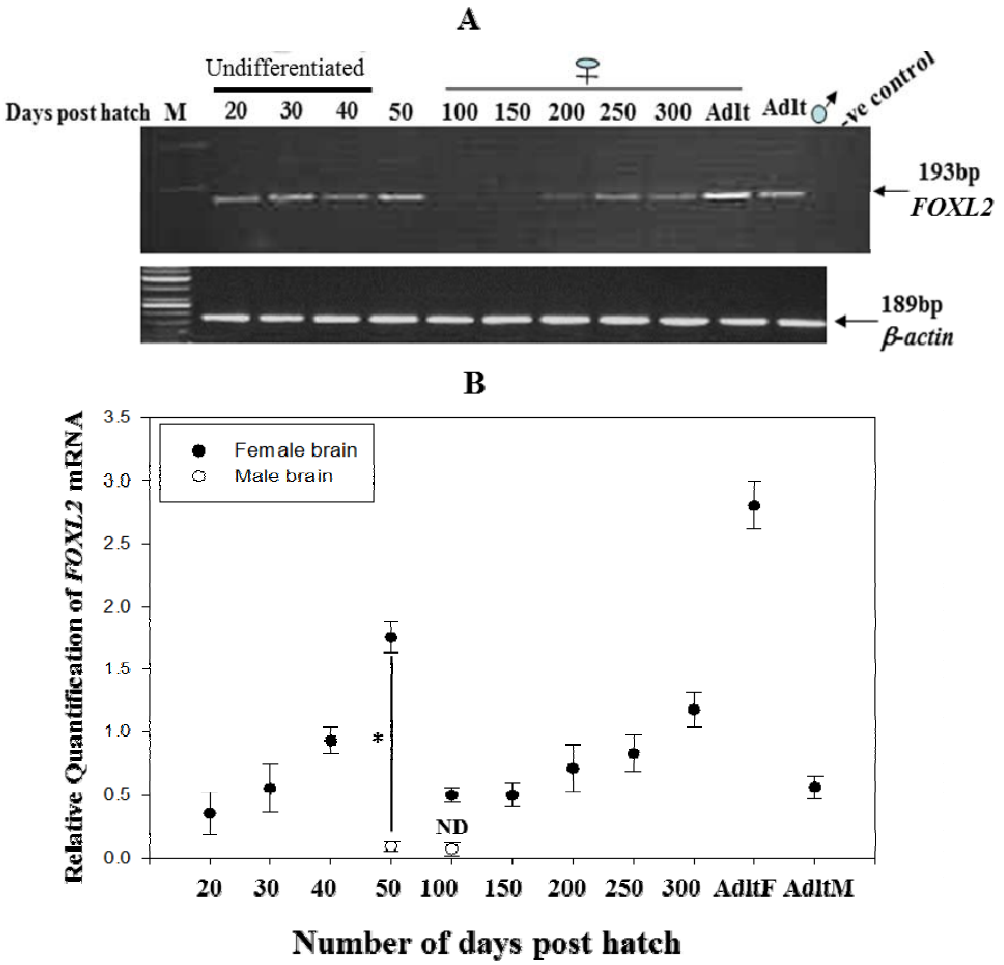

Fig.9. Ontogeny expression of *FOXL2* in brain of different age group male and female catfish. A) Semi-quantitative RT-PCR analysis *FOXL2* ontogeny expression. B) Changes in *FOXL2* mRNA levels during ontogeny in brain of male and female catfish using qRT-PCR. * indicates the significance at $P < 0.05$.

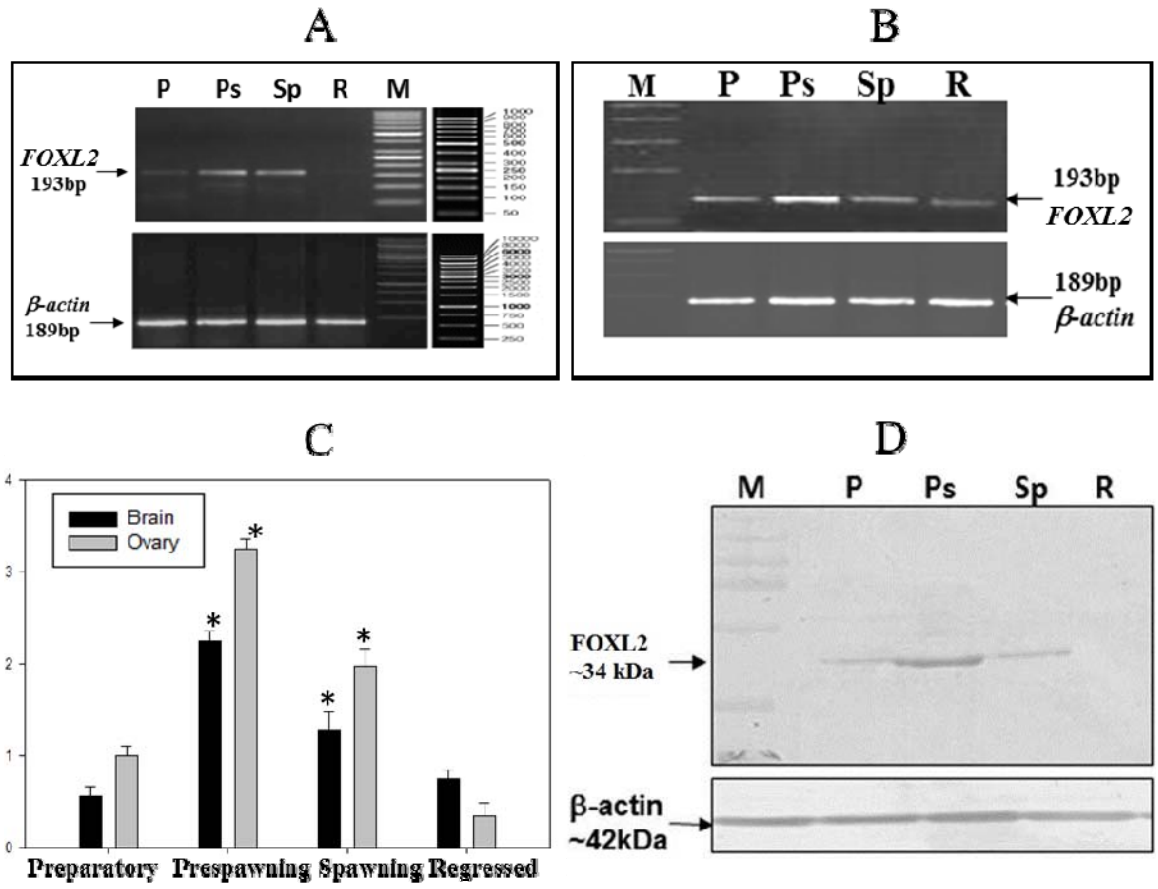

Fig.10. Expression of *FOXL2* during reproductive cycle of female catfish A), B), Semi-quantitative RT-PCR analysis of expression of *FOXL2* in brain and ovary of female catfish, respectively. P: Preparatory, Ps: Pre-spawning, Sp: Spawning, R: Regressed. C) Changes in *FOXL2* mRNA levels during different phases of ovarian cycle in catfish using qRT-PCR. * indicates the significance at $P<0.05$. D) Western blot analysis (upper panel) of FOXL2 in ovary protein (~34kDa) homogenate during different phases of ovarian cycle. Western blot using β-actin (~42kDa) antibody (lower panel) in ovary samples used as equal loading control.

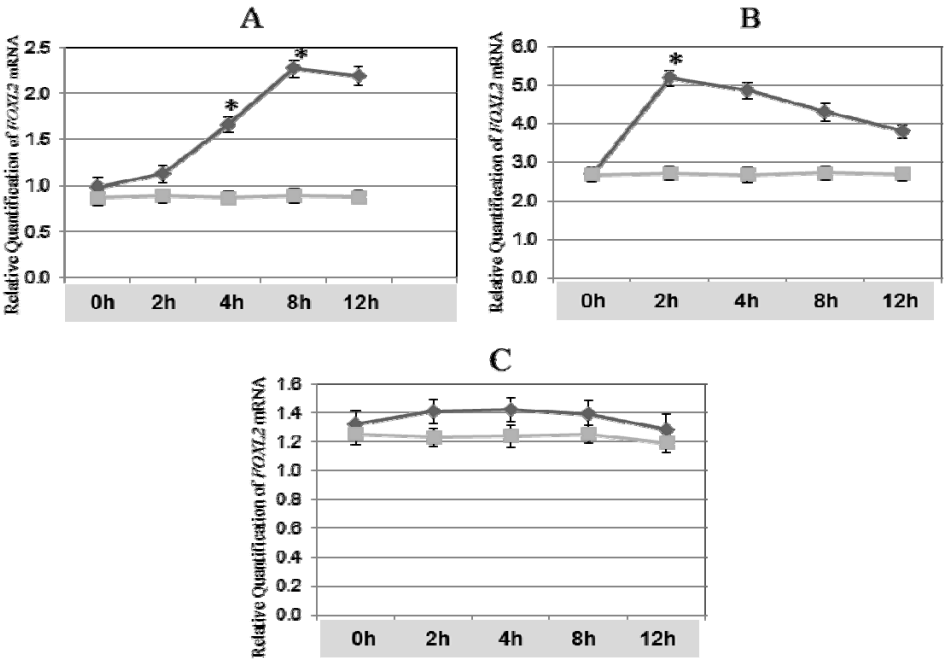

Fig.11. Changes in *FOXL2* mRNA levels in the oocytes at different time points after *in vitro* hCG treatment during different phases of catfish ovarian cycle**.** A) Preparatory phase, B) Pre-spawning phase and C) Spawning phase compared to respective saline treated controls. * indicates the significance at $P < 0.05$.

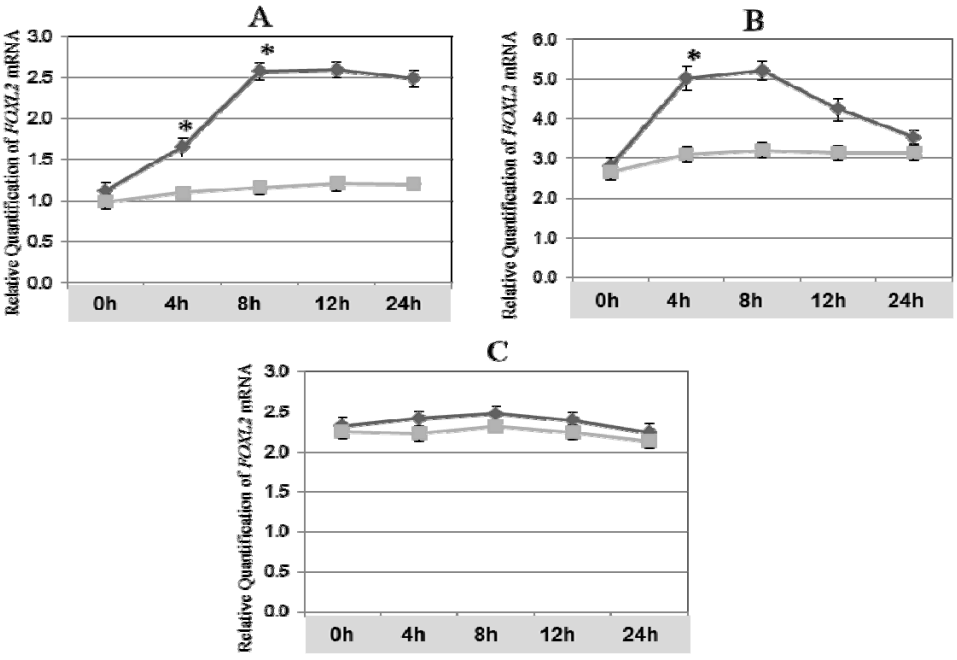

Fig.12. Changes in *FOXL2* mRNA levels in ovary at different time points after *in vivo*

hCG treatment during different phases of catfish ovarian cycle. A) Preparatory phase, B) Pre-spawning phase and C) Spawning phase compared to respective saline treated controls. * indicates the significance at $P < 0.05$.

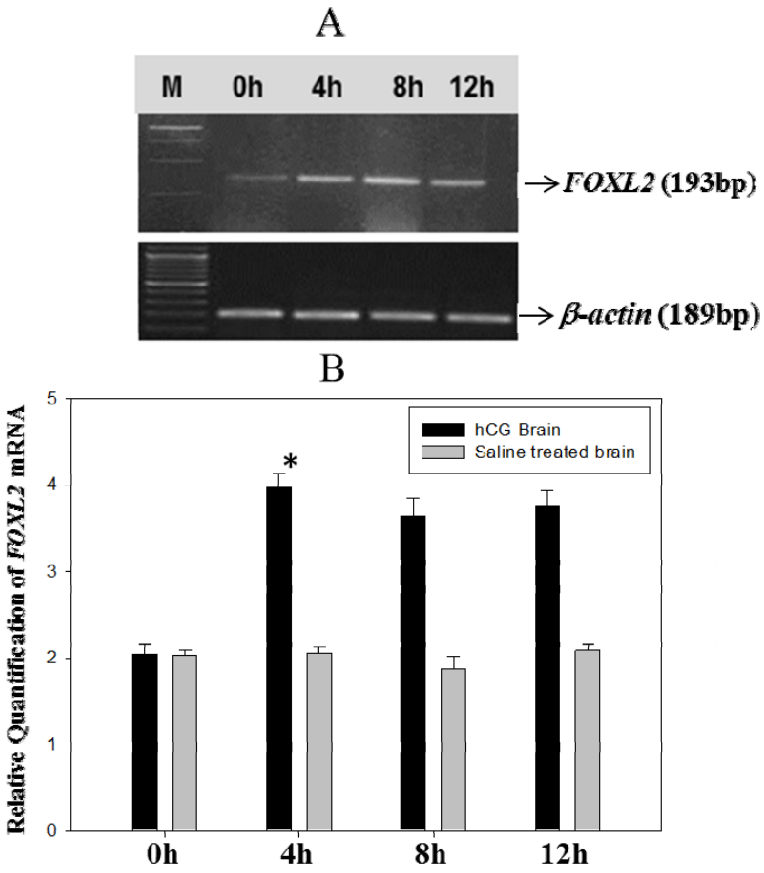

Fig.13. Expression of *FOXL2* in brain at different time points after *in vivo* hCG treatment during prespawning phase of ovarian cycle in female catfish. A) Semi-quantitative RT-PCR analysis of expression of *FOXL2*. Saline treated control was not shown as there was change in expression from 0h. B) Changes in *FOXL2* mRNA expression pattern in brain by qRT-PCR after *in vivo* hCG treatment during pre-spawning phase of ovarian cycle compared to saline treated controls. * indicates the significance at $P < 0.05$.

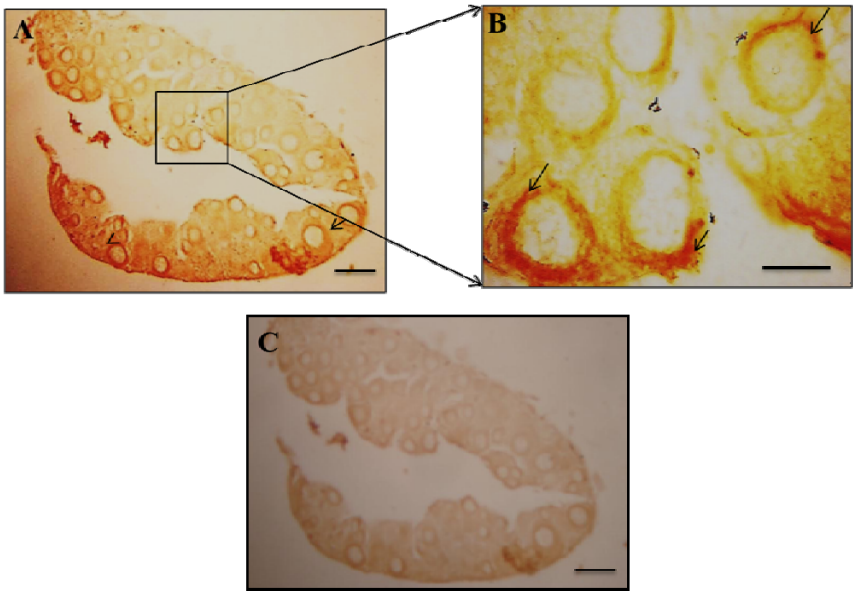

Fig.14. Immunocytochemical localization of FOXL2 in 100 dph catfish ovary. Arrow marks indicate immuno reactivity. No immuno reactivity was observed with peptide pre absorbed with FOXL2 antibody. Scale bar indicates 50µM.

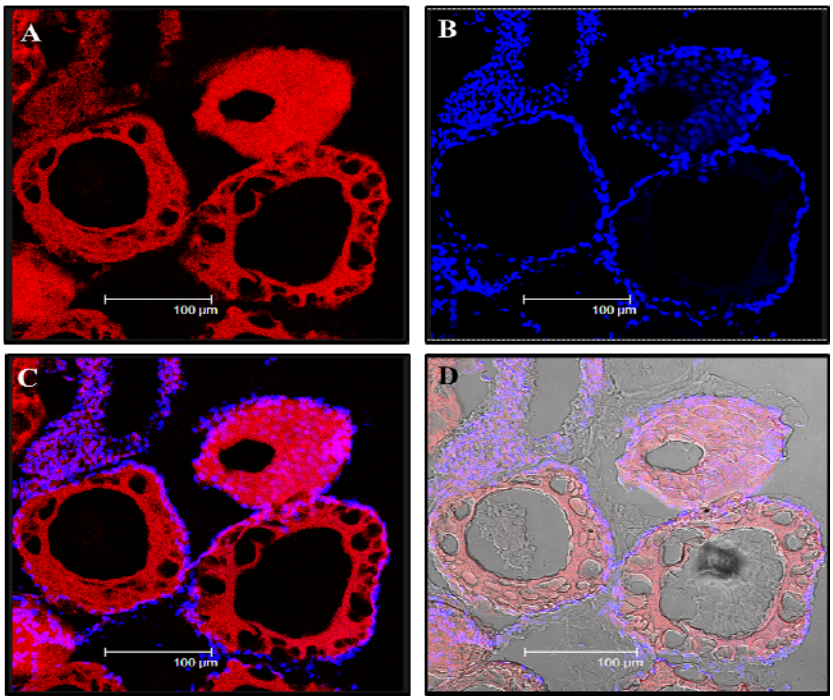

Fig. 15. Immunofluoroscence localization of FOXL2 in adult catfish ovary. A) Regions of follicular layer and granulosa cell layer showing the immunoreactivity (IR) with anti-

catfish FOXL2 antibody. B) The nuclear counter staining of oocytes with DAPI. C) Merge of the confocal images corresponding to A and B. D) Merge of phase contrast images with confocal images corresponding to A, B and C.

Discussion

The main focus of the current study is to elucidate the role of *FOXL2* in ovarian development and recrudescence using expression analysis. To begin with, we cloned the full length cDNA from the ovary using RACE strategy after obtaining a partial cDNA fragment using degenerate primers. Only one form of *FOXL2* had been identified in most of the mammalian species with a conserved DNA binding domain (Cocquet *et al.*, 2003). However, fish-specific whole-genome duplication has been hypothesized by Wittbrodt *et al.* (1998) which warrants for duplication of several genes. In spite of this, one form of *FOXL2* mRNA was identified in catfish like many other teleosts ranging from medaka, southern catfish and tilapia (Nakamoto *et al.*, 2006; Liu *et al.*, 2007; Wang *et al.*, 2007). Conversely, Baron *et al.* (2004) reported paralogs of *FOXL2* (*FOXL2a* and *FOXL2b*) in the rainbow trout. In the present study, we did not get any multiple forms of FOXL2 at any point of time when performed RACE using various stages of ovary. The phylogenetic analysis of *FOXL2* by neighbor-joining method revealed high homology of catfish FOXL2 with its counterpart in zebrafish. Multiple clades were seen among different teleosts indicating the divergence in FOXL2 domains except the conserved. Tissue distribution pattern revealed expression of *FOXL2* in ovary, brain, pituitary and gills. In mammals and birds, *FOXL2* is reported to be highly expressed in the ovary and is present even before morphological differentiation (Cocquet *et al.*, 2002; Govoroun *et al.*, 2004; Loffler *et al.*, 2003; Pannetier *et al.*, 2003). It is expressed in an ovarian-specific manner

in mouse embryos, chicken and turtle (Loffler *et al.*, 2003). Same is true for catfish where we could hardly find any expression of *FOXL2* in testis. These reports together with present study suggest its conserved role in vertebrate ovarian development. Our data at mRNA and protein levels, where in *FOXL2* is highly expressed in the ovarian tissue corroborates with the earlier reports in trout (Baron *et al.*, 2004), tilapia (Wang *et al.*, 2004) and southern catfish (Liu *et al.*, 2007). Nevertheless, in these species, the expression was negligible in testis (Baron *et al.*, 2004, Liu *et al.*, 2007, Wang *et al.*, 2004). In catfish, the expression of *FOXL2* in testis was barely detectable only in qRT-PCR. These results together indicate that there is no involvement of *FOXL2* during testicular development. The expression found in brain and pituitary denotes its role across brain–pituitary-gonadal axis during sex steroid synthesis as explained in mammals (Ellsworth *et al.*, 2006) and in teleosts (Wang *et al.*, 2007).

To further clarify the role of *FOXL2* in ovarian development, we analyzed the ontogeny of *FOXL2* which showed a clear pattern of sexual dimorphism during gonadal differentiation and development. A gradual increase from 0 to 40 dph in undifferentiated gonads and then a peak at 50 dph in female depicts an important role for this correlate in ovarian differentiation as the critical window of sex differentiation in catfish is between 35-50 dph (Raghuveer and Senthilkumaran, 2009). A similar pattern of *FOXL2* expression was seen in brain from 0 to 40 dph and an increase in 50 dph female brains but was almost not detectable in 50 and 100 dph male brain, however a low level expression was seen in adult male brain. Hence, we did additional studies in female brain which showed an increasing trend from 100 to 300 dph which peaked in adult female brain. Earlier reports in fish, showed a sexually dimorphic pattern in Nile tilapia (Wang *et*

al., 2004, 2007), rainbow trout (Baron *et al.*, 2004), and medaka (Nakamoto *et al.*, 2006). Further, Ijiri *et al.* (2008) suggested a close relationship between the expressions of *FOXL2*, and that of *CYP19A1* in XX gonads during early differentiation of tilapia. Maintenance of high expression of *FOXL2* till adulthood in female catfish also signifies its requirement in ovarian recrudescence like that of *CYP19A1*. Further, the immunolocalization studies in previtellogenic ovarian follicles (100 dph) and in adult ovary showed its localization in the follicular layer with an extended immunoreactivity towards cytoplasm. This might be due to high expression of *FOXL2*. Furthermore, *FOXL2* is known to be involved in the differentiation of the female supporting cell lineages, granulosa cells, and the formation/or maintenance of ovarian follicles in various vertebrates (Loffler *et al.*, 2003; Govoroun *et al.*, 2004; Wang *et al.*, 2004; Nakamoto *et al.*, 2006). FOXL2 localization pattern in tilapia and *Oryzias luzonensis* also confirms its localization in follicular layer, more specifically in granulosa cells (Wang *et al.*, 2004, Nakamoto *et al.*, 2009). Based on these, it is possible to depict that *FOXL2* might be an important transcription factor to drive ovarian growth. Further, *FOXL2* expression pattern during different phases of ovarian cycle was consistent with that of ovarian aromatase observed in our earlier study (Rasheeda *et al.*, 2010) implicating a role for FOXL2 in the regulation of aromatase vis-à-vis ovarian growth. In accordance to this, the expression of *FOXL2* was evident only in the previtellogenic and vitellogenic follicles and absent completely in the post-vitellogenic follicles of tilapia (Wang *et al.*, 2004). Interestingly, the seasonal changes of FOXL2 at the level of brain also showed a similar pattern which might indicate its importance not only in ovary but also in brain. Our study gains importance as the present report describe the expression of *FOXL2* in ovary and brain in

an annual breeder that undergoes a seasonal cycle. The variations in the expression of *FOXL2* at the level of transcript and protein during the ovarian cycle encouraged us to verify the effect of gonadotropins on *FOXL2* expression, *in vitro* and *in vivo* in different reproductive phases of adult female catfish. Earlier study from catfish have demonstrated the presence of LH receptor in ovary and brain and *in vitro* studies showed LH receptor transactivation by using various gonadotropins including hCG that prompted us to use the non-homologous gonadotropin, hCG instead of catfish LH (Vischer and Bogerd, 2003). Our study revealed enhanced expression of *FOXL2* upon hCG induction at preparatory and prespawning phases with no noticeable change during the spawning phase both *in vitro* and *in vivo* which are in consistent with aromatase transcript levels and activity in catfish (Rasheeda *et al.*, 2010). The ceased expression of *FOXL2* after hCG induction in spawning phase can be explained by the decreased necessity of aromatase during final oocyte maturation (Senthilkumaran *et al.*, 2004; Sreenivasulu and Senthilkumaran, 2009). Increase in the aromatase activity after *in vitro* treatment of gonadotropins in vitellogenic ovarian follicles has been demonstrated in several fish species (Nagahama *et al.*, 1991; Kagawa *et al.*, 2003; Senthilkumaran *et al.*, 2004). Earlier, Wang *et al.* (2007) demonstrated that FOXL2 up-regulates aromatase gene transcription in a female–specific manner by binding to the promoter as well as interacting with Ad4BP/SF-1 in tilapia. Considering this, our results on *FOXL2* expression after hCG induction and the finding of synergistic expression of *Ad4BP/SF-1* and *CYP19A1* in ovary of tilapia during vitellogenesis by Yoshiura *et al.* (2003) together confirm the interaction of *FOXL2, Ad4BP/SF-1* and *CYP19A1,* and their dependence on gonadotropins during ovarian recrudescence of teleosts. Having found differential expression of *FOXL2* after hCG

induction in ovary, we tested the changes in its expression at the level of brain only in the prespawning phase to confirm its role in brain. In our earlier report (Rasheeda *et al.*, 2010), hCG induction up regulated not only *CYP19A1* but also *CYP19A2* in a season-dependent manner. Corroborative changes in *FOXL2* expression in brain may propose a plausible role for the former to regulate *CYP19A2* in addition to *CYP19A1* directly or indirectly by binding to the DNA binding domain of a nuclear receptor family member. Further studies on this line may provide more interesting results to understand the regulation of both forms of aromatases in the context of *FOXL2*.

Conclusion

To summarize, we cloned full length *FOXL2* cDNA from the ovary of catfish and it showed high homology with zebrafish and moderately with other vertebrate counterparts. In view of all the data reported here, including the previous report on aromatase in the catfish, we find an overwhelming requirement of *FOXL2* during ovarian development and recrudescence. Results of *FOXL2* expression after *in vitro* and *in vivo* hCG induction further supports its role in the regulation of aromatases in ovary and brain, perhaps through gonadotropins. Sexual dimorphic expression and its early appearance designate *FOXL2* as an early marker for ovarian differentiation in catfish.

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Chapter IV

FTZ-F1 and FOXL2 synergistically up-regulate catfish brain aromatase gene transcription by specific binding to the promoter motifs

Abstract

Cytochrome P450 aromatase (CYP19) catalyzes the conversion of androgens into estrogens. Teleosts have two *CYP19* genes specific to ovary (*CYP19A1*) and brain (*CYP19A2*). Previous studies reported the regulation of *CYP19A1* expression by the members of NR5A nuclear receptor subfamily as well as a forkhead transcription factor, FOXL2. In the present study, we investigated the involvement of FTZ-F1, an NR5A subfamily member, and FOXL2 in the regulation of *CYP19A2* expression in the brain of catfish, *Clarias gariepinus*. Synchronous expression pattern of C*YP19A2*, *FTZ-F1* and *FOXL2* in the brain encouraged us to isolate the upstream region of *CYP19A2* in order to analyze the regulatory motifs. Promoter motif analysis revealed NR5A1/FTZ-F1 and FOXL2 binding nucleotide sequences in the 5'-flanking region of the *CYP19A2*. Transient transfection studies showed that FOXL2 and FTZ-F1 together, enhanced the transcriptional activity of *CYP19A2* gene in mammalian cell lines, and that a mutation in either of their putative binding sites within the *CYP19A2* promoter abolished this effect. Electrophoretic gel-mobility shift and chromatin immunoprecipitation assays further confirmed the binding of both the transcription factors to their respective cis-acting elements in the upstream region. This study is the first to report the transcriptional regulation of *CYP19A2* by FTZ-F1 and FOXL2, in a synergistic manner, in a teleost fish.

Introduction

Cytochrome P450 aromatase (CYP19) is the terminal enzyme in the steroidogenic pathway that catalyzes the conversion of C_{19} androgens to aromatic C_{18} estrogens in a complex with nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase in the endoplasmic reticulum (Simpson *et al.*, 1994, 2002). This enzyme is expressed in the gonad and brain in fish (Callard and Tchoudakova, 1997; Tchoudakova and Callard, 1998), but has

a broader distribution in mammals (Conley and Hinshelwood, 2001; Simpson *et al.*, 2002). In most vertebrates, including human and mouse, a single copy of *CYP19* is found whose tissue-specific expression is governed by use of a variety of tissue-specific promoters in combination with alternative splicing of 5'-untranslated exons (Simpson *et al.*, 1994; Conley and Hinshelwood, 2001). In contrast, with the possible exception of the Japanese eel (Ijiri *et al.*, 2003), most teleosts studied so far have been shown to possess two distinct forms of aromatase, ovarian form, *CYP19A1* and brain form, *CYP19A2* (Chang *et al.*, 2001; Kwon *et al.*, 2001; Chang *et al.*, 2005) due to a gene duplication event (Steinke *et al.*, 2006). *CYP19A1* is predominantly expressed in ovary, whereas the expression of *CYP19A2* is mostly in brain (Chang *et al.*, 2001; Tchoudakova *et al.*, 2001; Kwon *et al.*, 2001; Chang *et al.*, 2005; Rasheeda *et al.*, 2010). *CYP19A2* is implicated for neuroplasticity and neurogenesis and much recently examined for its role in sex differentiation and reproduction (Forlano *et al.*, 2001; Diotel *et al.*, 2010), while CYP19A1 is involved in ovarian differentiation and gametogenesis (Simpson *et al.*, 1994, 2002; Conley and Hinshelwood, 2001, Sudhakumari *et al.*, 2005). The presence of two distinct mRNAs of *CYP19* derived from separate gene loci that are principally expressed in brain and ovary direct to the study of their regulation at promoter level.

Expression of *CYP19A1* gene in teleost fish is modulated by the members of NR5A subfamily and forkhead box protein L2 (FOXL2) either independently or cumulatively. The NR5A subfamily belongs to nuclear receptor superfamily that share common DNA binding and transactivation properties (Nuclear receptor committee, 1999; Kuo *et al.*, 2005). A unified nomenclature system for nuclear receptors subdivided NR5A into NR5A1 and NR5A2 that includes fushitarazu factor 1 (FTZ-F1) and its close homologues Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) and liver receptor homolog 1 (LRH-1; Nuclear receptor committee, 1999). These are known to regulate gonadal *CYP19* expression

(Morohashi *et al.*, 1992; Lala *et al.*, 1992; Fayard *et al.*, 2004). Involvement of FTZ-F1 in the transcriptional regulation of *CYP19A1*has been reported in medaka (Watanabe *et al.*, 1999) and trout (Kanda *et al.*, 2006). Investigation in the Nile tilapia demonstrated transcriptional interaction of Ad4BP/SF1 with *CYP19A1* during vitellogenesis (Yoshiura *et al.*, 2003). SF-1 binding sites were identified in the upstream region of *CYP19A1* gene in zebrafish (Kazeto *et al.*, 2001) as well as goldfish (Tchoudakova *et al.*, 2001) suggesting its role as a likely candidate engaged in the regulation. In mammals too, NR5A receptors were shown to govern the *CYP19* transcription. Pezzi *et al.* (2004) demonstrated the altered *CYP19a* gene expression by LRH-1, the liver homologue 1, in rat testis. Carlone and Richards (1997) reported the SF-1 mediated constitutive expression of *CYP19A1* in gonadal cells. Meanwhile, there have been several studies implicating the fork head transcription factor (FOXL2), in *CYP19A1* regulation. FOXL2 is initially known as the earliest sex dimorphic marker in ovarian development and granulosa cell differentiation, and thus helps in the proper maintenance of ovarian function (Baron *et al.*, 2004). A pioneer study from Wang *et al.* (2007), for the first time provided the information that FOXL2 interacts with Ad4BP/SF1 to up regulate the *CYP19A1* expression in the Nile tilapia. Another evidence of FOXL2's interaction with SF-1 was provided in granulosa cells where synergistic action of both the transcription factors repressed *CYP17* transcription (Park *et al.*, 2010). Direct involvement of FOXL2 in the regulation of *CYP19A1* has also been elucidated in sheep ovary (Pannetier *et al.*, 2006). A specific somatic mutation in FOXL2 (C134W) in ovarian adult type granulosa cell tumors altered the regulation of *CYP19A1* (Fleming *et al.*, 2010).

Contrary to the well-documented regulation of *CYP19* gene in the gonadal tissues, only a few studies have addressed the regulation of *CYP19A2* at the promoter level. The analysis of brain form of *CYP19* genes from several teleost species underlined the presence of a well-conserved estrogen-responsive element (ERE) in the proximal promoter sequences

suggesting a direct effect of estrogen receptor (ER) on the transcriptional regulation of *CYP19A2* (Menuet *et al.*, 2002, 2003; Diotel *et al.*, 2010). However, the molecular mechanism of this potential regulation remain enigmatic given that ER expression in radial glial cells has never been reported in any fish species where *CYP19A2* was found to be highly expressed (Menuet *et al.*, 2002, 2003; Anglade *et al.*, 1994). Fewer studies on the regulation of *CYP19A2* as well as conflicting reports on the role of ER as a transcriptional regulator necessitate further studies to understand its regulatory mechanism.

In this context, present study was aimed to investigate whether transcription factors FTZ-F1 and FOXL2 bind to their respective cis-acting elements identified in the cloned promoter region of *CYP19A2* and regulate its expression in the catfish brain.

Materials and Methods

Experimental model

Air-breathing catfish, *Clarias gariepinus* were obtained from our laboratory aquaculture facility. The animals were sacrificed and brain tissue was dissected as explained in Chapter I. Identification of sex of the dissected fish at 50 days post hatch (dph) is fairly evident due to precise morphological distinction of gonadal tissues (Raghuveer *et al.*, 2011). To study the expression pattern of *CYP19A2*, *FOXL2* and *FTZ-F1*, brain tissue from female catfish were collected at 0 (<23 h post-hatch), 60, 120, 180, 240 and 300 dph.

qRT-PCR of *CYP19A2*, *FOXL2* and *FTZ-F1* in the brain of developing catfish

In order to study the expression pattern of *FOXL2*, *FTZ-F1* and *CYP19A2* in the developing catfish, total RNA (5 µg) was isolated from brain tissue separately from five fish (n=5). All the RNA samples were treated with DNAse I prior to first strand cDNA synthesis. For *FOXL2*, the primers were designed in the conserved DNA-binding forkhead domain. For

FTZ-F1, the primers were designed in the conserved ligand-binding domain. For *CYP19A2*, forward primer was located in intron-exon boundary of exon one and two and the reverse primer located in intron-exon boundary of exon two and three. Primers for *β-actin* used as endogenous control were designed from *β-actin* cDNA obtained from catfish (primers 1-8, Table.1). qRT-PCR was performed as explained in Chapter I. The real-time PCR results were presented as change in expression relative to control using target gene C_T values normalized to that of *β-actin* gene C_T values based on the comparative C_T method (Schmittgen and Livak, 2008).

Table 1. List of primers used for expression analysis of *CYP19A2*, *FTZ-F1* and *FOXL2*

S.N0	Primer	Sequence 5' to 3'	Purpose
1.	RTCYP19A2 Fw	CCAGGTCCATACTGGTTACTG	qRT-PCR
2.	RTCYP19A2 Rv	CACAGCAGATGACTTGCTTAG	qRT-PCR
3.	RTFTZ-F1 Fw	GGAGGAGCTCTGTCCTGTGTGCG	qRT-PCR
4.	RTFTZ-F1 Rv	GCTGCGTCTTATCGATGCCGCAG	qRT-PCR
5.	RTFOXL2 Fw	CATGGCTATACGCGACAGCTC	qRT-PCR
6.	RTFOXL2 Rv	CCAGTAGTTCCCCTTCCTCTC	qRT-PCR
7.	RTβ-actin Fw	ACCGAATGCCATCACAATACCAGT	qRT-PCR
8.	RTβ-actin Rv	GAGCTGCGTGTTGCCCCTGAG	qRT-PCR

Isolation of *CYP19A2* 5' flanking region by genome walking strategy

Genomic DNA was isolated from the brain tissue (Qiagen, GmbH, Germany).Genome walking library was constructed using Universal Genome Walker kit following manufacturer's protocol (Clontech, Moutainview, CA, USA). Briefly, aliquots of genomic DNA (25 µg) were digested overnight with each of the following restriction endonucleases,

EcoRV, *PvuII*, *DraI*, and *StuI*. Digested DNA aliquots were ligated separately to the Genome Walker adaptor and were subjected to a primary PCR amplification with the adaptor primer (AP1) and gene specific primer (GSP1). Touchdown PCR was used for the amplification. Cycling conditions were 94°C 30 sec, 72°C 3 min, 5 cycles, 94°C 30 sec, 68°C 30 sec, 72°C 3 min for 30 cycles. Secondary PCR amplification was carried out with the nested adaptor (AP2) and gene-specific primers (GSP2; primers 1 and 2, Table. 2). All the amplicons were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and the sequence was determined bi-directionally.

In silico analysis

Core promoter prediction was carried out using the software neural network promoter prediction program. Putative transcription factor binding sites were predicted using the Transcription Element Search System database (TESS), MatInspector and TRANSFAC Version 2.2 and by inspection for DNA sequences reported in other *CYP19A2* genes.

Plasmid constructs: *CYP19A2* reporter, *FOXL2* and *FTZ-F1* expression constructs

CYP19A2 5' flanking region progressive deletion constructs were amplified by PCR using restriction site attached gene specific primers (primers 3-10, Table. 2). Resultant constructs were cloned in *XhoI* and *KpnI* sites of pGL2-basic firefly luciferase vector (Promega). The identity of each construct was verified by double digestion and the absence of cloning artifacts was determined by nucleotide sequencing. Luciferase plasmids bearing a mutation in the FOXL2 and FTZ-F1 binding motifs were constructed by PCR-mediated mutagenesis using primers containing the mutated bases (primers 11 and 12, Table. 2). The reporter plasmid containing the mutated FOXL2 and the normal FTZ-F1 binding motifs was constructed by ligation of two fragments (using primers 5 and13 and primers 14 and 10, Table. 2) which consists these motifs using enzyme *Eam1104I*. The transcription factors

FOXL2 and *FTZ-F1* were sub cloned for expression into mammalian cell vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) from the original clones, using gene-specific open reading frame (ORF) primers (primers 1-4, Table. 3).

Table.2. List of primers designed for genome walking and for amplifying deletion constructs of *CYP19A2* gene

S.N0	Primer	Sequence 5' to 3'	Purpose/Position
1.	CYP19A2 Primary	CAGGAGAAGCAACAGTAATGCTG	Genome walking
2.	CYP19A2 Nested	CTAGCATCCACTGCAGCCACGAC	Genome walking
3.	Deletion construct 1 Fwd	ATGCCTTATCTCAGAGAAAATGACCTTTGAT	(-1978/+47)
4.	Deletion construct 2 Fwd	CTGCTTTATGACCTTTTTATACTGTATGGATTGTC	(-1942/+47)
5.	Deletion construct 3 Fwd	ATCAGCGCAGTAATTGGTTCCCG	(-1073/+47)
6.	Deletion construct 4 Fwd	AGCCCTTTCAGATGATGGATTAGGC	(-977/+47)
7.	Deletion construct 5 Fwd	AGTATCTGTGTCGCCGTAGCC	(-652/+47)
8.	Deletion construct 6 Fwd	CAGAGCCTTTGATTTGAAGGGCAT	(-284/+47)
9.	Deletion construct 7 Fwd	GCCAGAAGAGAAAAGGAGAGGAATCC	(-160/+47)
10.	Deletion construct Rev	CTAGCA TCCACTGCAGCCACGACG	(-124/+47)
11.	Mutant FTZ-F1 Fwd	AGTGCTCGGTCAGGCGTAAACGCCTC	(-1009/+47)
12.	Mutant FOXL2 Fwd	GCAGGAAGAATAGAGCAAAGCCGGCTAAAA	(-954/+47)
13.	Normal FTZ-F1+Mutant FOXL2-a	AACTCTTCAGAAGTGCCTTTGCTCTATTCTTCCTGC	Deletion mutants
14.	Normal FTZ-F1+Mutant FOXL2-b	AACTCTTCATTCAAAGGGGATAAAAGGCAAGAAAG	Deletion mutants

Cell culture, transient transfection, and luciferase assays

Human embryonic kidney 293 (HEK 293) and neuroglial (U251-MG) cell lines were obtained from the National Centre for Cell Science (Pune, India). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 20 U/ml pencillin, 20 µg/ml streptomycin and 50 ng/ml amphothericin B

in 5% CO_2 at 37°C. Fetal bovine serum and other cell culture reagents were purchased from Gibco-BRL (Invitrogen, Carlsbad, CA, USA). The cells were transfected in 24-well plates using lipofectamine reagent (Invitrogen) with the following plasmids: 1) 100 ng of deletion constructs (normal as well as mutated) of *CYP19A2* promoter cloned into pGL2-basic (firefly luciferse) vector 2) 100 ng of pcDNA3.1 expression plasmid containing the cDNAs encoding *FOXL2* and *FTZ-F1* or 250 ng of vector for overexpression of FOXL2 and FTZ-F1 3) 10 ng/well pRL-TK (*Renilla* luciferase) vector (Promega) was employed as an internal control. The confluency of both the cell lines was maintained at 90% at the time of transfection. Cells were washed in PBS 48 h after transfection and lysed in 100 µl luciferase lysis buffer. Luciferase reporter activity was determined with the dual-luciferase reporter assay system according to the manufacturer's instructions. Light intensity was measured using Turner Design 20/20 luminometer (Promega).

Electrophoretic gel-mobility shift assay

Preparation of nuclear extract from brain tissue and gel-mobility shift assay was performed by following methods described earlier (Watanabe *et al.*, 1999; Yoshiura *et al.*, 2003; Wang *et al.*, 2007) with few modifications. Briefly, 20 µg of nuclear extract was incubated with 15 f mol of [^{32}P] end labelled double stranded wild or mutated *FOXL2* and *FTZ-F1* oligonucleotides (primers 5-8, Table. 3) in the presence of 0.5 µg of poly dI:dC in a binding buffer (20 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.4 mM DTT and 5% glycerol) for 30 min at 37 °C. The DNA-protein complex formed was separated from free oligonucleotide on a 6% native polyacrylamide gel. For supershift, 1 µg of FOXL2/FTZ-F1 antibody was incubated with the nuclear extract at room temperature for 15 min prior to addition of radiolabelled probe.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed following the protocol of Turner *et al.* (2006) with few modifications. Briefly, 25 mg of homogenized brain tissue was cross-linked with formaldehyde supplemented with protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin), centrifuged for 15 min at 450Xg and the cell pellet was suspended in lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 8.0, 1% Triton-X-100, 0.1% SDS, 0.5% sodium deoxycholate with protease inhibitors). Resuspended pellet was sonicated on ice with 10 X 15 s bursts with a 30 s pause between each sonication. The sonicated samples were diluted with lysis buffer. Samples were precleared for 1 h at 4 °C using pre-immune serum and protein-a agarose beads. Pre-cleared extracts were then incubated with either control IgG or FOXL2/FTZ-F1 antibodies or without the addition of any antibody (no antibody control). Washes were carried out according to the protocol given in Turner *et al.* (2006). In order to exclude a potential contamination with non-specifically adsorbed DNA, we set up a parallel immunoprecipitation experiment with the ORF region of *CYP19A2* which is not the target of both FOXL2 and FTZ-F1. The sequences of the primers used for ChIP assays for detection of FOXL2 and FTZ-F1 binding sites are mentioned in Table 3. PCR conditions were as follows: 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 20 sec and the final step at 72 °C for 2 min. qRT-PCR of the immunoprecipitated samples were performed and the values were plotted as percent input.

Statistical analysis

Data were presented as mean ± SEM. Differences between groups were evaluated using Students'*s*t test or one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis. For each test, *P* value of <0.05 [*] and <0.001 [**] were considered significant.

Table. 3. List of primers used for transcriptional analysis

S.N0	Primer	Sequence 5' to 3'	Purpose
1.	pcDNA-FOXL2 ORF Fwd	GAGATGATGGCCGCCTACCTAGGC	Mammalian cell expression
2.	pcDNA-FOXL2 ORF Rev	GATCCGGGTGTGTAGAGCGGAGTG	Mammalian cell expression
3.	pcDNA-FTZ-F1 ORF Fwd	GTGATGGAGTTCACCACAGAAGAGGAT	Mammalian cell expression
4.	pcDNA-FTZ-F1 ORF Rev	GATGGTGGTGGCTCTCTTGGCATG	Mammalian cell expression
5.	FOXL2 normal Oligo	AGGCAGGAAGAATAGAGCAAAGACAAATAAAAGGGGATA	EMSA
6.	FOXL2 mutated Oligo	AGGCAGGAAGAATAGAGCAAAGCCGGCTAAAAGGGGATA	EMSA
7.	FTZF1 normal Oligo	GGCTGACCTGCCTCTTTAAAAGCCCTTTCAGATGATGGATTA	EMSA
8.	FTZF1 mutated Oligo	GGCGTAAACGCTCTTTAAAAGCCCTTTCAGATGATGGATTA	EMSA
9.	FTZ-F1 Fwd	GACCAGTAAATGTTTTGACCGAACTG	ChIP
10.	FTZ-F1 Rev	CCTGCCTAATCCATCATCTGAAAGGGC	ChIP
11.	FOXL2 Fwd	GCCCTTTCAGATGATGGATTAGGCAGG	ChIP
12.	FOXL2 Rev	CTTGTGCATGTGAAAGACCATTGTAG	ChIP
13.	CYP19A2 ORF Fwd	CAGCTGACCTGCAGAAGTTGTCAG	ChIP
14.	CYP19A2 ORF Rev	GCTTTGGAAAAGAACTCCGTGCTC	ChIP

Results

CYP19A2, *FOXL2* and *FTZ-F1* expression in the developing brain of female catfish

Expression of *CYP19A2*, *FOXL2* and *FTZ-F1* was monitored simultaneously at specific intervals in the developing brain of female catfish from 60 dph. A strikingly similar expression profile of these geneswas observed at 60, 120, 180, 240 and 300 dph that provides a clue for the regulation of *CYP19A2* by FOXL2 and FTZ-F1 (Fig. 1). The expression of these genes was maximum at 60 dph followed by a gradual decline till 240 dph. Thereafter, an abrupt increase in the expression was observed in the adult.

Structure of 5' flanking region of *CYP19A2* gene

Using genome walking strategy, a PCR product of 1978 bp was amplified and cloned that corresponds to 5' flanking region of *CYP19A2* (Fig. 2). The upstream analysis revealed FTZ-F1 and FOXL2 binding sequence, an estrogen binding element (ERα), Estrogen response element half site (ERE-half site), SOX9 and CREB binding sites, glucocorticoid receptor (GR) binding elements and a TATA box (Fig. 3). The putative transcription start site of *CYP19A2* was assumed at -33 position which was obtained by screening and sequencing of the single transcript hybridized from the catfish brain cDNA library constructed in our laboratory (Chapter I).

Identification of functional FOXL2 and FTZ-F1 binding sites in the upstream region of *CYP19A2*

To assess the functionality of FOXL2 and FTZ-F1 binding sites, a series of PCR based deletion constructs were cloned into pGL2 vector and were transfected into U251-MG (neuroglial) cell lines and luciferase activity was measured. The series of progressive PCR based deletion mutants generated are shown in (Figs. 4 and 5). The construct that contained the FOXL2 binding sequence (-977/+47) showed a significant promoter activity in both the cell lines when compared to the control (Fig. 6). The luciferase activity of the construct that harboured FTZ-F1 (-1073/+47) binding site was more prominent. The noteworthy point here is that this construct also contained FOXL2 binding sequence. The constructs with CREB, TATA box and ERE-half binding elements showed negligible promoter activity. The other two reporter constructs, one with GR binding site and the whole fragment did not alter the promoter activity further with respect to FTZ-F1 reporter construct. Similar results were obtained in HEK293 cell line (Fig. 7). These results demonstrate that both FOXL2 and FTZ-F1 binding elements are functional in the *CYP19A2* promoter region.

Activation of *CYP19A2* by FOXL2 and FTZ-F1 in a synergistic manner

A significant change was observed in the luciferase activity of the reporter construct that contains FOXL2 binding element but was not prominent, which raised question on the ability of FOXL2 to alter the *CYP19A2* transcription single-handedly. In other words, the activity of the promoter was probably due to the participation of both FOXL2 and FTZ-F1. To determine this and also to find the precise location to which these transcription factors bind to activate the promoter activity, the reporter constructs containing these elements were mutated and transfected into U251-MG cells (Fig. 8). When compared to the experiment with the wild-type promoter, the activation of *CYP19A2* promoter by FOXL2 as well as FTZ-F1 diminished significantly when the candidate sequences were mutated. There was also an appreciable decrease in promoter activity observed in the presence of reporter construct that carried normal FTZ-F1 and mutated FOXL2 binding elements. These results clearly indicate the necessity of both the transcription factors for upregulation of the *CYP19A2* expression. This study has also resulted in finding of core elements ACAAATA and TGACCT to which FOXL2 and FTZ-F1 bind in the upstream of *CYP19A2*, respectively. Further, the requirement of both FOXL2 and FTZ-F1 for the promoter activation was confirmed by transient transfection of increased concentration of *FOXL2* and *FTZ-F1* expression vectors (either alone or in combination) into neuroglial cells. Upon co-transfection of both the expression vectors, the luciferase activity of the construct containing both FOXL2 and FTZ-F1 binding sequences increased drastically when compared to the construct that contained only FOXL2 response element (Fig. 9). The promoter activity of whole fragment remains unaltered compared with that of the fragment that contains both FOXL2 and FTZ-F1 binding elements.

Confirmation of FOXL2 and FTZ-F1 binding on *CYP19A2* promoter

In order to confirm the *in vitro* and *in vivo* interaction of FOXL2 and FTZ-F1 to their binding sites on the promoter of *CYP19A2*, EMSA and ChIP assays were performed. Gel-shift experiments were performed using normal and mutated probes designed according to the sequences between -956 and -917, and -997 and -955 for FOXL2 and FTZ-F1 respectively. FOXL2 or FTZ-F1 proteins present in the nuclear extract bound to the radiolabelled oligonucleotides, resulting in the formation of specific band of protein/DNA complex. This did not occur when the mutated probe was used. Furthermore, the band was supershifted by the addition of either FOXL2 or FTZ-F1 antibodies (Fig. 10A and B). The direct interaction of FOXL2 as well as FTZ-F1 to their respective binding sites was further verified using chromatin immunoprecipitation. Immunoprecipitation was carried out using anti-FOXL2 and anti-FTZ-F1 antibodies. No amplification was obtained when control IgG was used and in no antibody control. On the other hand, intense PCR amplification was obtained with primers specific for the brain promoter of *CYP19* when the chromatin was immunoprecipitated either with FOXL2 or FTZ-F1 antibodies (Fig. 11A and B). Further, primers failed to amplify *CYP19A2* ORF region when immunoprecipitated in the parallel experiment (Fig. 11C).

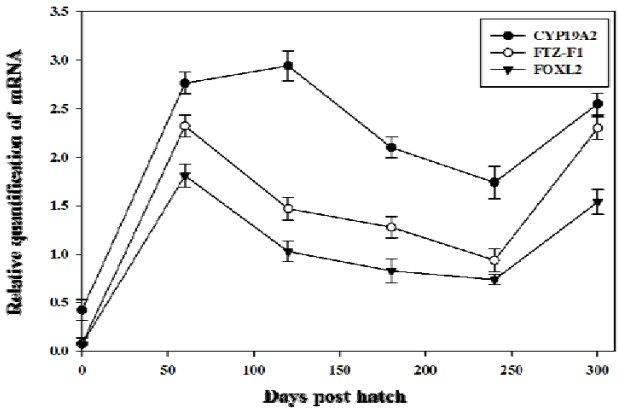

Fig.1. Changes in *CYP19A2, FOXL2* and *FTZ-F1* mRNA levels in developing female catfish brain. qRT-PCR analysis carried out in 0, 60, 120, 180, 240, 300 dph catfish.

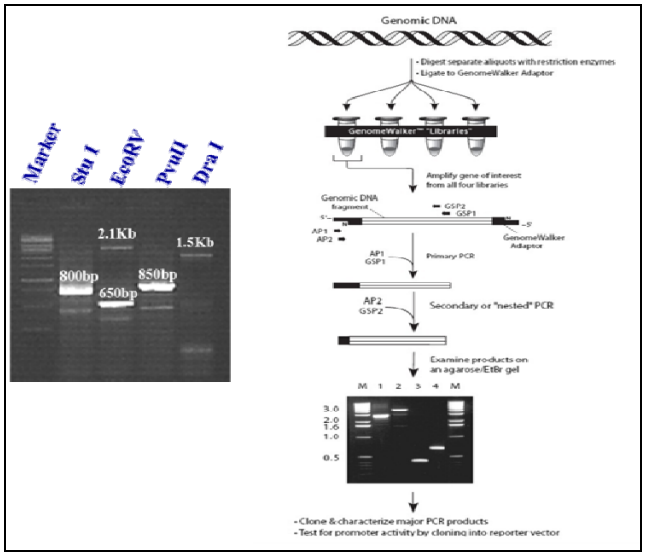

Fig.2. PCR amplifications using *CYP19A2* gene specific primers in catfish genome walking library. Numbers over each band represents the size of band in base pairs. Right panel is schematic representation of genome-walking strategy.

Fig.3. Nucleotide sequence of the 5' flanking region of the *CYP19A2* gene. The putative transcription start site is depicted by an arrow and the nucleotide sequence is numbered on the left. The potential transcription binding sites are underlined and labeled.

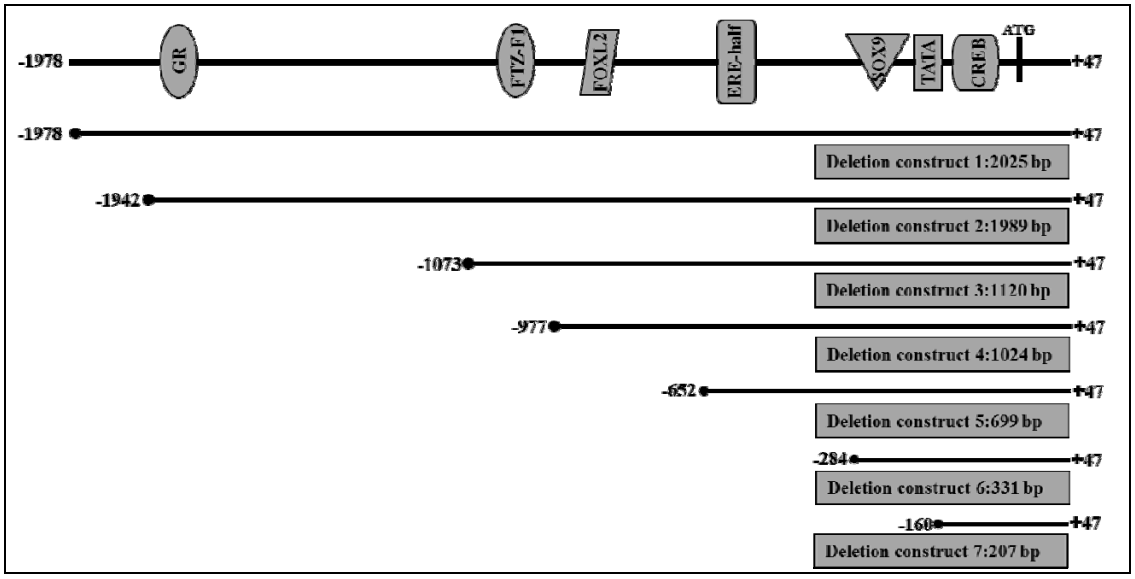

Fig.4. Schematic representation of PCR based progressive deletion constructs of *CYP19A2* promoter.

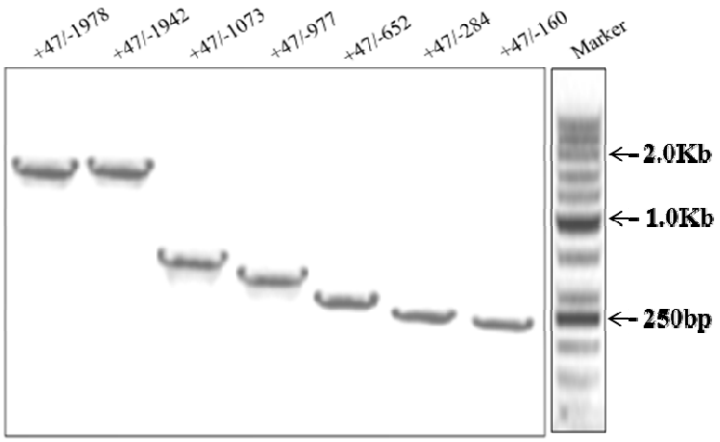

Fig. 5. PCR amplification of deletion constructs cloned into pGL2 vector.

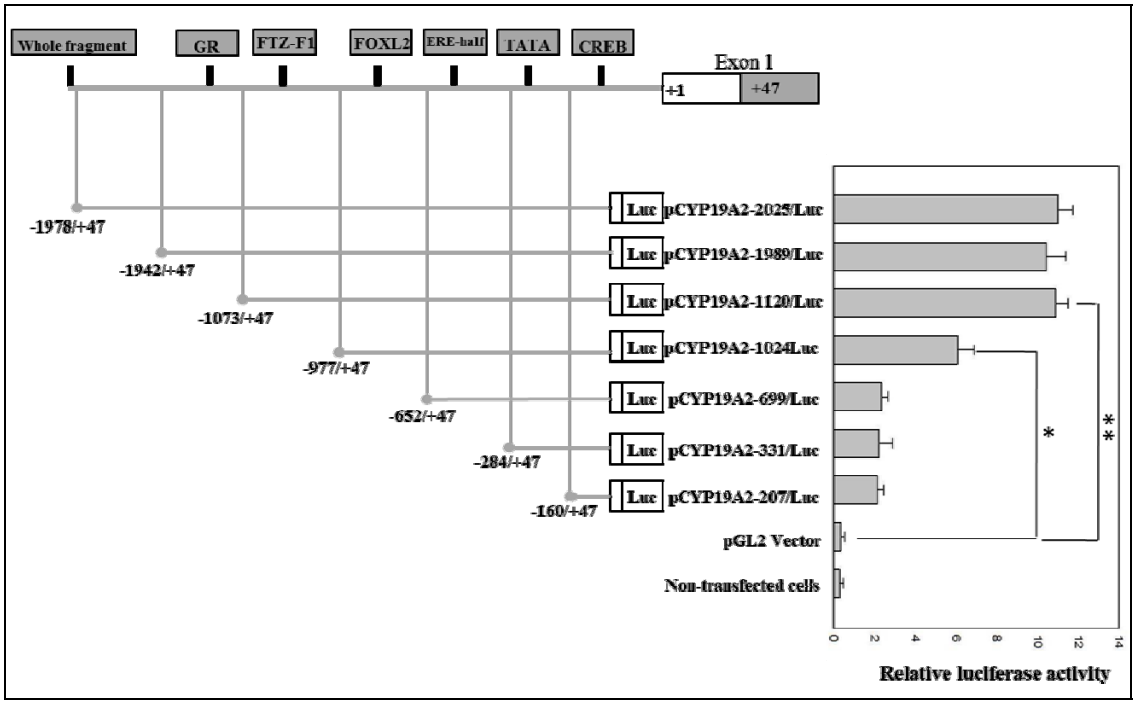

Fig.6. Functional analysis of different *CYP19A2* promoter constructs co-transfected with pRL-TK into U251-MG neuroglial cells. Luciferase activity of *CYP19A2* promoter constructs (100 ng each) co-transfected with FOXL2 and FTZ-F1 (100 ng each) expression vectors into the cell lines. The enzyme activity is presented as relative to the activities measured for *Renilla* luciferase. The data represent the mean ± SEM. from three independent experiments. *P* value of <0.05 [*] and <0.001 [**] were considered significant.

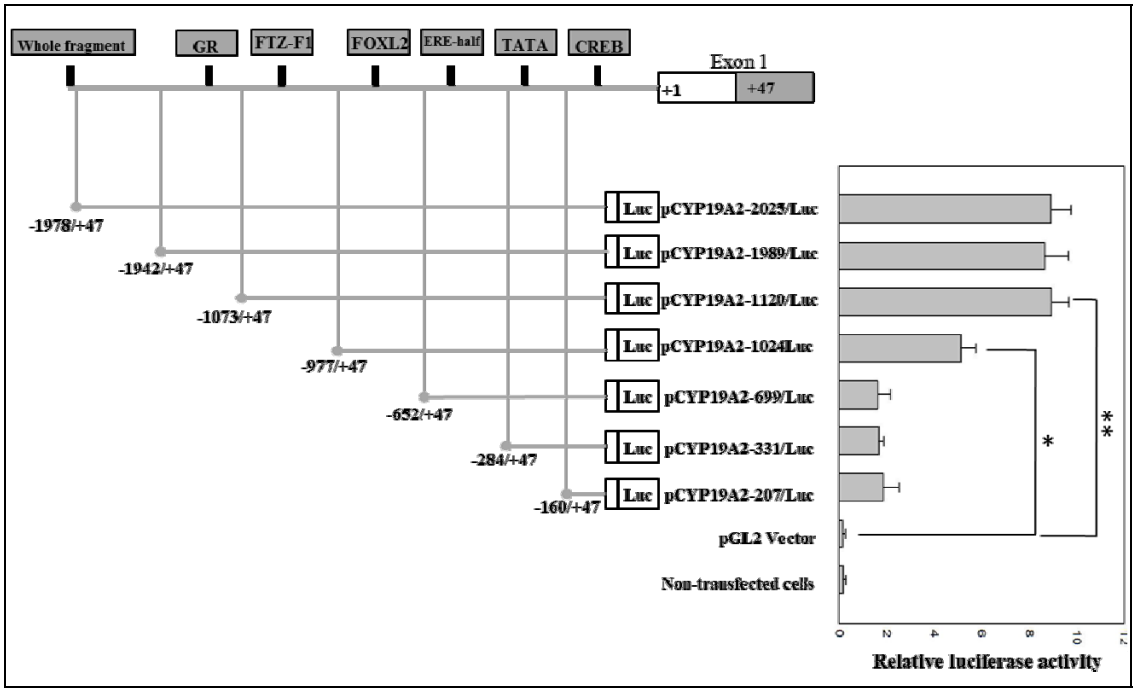

Fig.7. Functional analysis of different *CYP19A2* promoter constructs co-transfected with pRL-TK into HEK 293 cell line. Other detalils are as in Fig. 6.

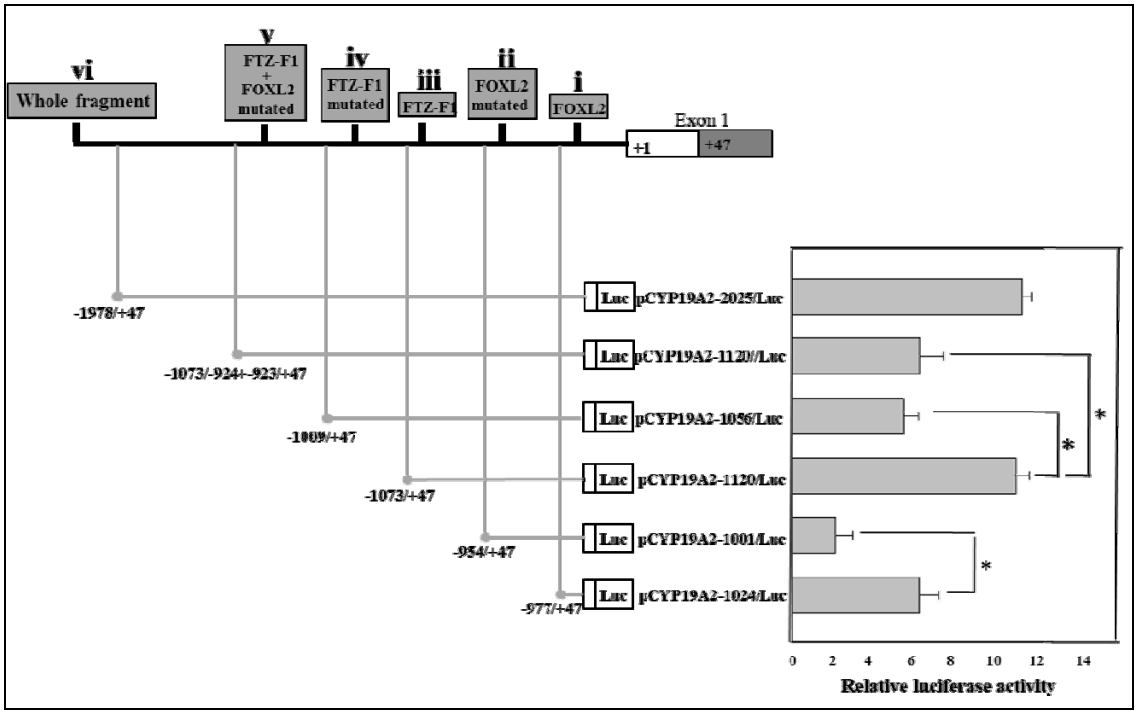

Fig.8. Effect of deletion mutants of FOXL2 and FTZ-F1 binding sites on CYP19A2 promoter activity in U251-MG cells. Reporter constructs (100 ng each) containing either FOXL2 i)

normal FOXL2 ii) mutated FOXL2 iii) normal FTZ-F1 iv) FTZ-F1 mutated; v) FTZF1 normal+FOXL2 mutated binding sites as well as vi) whole fragment were co-transfected with FOXL2 and FTZ-F1 expression vectors (100 ng each) into neuroglial cells. * at *P* <0.05 is considered significant.

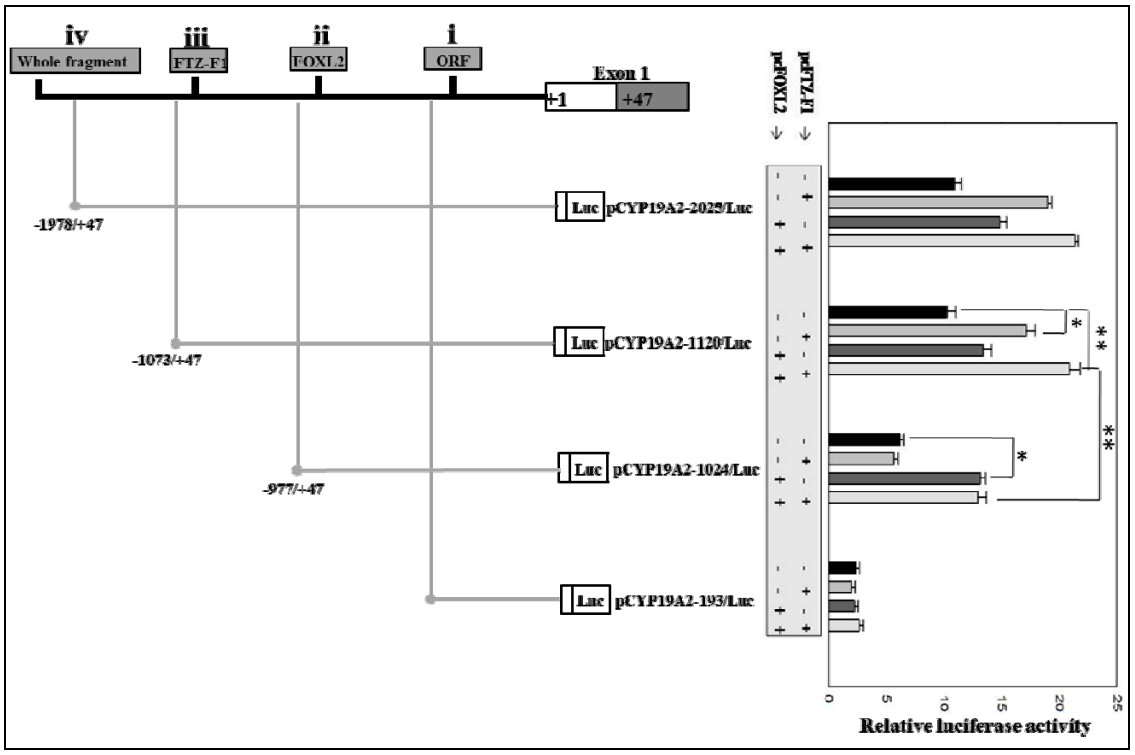

Fig.9. Effect of overexpression of FOXL2 and FTZ-F1 on *CYP19A2* promoter activity. FOXL2 or FTZ-F1 plasmids alone or together (250 ng each) were co-transfected along with i) *CYP19A2* ORF ii) FOXL2 iii) FTZ-F1 or iv) whole fragment reporter constructs (100 ng each). -/- represents 100 ng of each expression vector; -/+ represents increased concentration of either expression vectors; +/+ represents increased concentration of both the vectors. *P* value of <0.05 (*) and <0.001 (**) were considered significant.

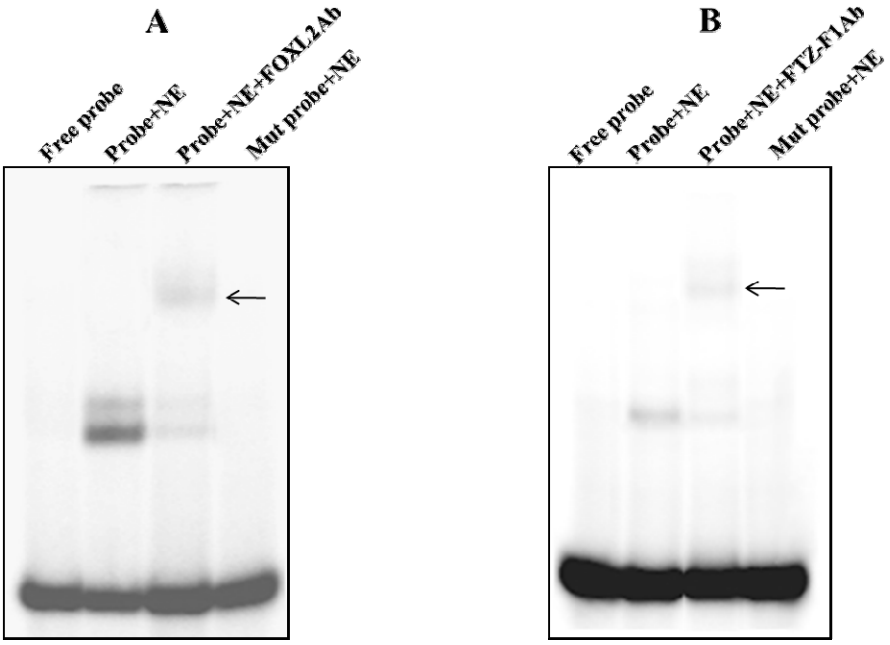

Fig.10. Autoradiograms showing electrophoretic mobility gel shift of brain nuclear extracts. Gel shift assays showing the binding of A) FOXL2 and B) FTZ-F1 (in nuclear extract) to their respective oligomers (probe) synthesized from the upstream nucleotide sequence of *CYP19A2*. Abbreviations: Nuclear extract (NE), Antibody (Ab) and Mutant (Mut). Arrows indicate the supershift observed upon addition of respective antibodies.

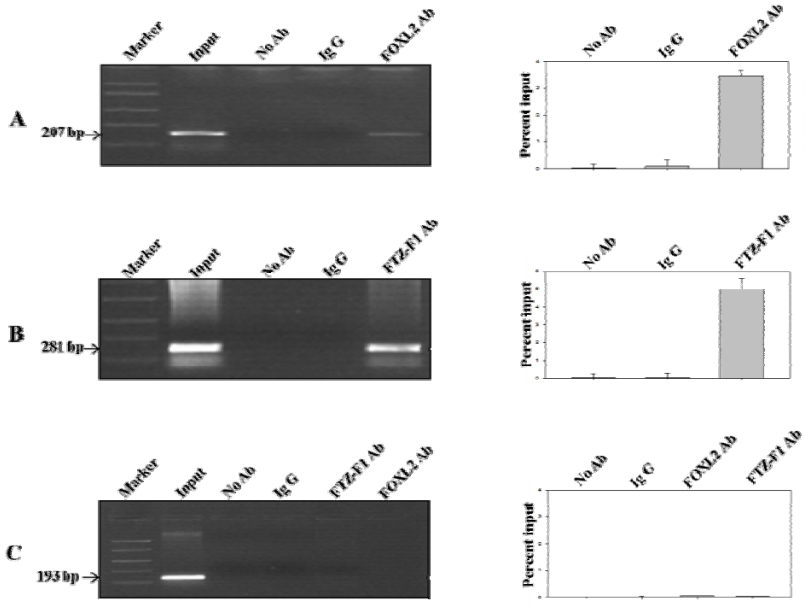

Fig.11. Chromatin immunoprecipitation assay. Amplification of the *CYP19A2* promoter was

positive only in the chromatin immunoprecipitated with either A) FOXL2 or B) FTZ-F1 antibody. No signal was detected either with the control IgG or no antibody control. Also, no amplification of DNA fragment from the C) ORF region of *CYP19A2* was detected. Input DNA was used as loading control in semi-quantitative PCR. For qRT-PCR (right panel), each ChIP DNA fraction Ct value was normalized to the input DNA ($1/10^{th}$ of DNA used in ChIP assays) fraction Ct value. % input was calculated for each ChIP fraction and the values were plotted. The data represent the mean ± SEM from three independent experiments.

Discussion

The extremely high aromatase activity in the brain of teleosts including catfish compared to that of adult mammals makes them intriguing with respect to brain aromatization (Forlano *et al.*, 2001). Initially, we obtained a good correlation of *CYP19A2* expression pattern with those of *FOXL2* and *NR5A1/FTZ-F1*during the brain development. This observation hinted at a plausible involvement of these two transcription factors in the regulation of *CYP19A2* expression. Although *CYP19A2* is well studied in teleosts (Callard and Tchoudakova, 1997; Chang *et al.*, 2001; Kwon *et al.*, 2001; Kazeto and Trant, 2005; Rasheeda *et al.*, 2010), there are no reports so far that show the presence of all the three transcripts i.e., *CYP19A2*, *FOXL2* and *FTZ-F1* in a single study. However, there are few evidences in the recent past predicting the role of FOXL2 and NR5A members in the brain. In mammals, mutation in FOXL2 has resulted in eyelid malformations and craniofacial abnormalities (Benayoun *et al.*, 2008). In addition to ovary, *FOXL2* expression was detected in the brain of Nile tilapia (Wang *et al.*, 2004). SF1 was abundant in the hypothalamus and the anterior pituitary gland in mice (Parker *et al.*, 2002). Lately, in medaka, *Oryzias latipes*, LRH-1 was shown to act as an activator of *CYP19A2* (Matsuyama *et al.*, 2007). The involvement of FOXL2 and NR5A members in the regulation of *CYP19A1* are relatively well studied and are conserved throughout vertebrates

from fish to mammals (Pezzi *et al.*, 2004; Pannetier *et al.*, 2006; Wang *et al.*, 2007). Of late, the regulation of few other genes by the interaction of FOXL2 and NR5A receptors is shown in mammals (Park *et al.*, 2010; Yang *et al.*, 2010). Based on these reports it is clear that FOXL2 and NR5A members are expressed in both brain and ovary and their involvement is prerequisite to regulate either forms of *CYP19*s or other reported genes. Hence, a probable common regulatory unit controlling *CYP19* gene expression in both the tissues cannot be ruled out. This view was strengthened further when we identified putative FOXL2 and FTZ-F1 binding sites that matched well with their known consensus sequences, 5'-(G/A)(T/C)(C/A)AA(C/T)A-3' (Menuet *et al.*, 2005) and 5'-TGACCT-3' (Pezzi *et al.*, 2004; Kanda *et al.*, 2006) respectively, in the upstream region of *CYP19A2*. Transient transfection studies using two different cell lines demonstrated that FOXL2 alone is a weak transcriptional activator on *CYP19A2* promoter. However, in the presence of FTZ-F1 activation of *CYP19A2* promoter enhanced greatly in a synergistic manner. This finding is endorsed by previous reports that showed the likely interaction of FOXL2 and NR5A1 in the Nile tilapia (Wang *et al.*, 2007), mice (Yang *et al.*, 2010) and human granulosa cells (Park *et al.*, 2010) to regulate the transcription of *CYP19A1*, *melanocortin2receptor* and *CYP17* genes, respectively. Collectively, these data suggest that the interaction between FOXL2 and NR5A1 plays a decisive role in transcriptional activity of *CYP19* genes (both brain and gonadal forms). Further, the confirmation of binding of FOXL2 and FTZ-F1 to their respective cis-acting elements demonstrated through the EMSA and ChIP analyses binding assays provides more substantial proof of the synergism between FOXL2 and FTZ-F1 in the regulation of *CYP19A2* transcription.

In this context of this study, it is important to mention that ERα and ERE-half motifs were identified in the proximity of FTZ-F1 motif in the upstream region of *CYP19A2* of catfish, which were in basal state. However, induction of estrogen (E_2) or analogues or xenobiotic

compounds with estrogenic activity might promote the transcription of CYP19A2. In addition, the E_2 dependent regulation was specific to radial glial cells (Anglade *et al.*, 1994; Menuet *et al.*, 2002; 2003). Contrary to this, data on the expression of ER in fish have not supported the assumption that radial glial cells express ER (Kishida and Callard, 2001; Menuet *et al.*, 2005; Diotel *et al.*, 2010). In a different study, a GxRE motif in the proximity of ERE was shown to play an important role in E_2 dependent regulation of *CYP19A2* in these cells (Le Page *et al.*, 2008). The GxRE motif contains the core sequence AGGTCA that is acknowledged to recruit different types of NR5A members (Pezzi *et al.*, 2004; Kanda *et al.*, 2006; Diotel *et al.*, 2010). In addition, none of the nonependymal ER expressing cells observed in the brain of zebrafish expresses aromatase (Kishida and Callard, 2001; Menuet *et al.*, 2005). These data suggests that ERs might not be responsible for the expression of brain aromatase at all conditions. The combined action of FTZ-F1 and FOXL2 described in this study provide some clarity for the regulation of expression of brain aromatase.

In conclusion, we demonstrate the synergistic effect of FOXL2 and FTZ-F1 by binding to the upstream region of *CYP19A2* for the activation of transcription. This regulatory mechanism most likely drives the brain aromatization in *C. gariepinus*. Further studies on the regulation of *CYP19A2* in other teleosts involving these two transcription factors might enlighten our findings.

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Chapter V

Effects of estradiol analogues, diethylstilbestrol and ethynyl estradiol on the expression of aromatases and their transcription factors in the brain and ovary of catfish, *Clarias gariepinus,* during ovarian differentiation

Abstract

Estradiol (E_2) directly influences ovarian development and sex differentiation in teleosts and its appropriate biosynthesis is required for reproductive success. Alteration in the expression of steroidogenic enzyme genes alters E_2 production. Few transcriptional regulatory elements in the 5'flanking regions of cytochrome P450 aromatase (CYP19) genes make them responsive to external estrogens and hence vulnerable for endocrine disruption. In the present study, we treated the juvenile air-breathing catfish, *Clarias gariepinus* (3 days post hatch [dph]) with estradiol analogues, diethylstilbestrol (DES) and ethynylestradiol (EE_2) and changes in expression pattern of aromatases were analyzed. Both DES and EE_2 enhanced the expression of both *CYP19A1* and *CYP19A2* and also aromatase activity in ovary and brain of 50 dph fish, but later on after withdrawal of treatment, this effect subsided. Similar changes were observed in the transcript levels of *FOXL2* and *FTZ-F1*. Histologically, the ovaries of DES and EE_2 treated fish showed precocious oocyte maturation and atresia at an early stage. Though the CYP19s expression and activity returned to normalcy in 250 dph and 350 dph fish, histologically ovaries were not normal. Morphologically, DES treatment showed more deleterious effects like rudimentary or underdeveloped ovaries, but growth retardation was a common phenomenon observed in both DES and EE_2 treated fish when compared to control. Based on these results, it is possible to suggest that endocrine disruptors possessing estrogenic property can alter ovarian development in teleosts even with minimal dosage and exposure time.

Introduction

Estrogens, especially estradiol-17β (E_2), play an important role in the ovarian development, growth, sexual differentiation and reproduction in all vertebrates. Endogenous sex steroids have been key regulators of sexual differentiation in all vertebrates. E_2 is required during ovarian differentiation and reproduction, and its deficiency or depletion leads to maleness or sterility, ascertaining its role in diverse vertebrate form in maintaining the androgen-estrogen homeostasis (Hayes, 1998). In lower vertebrates including teleosts, E_2 directly influences a number of developmental and reproductive events including, hepatic vitellogenin (the precursor of yolk protein) and egg membrane protein synthesis, oocyte growth (Wallace, 1985), germ cell development (Billard, 1992), gonadal sex differentiation (Wibbels *et al.*, 1998), and reproductive behavior (Bjerselius *et al.*, 2001). This property of E_2 makes physiological systems of several vertebrates vulnerable if exposed to E_2 mimicking compounds at an inappropriate time of development. A number of chemicals released in to the aquatic environments have the potential to alter the function of the endocrine system of animals by targeting the sex steroid receptors mediated signaling mechanisms (Andersen *et al.*, 2003). This may further lead to the improper development of the reproductive system of the animals in the aquatic environments (Baek *et al.*, 2003). In fishes, this may result in reduced gonad size or feminization of genetic male fish, skewed sex ratios, impaired gametogenesis or altered adult sexual maturity, delayed ovulation and spawning, decreased fertility and egg production (Bjerselius *et al.*, 2001). It is also evident that appropriate and timely changes in E_2 biosynthesis are required for reproductive success. Changes in the expression of genes encoding sex steroidogenic enzymes could negatively

regulate E_2 production. Hence, a study on the effect of estradiol analogues on these enzymes could be interesting.

Among the steroidogenic enzymes, aromatase (CYP19) is the rate-limiting enzyme in ovarian steroidogenesis and it regulates the levels of E_2 within passable ranges for ovarian differentiation (Trant *et al.*, 2001). In mammals, there is a single *CYP19* gene that is expressed in a variety of tissues, is mediated through tissue-specific promoters and is characterized by alternative splicing of transcripts (Simpson *et al.*, 2002). Teleosts, in contrast, have two structurally distinct *CYP19* isoforms *CYP19A1* (ovarian form) and *CYP19A2* (brain form). These gene products have been identified in several species of teleosts, including goldfish (Tchoudakova and Callard, 1998), zebrafish (Trant *et al.*, 2001; Kishida and Callard, 2001), tilapia (Kwon *et al.*, 2001) and catfish (Rasheeda *et al.*, 2010; Chapter I). Recently, a number of consensus transcriptional regulatory elements were identified in the 5'flanking regions of zebrafish *CYP19A1* and *CYP19A2* genes (Kazeto *et al.*, 2001) that are responsive to estradiol analogues, which suggest that *CYP19* genes would make excellent transcriptional targets for endocrine disruption. In addition, the involvement of the *CYP19A2* on reproductive physiology through the brain–pituitary–gonadal-axis has been suggested in fish (Cavaco *et al.*, 2001). However, the molecular effects of estradiol analogues on the expression of CYP19s and their related transcription factors (*FOXL2* and *FTZ-F1*), have received little attention. In the present study, we intend to analyze these correlates during ovarian differentiation, after treatment with non-steroidal analogue, diethylstilbestrol (DES) and steroidal analogue, ethynylestradiol (EE_2).

Materials and methods

Catfish breeding and sampling

Air-breathing catfish, *Clarias gariepinus* is bred and reared in our laboratory aquaculture facility and the conditions were described earlier in Chapter I. The hatchlings were kept in the plastic tubs supplied with circulating filtered water under natural photoperiod and ambient temperature. After breeding, for the treatment experiments, the hatchlings from each group (15-30 fish) were maintained in individual aerated plastic tubs with a capacity of 15 L. They were fed with live tubeworms *ad libitum* till they grew to fingerling size (~5-6 mm). Later on pelleted food was provided along with live tube worms. Water was changed twice a day after feeding and also after the treatment of sex steroid analogs. Care was taken during water change and at no time fish was exposed under water-less or non-aerated conditions. Both control and treated groups were handled similarly.

Treatment of juvenile catfish with estradiol analogues

Chemicals used in the study, diethylstilbestrol DES (3, 4-bis [4-hydroxyphenyl]-3-hexene), 99.0% purity, ethynylestradiol EE_2 (17β-ethynyl-1, 3, 5[10]-estriene-3, 17β-diol), 98.0% purity, were obtained Sigma (St Louis, MO). The chemical structures of E_2 and its analogues are given in Figure 1. Three days post hatch (dph) juvenile catfish obtained by *in vitro* fertilization were treated with estradiol analogues DES and EE_2 up to 50 dph (Fig. 2). The dosage of treatments of DES (10ng/l) and EE_2 (50ng/l) were chosen based on pilot studies. Further the dosage of EE_2 used in the present study is comparable to previous studies in catfish (Raghuveer and Senthilkumaran, 2009). Dosage standardization for DES treatment was done considering

different parameters like age of fish and mortality. We used slightly higher concentration of E_2 analogs for treatments to understand the severity and hence, the results cannot be compared directly to water pollution by sex steroid analogs. Both DES and EE_2 used in the study were first dissolved in absolute alcohol and then air-dried to dissolve it in fish physiological saline (0.90% w/v of NaCl). These were then used for treating the fish. The treatment was withdrawn after 50 days and fish of 50, 250 and 350 dph from both control and treated groups were anesthetized in ice-cold water, weighed and sacrificed. Brain and gonads were dissected out and processed for total RNA preparation and histological analysis.

qRT-PCR for expression studies

Methodology for total RNA isolation and first strand cDNA synthesis from brain and ovary of female catfish are given in Chapter I. Expression of *CYP19A1, CYP19A2, FOXL2* and *FTZ-F1* mRNA were analyzed by qRT-PCR in control and fishes treated with DES and EE_2. The brain and ovary tissues were dissected out using fine forceps under a stereozoom microscope (Leica, Wetzlar, Germany), after morphological (also histological) identification of testis and ovary under sterile conditions. From histological observation it was evident that the critical period of sex differentiation was around 35-50 dph when the gonads get morphologically distinguishable in air-breathing catfish (Raghuveer and Senthilkumaran, 2009). Hence, the first batch of control and treated juvenile catfish were harvested at 50 dph, which permitted us to unambiguously identify and isolate the female ovary and brain for expression and enzyme activity analyses. Real time PCR primers for each gene were designed from intron exon boundaries for *CYP19A1* and *CYP19A2* and for *FOXL2* and *FTZ-F1* from the conserved fork head domain and DNA binding

domain, respectively. Primers for *β-actin* used as the reference gene were designed from sequence obtained by cloning the catfish *β-actin*. All the primers used for the study are given in Table 1. The standardization and methodology followed for qRT-PCR are as given in Chapter I. For total RNA isolation (1µg) from 50 dph fish, the brain and gonadal tissues from 5-10 juveniles were dissected and pooled to have 5 biological samples (brain/ovary tissue from 4-5 juvenile fish constitute one sample, n=5). The tissue samples were then immediately snap-frozen in liquid N_2 and kept at -80ºC for subsequent analysis.

Table.1. List of primers used for expression analysis of *CYP19A1*, *CYP19A2*, *FOXL2* & *FTZ-F1*.

S. No.	Primer	Sequence 5' to 3'	Purpose
1.	CYP19A1 Fw	AGGTCCCTGGTTTTGTCTG	qRT-PCR
2.	CYP19A1 Rv	TGCAGATGGCCTGCTGAGG	qRT-PCR
3.	CYP19A2 Fw	GGTCTGGGGATTGGGACAGC	qRT-PCR
4.	CYP19A2 Rv	CACAGCAGATGACTTGCTTAG	qRT-PCR
5.	FOXL2 Fw	CATGGCTATACGCGACAGCTC	qRT-PCR
6.	FOXL2 Rv	CCAGTAGTTCCCCTTCCTCTC	qRT-PCR
7.	FTZ-F1 Fw	GGAGGAGCTCTGTCCTGTGTGCG	qRT-PCR
8.	FTZ-F1 Rv	GCTGCGTCTTATCGATGCCGCAG	qRT-PCR
9.	β-actin-Fw	ACCGAAGTCCATCACAATACCAGT	qRT-PCR
10.	β-actin-Rv	GAGCTGCGTGTTGCCCCTGAG	qRT-PCR

Changes in aromatase activity in ovary and brain in control and treated catfish

The aromatase activity in ovary and brain of catfish collected after treatment in different age group fishes was assayed as per the method described (Tsai *et al.*, 2000) with minor modifications. The brief methodology is mentioned in Chapter. I.

Histological studies

For histological examination, brain and gonadal tissues were fixed in freshly prepared Bouin's fixative (saturated picric acid: formaldehyde: glacial acetic acid at the proportion of 15: 5: 1) for 1–2 h at room temperature, then processed with graded alcohol series and embedded in paraplast (Kendall, Mansfield, MA, USA). Sections of 6μm thickness were cut with a rotatory microtome (Leitz, Wetzlar, Germany) and stained with hematoxylin–eosin following a series of dehydration, rehydration steps through graded concentrations of ethanol and xylene. All the photomicrographs for histological analysis were taken with Olympus CX41 light microscope fitted with a digital camera.

Statistical Analysis

Data analysis was carried out using one-way ANOVA followed by Tukey's test. The entire experimental procedures was repeated thrice and the results expressed as mean ± SEM of three replicates for both ovarian and brain samples collected individually from five fish (n=5). Difference among groups was considered significant at $P < 0.05$.

Results

Morphological examination of control and treated catfish

The representative photographs of the treatment of juvenile fish with DES and EE_2 are shown in Fig. 2A, B and C. In DES treated fish, after 27 days, curved tail phenotype and water retention was observed which disappeared after with drawl of treatment in 100 dph treated fish (Fig. 3 and 4). Stunted growth was observed in EE_2 treated fish when compared to respective controls age wise shown in Fig. 5A and B. The decrease in both height and weight was evident. By morphological observation there was no clear difference between control and treated ovaries in EE_2 treated fish at 250 dph but rudimentary or underdeveloped ovaries were observed in most of the DES treated fishes (Fig. 6).

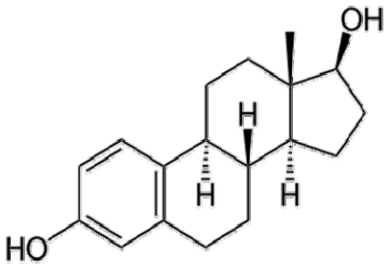
Expression of *CYP19A1, CYP19A2, FOXL2* and *FTZ-F1* by qRT-PCR

The expression of *CYP19A1* in the ovary of DES and EE_2 treated fish showed a significant increase in the transcript levels when compared to control in 50 dph fish however there was no significant difference observed in the expression after the withdrawal of treatment in 250 dph and 350 dph fish (Fig. 7A). The pattern of expression of *CYP19A2* in brain of DES and EE_2 treated fish collected at similar time points from the same fish simultaneously was almost analogous to *CYP19A1* in ovary (Fig. 8A). The expression of transcription factors, *FOXL2* and *FTZ-F1* by qRT-PCR revealed a similar expression pattern to that of *CYP19A1* and *CYP19A2* in ovary and brain, respectively (Fig. 9A and B). The aromatase activity studies in both brain and ovary of control and treated fish corroborated well with the transcript data (Fig 7B and 8B).

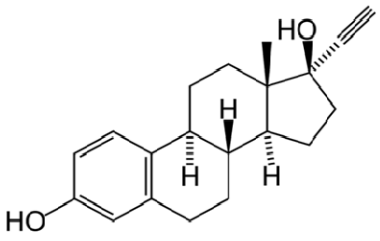
Histological examination of EE_2 and DES treated catfish

The hematoxylin and eosin staining of ovarian sections in control and treated fish at 100 dph revealed that ovarian sections from control fish showed more pre-vitellogenic and vitellogenic oocytes with less number of post-vitellogenic oocytes while EE_2 treated females exhibited precocious oocyte development with higher number of post-vitellogenic oocytes compared to controls (Fig. 10A and D). Similar kind of histology to that of 100 dph was observed in 250 dph ovarian section when compared to control (Fig. 10B and E). In ovarian sections of 350 dph fish, more number of oocytes underwent atresia where there was degeneration and subsequent resorption of oocytes (Fig. 10C and F).

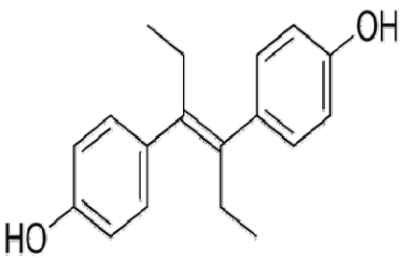
The histological analysis was difficult in DES treated catfish as most of the fishes had either rudimentary or underdeveloped ovaries. However, hematoxylin-eosin staining of the ovarian sections obtained from DES treated 100 dph fish (Fig. 10G) more number of previtellogenic and immature oocytes were observed when compared to control. In 250 dph DES treated fish, very few vitellogenic oocytes were seen among which later underwent atresia by 350 dph (Fig. 10H and I).



Estradiol-17β



Ethynyl estradiol (EE_2)



Diethylstilbestrol (DES)

Fig.1. Chemical structures of estradiol and its analogues

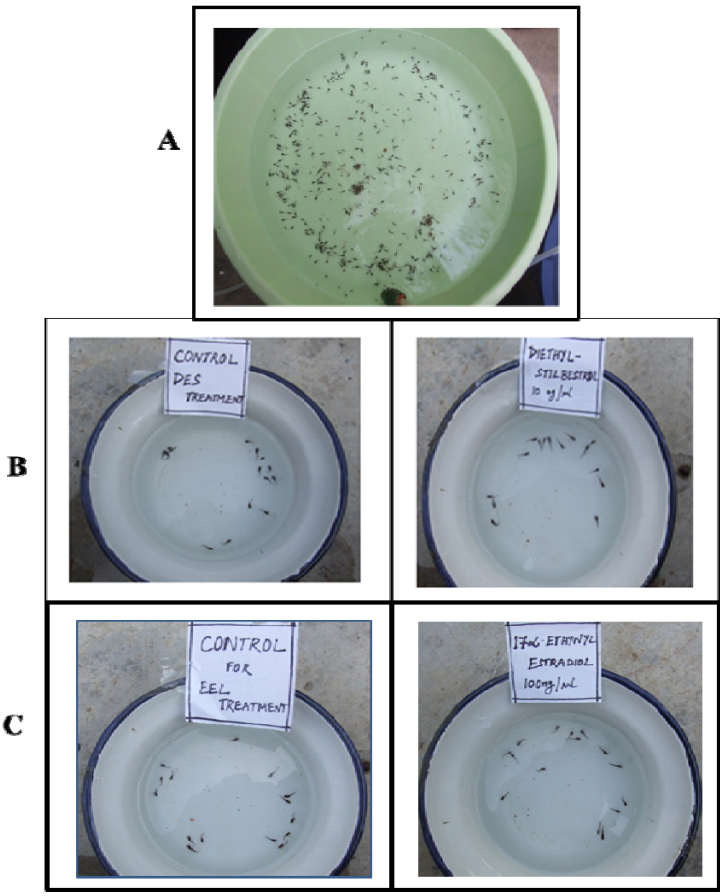
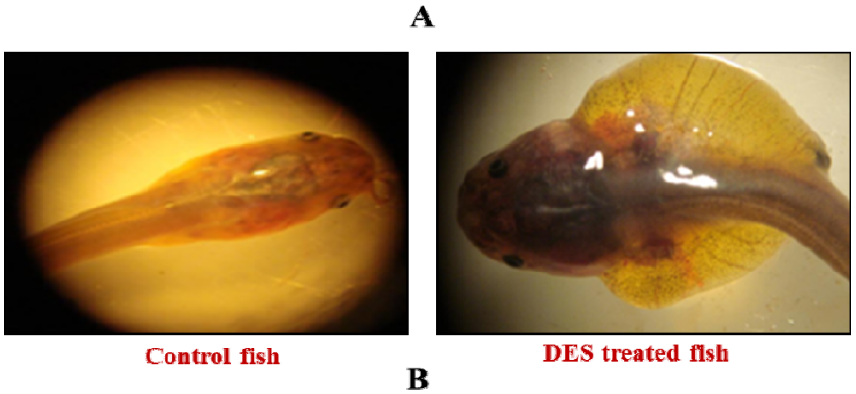


Fig.2. Representative photographs showing treatment of juvenile catfish A) 3 dph, B) Control and DES treated catfish at 10 dph C) Control and EE_2 treated catfish at 10 dph.

Fig.3. Representative photograph showing curved tail phenotype in DES treated catfish when compared to control at 27 dph.



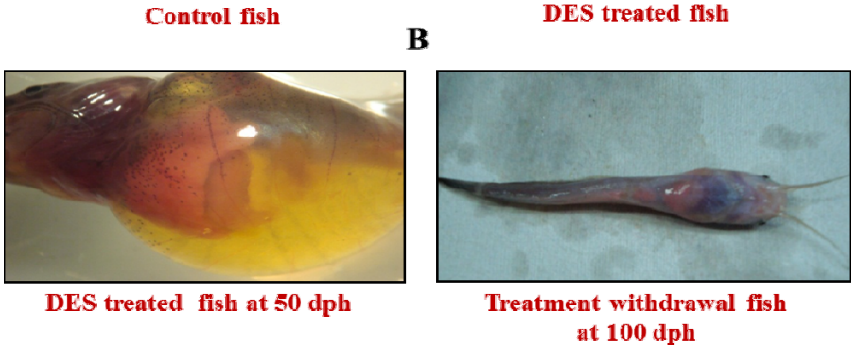


Fig.4. Representative photographs showing water retention in DES treated catfish. A) DES treated fish when compared to control at 50 dph. B) Recovery of water retention in 100 dph DES treated fish.

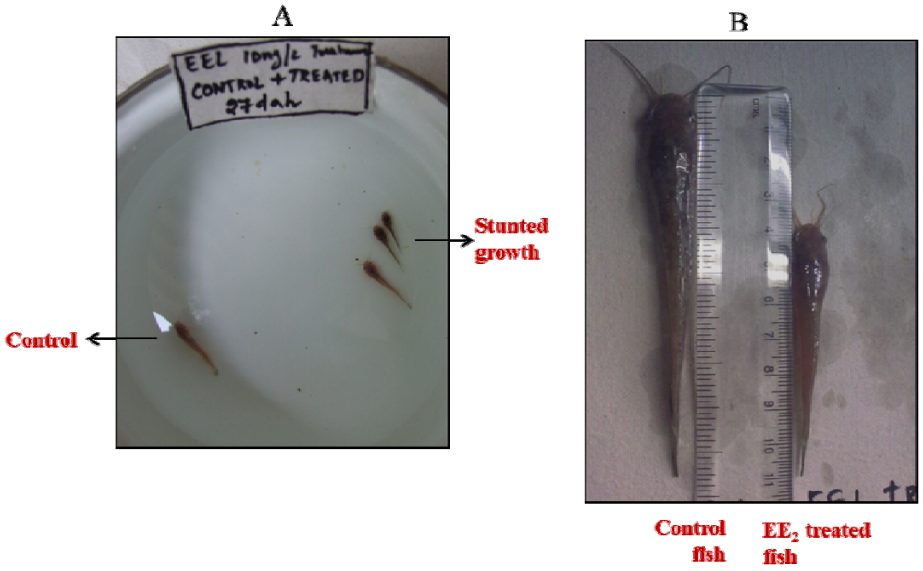


Fig.5. Representative photographs showing effect of EE_2 treatment on growth A) 27 dph fish and B) 1 year old fish.

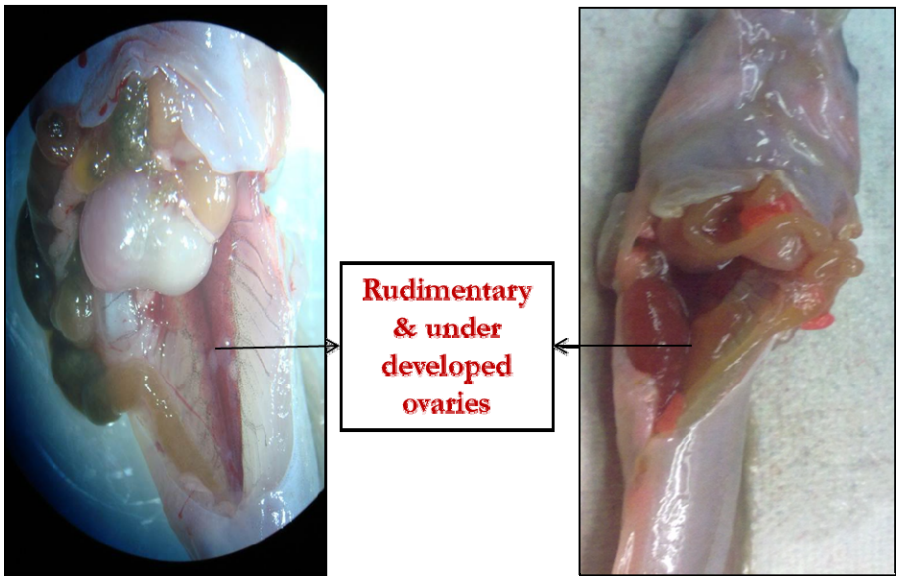


Fig.6. Representative photograph showing effect of DES treatment. Rudimentary and under developed ovaries in 1 year old DES treated catfish.

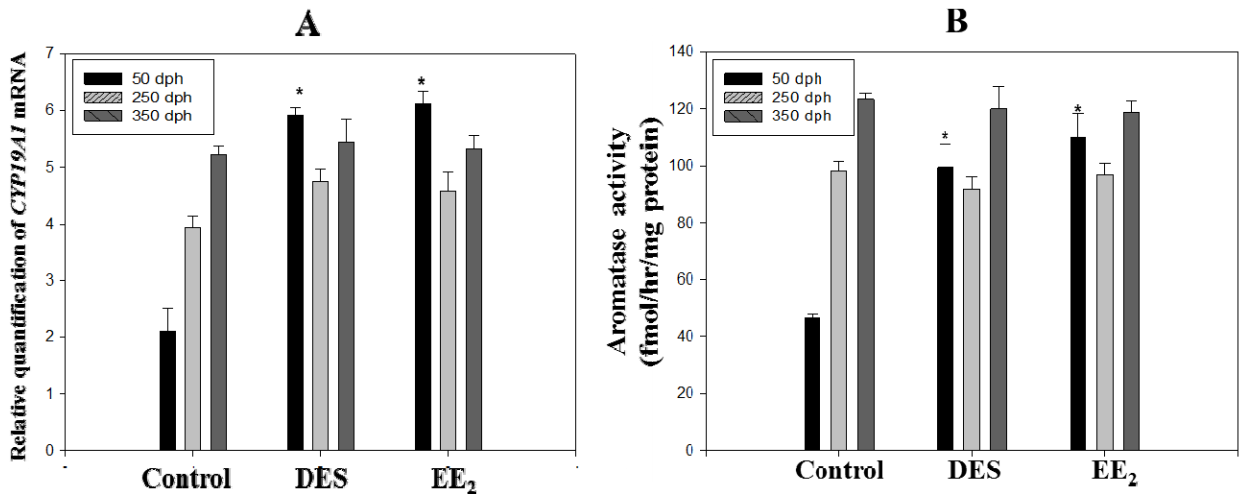


Fig.7. Changes in *CYP19A1* expression in DES and EE_2 treatment in catfish ovary**.** A) qRT-PCR analysis of *CYP19A2* mRNA levels B) Changes in aromatase enzyme activity in ovary of DES and EE_2 treated fish. Data from qRT-PCR were expressed as mean ± SEM. Significance between groups were tested by ANOVA followed by Tukey-Kramer's test using sigma plot 11 software. * indicates the significance at $P<0.05$.

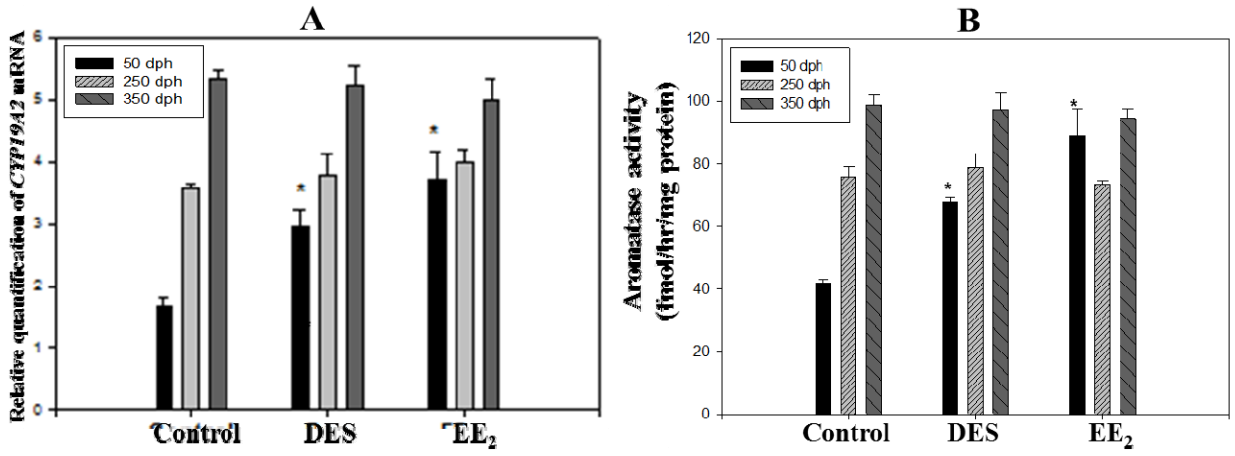


Fig.8. Changes in *CYP19A1* expression in DES and EE_2 treatment in female catfish brain**.** A) qRT-PCR analysis of *CYP19A2* mRNA levels B) Changes in aromatase enzyme activity in brain of DES and EE_2 treated fish. Other details as in Fig.7.

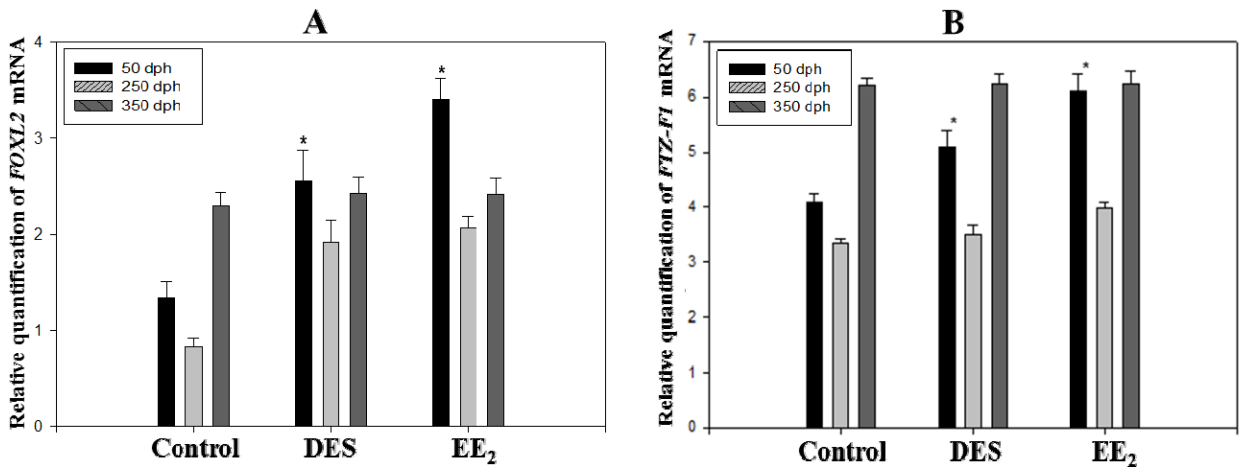


Fig.9. Changes in *FOXL2* and *FTZ-F1* expression after DES and EE_2 treatment in female catfish A) qRT-PCR analysis of *FOXL2* mRNA levels in brain B) qRT-PCR analysis of *FTZ-F1* mRNA levels in ovary. Other details as in Fig. 7.

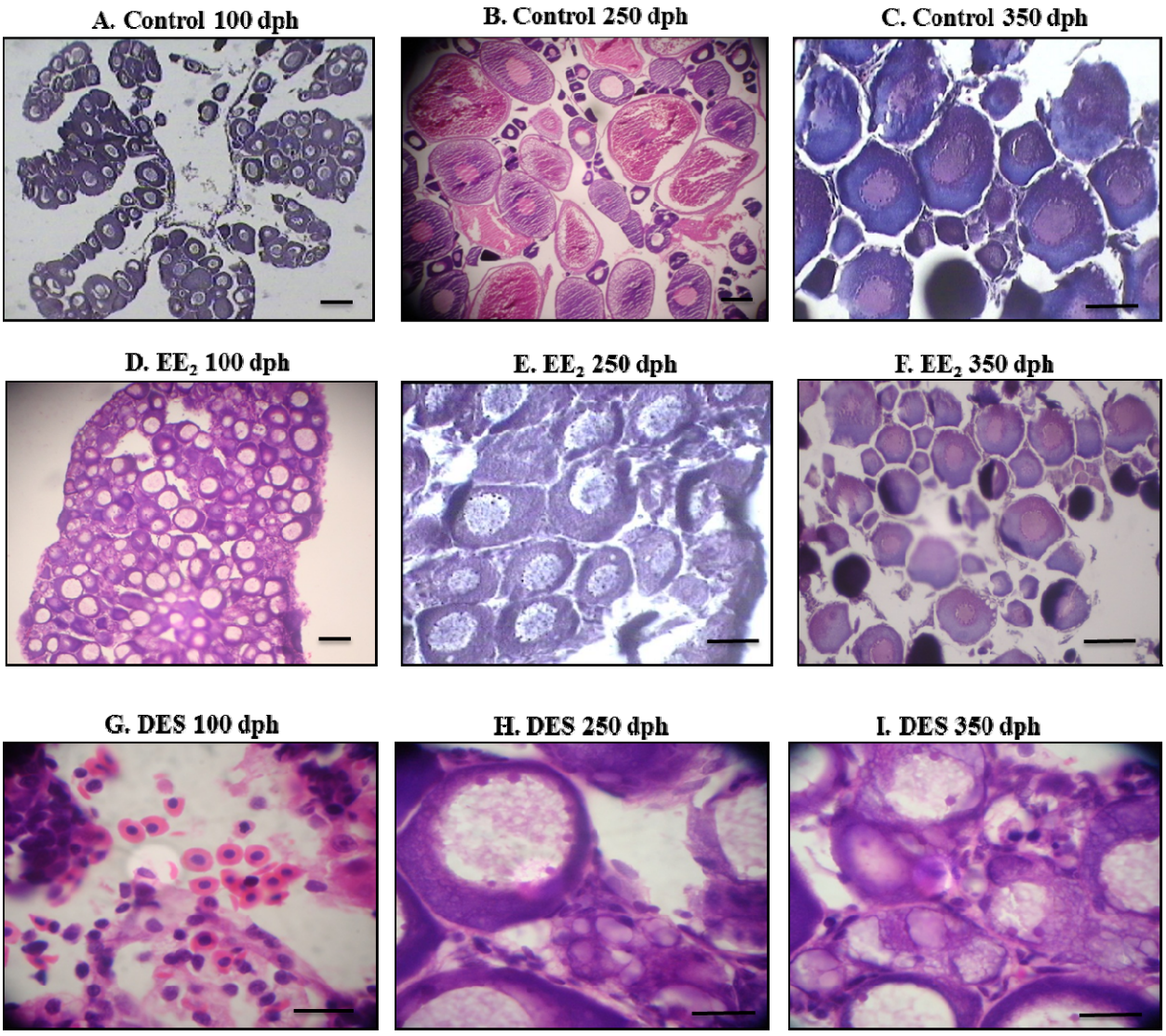


Fig.10. Histology of control, EE_2 treated and DES treated catfish. A), B), C) Control catfish ovary at 100, 250, 350 dph. D), E), F) EE_2 treated catfish ovary corresponding to control. G), H), I) DES treated catfish ovary corresponding to control. Scale bar indicates 50μM.

Discussion

Various pesticides/ pharmaceutical compounds discharged in the environment are known to have estrogenic property by virtue of their ability to bind to estrogen receptor (ER) to mimic or block estrogen dependent biological responses and they alter the production or availability of natural hormones. Treatment with sex steroids or their analogs during early development or juvenile phase often lead to sex reversal and skewing of mixed sex population or genetic males to female sex population irrespective of the genetic sex (Kobayashi *et al.*, 2003; Nagahama, 2005; Raghuveer *et al.*, 2005). This effect was more evident when steroid treatment is used during the critical period of sex determination/differentiation (see Nagahama, 2005; Raghuveer *et al.*, 2005). Earlier study in catfish where sex reversal was evident only when EE_2 treatments were given intermittently (Raghuveer *et al.*, 2005). In the present study, we chose to treat juvenile catfish with acceptable doses DES and EE_2 (estradiol analogues) continuously for 47 days in order to study their effects during and after ovarian differentiation. These synthetic estrogens are well known to bind to fish ER, *in vitro* (Loomis and Thomas, 1999) and to elicit estrogenicity in many different vertebrate species and test systems (Andersen *et al.*, 1991).

Morphological changes

As discussed earlier, DES and EE_2 are potent synthetic estrogens and prototypic endocrine disruptors. During the early developmental stages of fish and other vertebrates, the differentiation of tissues and cells (such as the gonads) into organs with the proper structure and capability of responding to external and internal cues is controlled, at least in part, by the hormones of the brain-pituitary-gonadal axis (Watanabe *et al.*, 2009). It is well known that

estrogen has organizing effects in the developing central nervous system (Bakker and Baum, 2008) and hence the external estrogens might affect the development on the whole of the juvenile catfish. We observed high degree of mortality (about 10-20%) initially in DES treated fish which decreased later on. An interesting phenomenon of water retention was evident in the DES treated fish which however disappeared in the later stages after withdrawal of the treatment. Water retention may be explained as a consequence of DES treatment. Curved tail phenotype was also evident in DES treated fish in the initial days of treatment which can be attributed to a contaminant or to the conversion of DES to E_2 via the actions of 17β-hydroxysteroid dehydrogenases (Kishida *et al.*, 2001). Although these kinds of deformities were not observed in EE_2 treated fish, growth retardation was a common developmental abnormality in both EE_2 and DES treated fish. The majority of evidence is consistent with estrogen specificity of these developmental defects in catfish are in agreement with an earlier studies in zebrafish (Kishida *et al.*, 2001) and *Xenopus* (Nishimura *et al.*, 1997), where embryos treated with 10 μM E_2 and DES, but not estradiol-l7α, 5α-dihydrotestosterone or testosterone, had higher mortality and malformations including small head, large abdomen and crooked vertebrae. DES and EE_2 were also known for their sexual disruption and sex reversal capabilities in fish (Raghuveer *et al.*, 2005; Yang *et al.*, 2008; Paul-prasanth *et al.*, 2011) however, it remains unresolved how estrogenic chemicals disrupt the default genetic sex determination program of the genetic males and initiate formation of ovaries. The sex ratio was more proportionate towards females in both DES and EE_2 treated fish. Dissection of DES treated fish after one year revealed most of the fish having either underdeveloped or rudimentary ovaries in most of the female fish. Such anomalies were not observed in EE_2 treated fish when checked morphologically. Taken together, we can

understand that in any dosage even if it was lower, long term exposure of juvenile fish to DES leaves perennial inhibitory effect on fish developmentally and morphologically and moreover, steroidal analogue EE_2 can be more are useful for the sex change studies rather than the non-steroidal analogue, DES.

Expression of aromatases and its transcription factors

Though several other enzymes are involved in the estrogen biosynthetic pathway, aromatization is the ultimate step where CYP19 catalyzes the conversion of androgens to estrogens. The CYP19 isoforms play a key role in the sexual differentiation and regulation of the reproductive cycle in diverse teleost species and hence alteration in their expression and/or activity may lead to diverse disturbances of the above mentioned processes. Sensitivity of CYP19s to estrogens/xenoestrogens can be explained by the presence of a well-characterized ERE and ERE half in the proximal promoter region of aromatases where estrogen receptors bind and transcriptionally regulate the aromatases (Kazeto *et al.*, 2004). Well characterized ERE-halves were also earlier identified in both CYP19 promoters in medaka, goldfish, zebrafish, rainbow trout, gilthead seabream and catfish (Tanaka *et al.*, 1995; Callard *et al.*, 2001; Kazeto *et al.*, 2001; Tchoudakova *et al.*, 2001; Tong and Chung, 2003; Chang *et al.*, 2005; Kanda *et al.*, 2006; Wong *et al.*, 2006; Chapter IV). Hence, the effect of E_2 analogues on the expression of both forms of CYP19 and its activity in brain and gonads is of greater importance in the present study. By qRT-PCR analysis, there was an initial increase in the mRNA levels of both *CYP19A1* and *CYP19A2* in ovary and brain, respectively, at 50 dph but after withdrawal of the treatment there was no significant difference in the expression levels at 250 and 350 dph, when compared to

control. Similarly, increased levels of *CYP19A1* transcripts were observed in medaka and the Atlantic salmon following exposure to EE_2 (Scholz and Gutzeit, 2000; Lyssimachou *et al.*, 2006). In zebrafish, Kishida *et al.* (2001) have shown that EE_2 and DES up regulate *CYP19A2* but not *CYP19A1* and earlier studies using aromatase inhibitors for sex reversal in flat head minnow (Villeneuve *et al.*, 2006) and medaka (Tompsett *et al.*, 2009) have also suggested a negative feedback relationship between estrogen levels and *CYP19A1*. Based on these results it is clear that though ERE present in the promoter region of *CYP19A2* in catfish (Chapter IV) may not have functional role in the transcriptional regulation of *CYP19A2* in normal conditions, it might get activated under estrogen inducible conditions.

Although estrogen is generally regarded as a circulating hormone derived from the gonads, certain neural actions of estrogen are dependent on aromatization of circulating androgen to estrogen in the brain itself. Because of its relatively poor binding affinity for the plasma sex steroid binding globulin in fish (Pasmanik and Callard, 1986), DES may have greater access than E_2 to CNS target sites, as reported in rodents (McEwen, 1994) are mediated by classical nuclear estrogen receptors ER, which regulate transcription of target genes (McEwen, 1994). This can be explained by showing that DES and EE_2 can mimic E_2 induced upregulation of *CYP19A2* mRNA in zebrafish embryos (Kishida and Callard, 2001). Consistent with these results several others reported that treatment with estrogens or aromatizable androgens increases the levels of *CYP19A2* mRNA, due to the presence of ERE element in the promoter motifs of this gene (Gelinas *et al.*, 1998; Kishida *et al.*, 2001; Kazeto *et al.*, 2003, 2004; Menuet *et al.*, 2005; Andersen *et al.*, 2006; Kallivretaki *et al.*, 2006). Corroborative results of aromatase activity analysis in ovary and brain of in DES and EE_2 treated juvenile catfish belonging to similar age

groups, further validated our findings and those of others at mRNA level. Studies in several teleosts like the Nile tilapia (Tsai *et al.*, 2000), medaka (Contractor *et al.*, 2004) and zebrafish (Andersen *et al.*, 2003) showed that exposure to estrogen analogues increased aromatase activity in the respective female brains. Though our results with regard to *CYP19A2* in catfish corroborate with the earlier reports, the overall discrepancies on the external estrogens effect on *CYP19A1* gene expression seem to differ depending on the developmental stage, tissue type, species and chemicals. Nevertheless, it remains to be proved experimentally whether they can directly suppress the transcription of *CYP19A1*.

In order to check the influence of DES and EE_2 on potential transcription factors of *CYP19*'s, we analyzed *FOXL2* and *FTZ-F1* mRNA by qRT-PCR in ovary and brain, respectively. The expression remained similar to that of aromatases expression, suggesting their involvement in sex differentiation or developmental processes like that of aromatases. Furthermore, teleost *FTZ-F1* has been considered as a likely candidate to regulate several genes involved in steroidogenesis, and it has been suggested that teleost *FTZ-F1* may be involved in feminization of developing testis in response to external estrogens. No xenoestrogens have been suggested to have a specific impact on FTZ-F1-driven regulation in teleosts, but the Ad4BP/SF-1 (FTZ-F1 homologue) site was shown to be partially responsible for cAMP-stimulated transcription of human aromatase CYP19 (Michael *et al.*, 1995). Moreover, synergistic transcriptional action of Ad4BP/SF-1 and liganded ERα has been shown (Le Drean *et al.*, 1996). Thus, estrogenic mimics that can bind to ER and/ or have an impact on cAMP accumulation in the cells may also affect *FTZ-F1* regulation of *CYP19* transcription in teleosts. Further the involvement of *FOXL2* in *CYP19A1* transcription was shown to be indirect by interacting with *Ad4BP/SF-1* to form a

hetero-dimer which eventually enhances the *Ad4BP/SF-1* mediated *CYP19A1* transcription (Nakamoto *et al.*, 2006; Wang *et al.*, 2007). Hence, estrogen analogues might have a similar effect on *FOXL2* expression like that of aromatases. Taken together, this implies the possibility of direct influence of estradiol analogues on the aromatase gene expression and, consequently aromatase activity in the brain and ovary through up-regulation of expression of their transcription factors during sex differentiation. However, further studies are needed to clarify the exact mechanisms of estrogenic substances' influence on the *CYP19* expression, in order to evaluate possible consequences of estrogenic exposure with regard to these gene and their functions. Furthermore, the diminishing effect of DES and EE_2 on the expression of *CYP19s* after the withdrawal of treatment incites us for histological analysis of ovary and gonads of control and treated groups.

Histological analysis

The hematoxylin and eosin staining of ovarian sections from control fish in different age groups showed all the oocyte developmental stages whereas EE_2 treated females exhibited precocious oocyte development with more number of post-vitellogenic oocytes compared to controls, although there are earlier reports on estradiol-17β to be ineffective in inducing fish oocyte maturation (Young *et al.*, 1982; Trant and Thomas, 1988) and even inhibitory in several teleost species (Andersen *et al.*, 2003). Ovary sections of DES treated fish showed precocious oocyte maturation and atresia supporting which Baek *et al.* (2003) and Tokumoto *et al.* (2004) have earlier shown that DES can induce oocyte maturation and DES triggered germinal vesicle break down depending on the stage of oocyte development in goldfish and zebrafish. These

observations indicate that treatment of estrogen analogues DES and EE_2 were effective on expression of CYP19s, however the affect of treatment was more deletorius on developing ovaries and non-steroidal analogues like DES effect ovarian development in teleosts even with minimal dosage and exposure time.

Conclusion

Our study warrants that CYP19s (*CYP19A1* and *A2*) genes and their transcription factors, can be considered as excellent biomarkers for endocrine disruptor studies. Though estrogens can be best used for getting mono sex populations, usage of short term pulse treatments, rather than continuously, and also at lower doses needs to be considered. Treatment of estrogen analogues DES and EE_2 were effective to enhance the expression of aromatases in ovary and brain, however withdrawal of treatment brought the effect to normalcy but histologically the ovaries were never normal. Steroidal analogues are useful for the sex change studies rather than the non-steroidal analogue, as DES creates more developmental anomalies when compared to EE_2. A more detailed dose response studies would be necessary to determine if relative potencies of these compounds can be predicted by their *in vitro* ER binding affinities in fish.

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Consolidated Summary

In the present study, we examined the expression pattern of cytochrome p450 aromatase (brain form; *CYP19A2*) and two different transcription factors, namely, fushi tarazu factor 1 (*FTZ-F1*) belonging to NR5A nuclear receptor subfamily and fork head transcription factor L2 (*FOXL2*) during ovarian differentiation and development in the brain of catfish, *Clarias gariepinus*. In order to elucidate their role in ovarian recrudescence, we also analyzed the expression of these genes during reproductive cycle and after *in vivo* hCG induction by qRT-PCR. Further, we extended our study to investigate the role of these transcription factors in the regulation of *CYP19A2*. Attempts were also made to understand the effect of estradiol analogues, diethylstilbestrol (DES) and ethynylestradiol (EE_2) on the expression of *CYP19A1*, *CYP19A2*, *FTZ-F1* and *FOXL2* in the juvenile catfish during ovarian differentiation. Their effects were also studied histologically. These aspects were studied as five major chapters and the results and important findings were summarized below.

Chapter I. Cloning, expression and enzyme activity analysis of brain aromatase in the air-breathing catfish, *Clarias gariepinus*, during ovarian development, recrudescence and after *in vivo* hCG induction

In the present study, we analyzed the importance of *CYP19A2* during ovarian development and recrudescence of the North African/air-breathing catfish, *Clarias gariepinus*. We cloned *CYP19A2* (1786 bp) from the brain, which showed only 47% homology with *CYP19A1* of the catfish. Characterization of encoded protein of *CYP19A2* in non-steroidogenic COS-7 cells could efficiently catalyze the aromatization reaction by producing estradiol-17β (E_2) from testosterone. Tissue distribution pattern revealed preferential expression of *CYP19A2* in brain. Quantitative real-time PCR analysis of *CYP19A2* in brain revealed high levels of transcripts in the

prespawning phase of ovarian cycle. Ontogeny studies displayed sexual dimorphism, with an early expression of *CYP19A2* in female brain. Phase-dependent rise in the expression and enzyme activity of aromatase in brain after human chorionic gonadotropin (hCG) treatment revealed the stimulatory role of gonadotropin during preparatory and prespawning phases. Lack of influence of hCG treatment during spawning phase endorsed it further. A good correlation of expression and enzyme activity suggests a plausible role of *CYP19A2* during ovarian differentiation and ovarian cycle, either directly or indirectly, through hypothalamo-hypophyseal gonadal axis.

Chapter II. Cloning, expression and ontogeny of brain *FTZ-F1* in the catfish, *Clarias gariepinus*, during ovarian development, recrudescence and after *in vivo* hCG induction

FTZ-F1 encodes an orphan nuclear receptor belonging to the nuclear receptor family 5A (NR5A) that include adrenal 4-binding protein or steroidogenic factor-1 (Ad4BP/SF-1), Liver receptor homologue 1 (LRH-1). All these transcription factors are known to play a pivotal role in the regulation of CYP19s. Present study aimed to understand the possible role of *FTZ-F1* in regulating *CYP19A2* during development, recrudescence and after hCG induction in an annually breeding teleost model, *C. gariepinus*. To accomplish this, we cloned the full length cDNA of *FTZ-F1* from the brain of catfish and its developmental expression was analyzed. Its expression was evident during early stages of development, which showed maximum levels during gonadal differentiation in the female brain. The tissue distribution pattern both at transcript and protein levels revealed its expression to be prominent in brain along with liver, kidney and testis. The expression pattern of *FTZ-F1* during reproductive cycle and after *in vivo* hCG induction

analogous to that of *CYP19A2*, indicating its direct involvement in recrudescence. Based on our previous studies (Chapter 1) and together with present data, it is possible to implicate *FTZ-F1* as a potential transcriptional regulator of teleostean *CYP19A2*.

Chapter III. Cloning and differential expression of *FOXL2* during ovarian development and recrudescence in the catfish, *Clarias gariepinus*

FOXL2 is a member of the forkhead/HNF-3-related gene family of transcription factors which provides tissue-specific gene regulation. It is known to regulate *CYP19A1* that plays a crucial role in ovarian differentiation. To understand the role of *FOXL2* in ovarian development and recrudescence, we cloned the full-length cDNA of *FOXL2* and studied its spatio-temporal expression both at transcript and protein levels in the air-breathing catfish, *C. gariepinus*. Based on its deduced amino acid sequence, an antigenic peptide conjugated with a carrier protein was synthesized and used for raising antibody that cross-reacted specifically with FOXL2. Tissue distribution pattern of *FOXL2* revealed its presence prominently in ovary and female brain. Highest expression of *FOXL2* was observed in prespawning phase both in ovary and brain during reproductive cycle indicating an important role for this correlate in ovarian recrudescence. Treatment of hCG, *in vitro* and *in vivo*, induced the expression of ovarian *FOXL2* during preparatory and prespawning phases. Similar type of enhanced expression was evident in the brain after hCG-induction during the prespawning phase. The ontogeny of *FOXL2* showed a sexual dimorphic expression pattern in gonads and brain. Based on our previous studies, the expression pattern of *FOXL2* was found to be synchronous not only with that of *CYP19A1* but also with *CYP19A2*. Taken together, present study substantiates the role of *FOXL2* in the

regulation of CYP19s in teleosts and also designates *FOXL2* as a potential marker for ovarian differentiation in catfish.

Chapter IV. FTZ-F1 and FOXL2 synergistically up-regulate catfish brain aromatase gene transcription by specific binding to the promoter motifs

CYP19 catalyzes the conversion of androgens into estrogens. Teleosts have two *CYP19* genes, expressed specifically in ovary (*CYP19A1*) and brain (*CYP19A2*). Previous studies reported the regulation of *CYP19A1* expression by the members of NR5A nuclear receptor subfamily as well as, FOXL2. In the present study, we investigated the involvement of FTZ-F1 and FOXL2 in the regulation of *CYP19A2* expression in the brain of catfish, *C. gariepinus*. Synergistic expression of C*YP19A2*, *FTZ-F1* and *FOXL2* in the developing female brain of catfish encouraged us to isolate the upstream region of *CYP19A2* in order to analyze the regulatory motifs. Promoter motif analysis revealed NR5A1/FTZ-F1 and FOXL2 binding nucleotide sequences in the 5'-flanking region of the *CYP19A2*. Transient transfection studies showed that FOXL2 and FTZ-F1 together, enhanced the transcriptional activity of *CYP19A2* gene in mammalian cell lines, and that a mutation in either of their putative binding sites within the *CYP19A2* promoter abolished this effect. Electrophoretic gel mobility shift and chromatin immunoprecipitation assays, further confirmed the binding of both the transcription factors to their respective cis-acting elements in the upstream region. This study is the first to report the regulation of *CYP19A2* by FTZ-F1 and FOXL2, in a synergistic manner, in a teleost fish.

Chapter V. Effects of estradiol analogues, diethylstilbestrol and ethynyl estradiol on the expression of aromatases and their transcription factors in the brain and ovary of catfish, *Clarias gariepinus*, during ovarian differentiation

Sex steroid, E_2 is known to influence development and sex differentiation in teleosts. Its appropriate biosynthesis is required for reproductive success and development. Alteration in the expression of steroidogenic enzyme genes modulates E_2 production. Few transcriptional regulatory elements in the 5'flanking regions of CYP19 genes make them responsive to external estrogens and hence, vulnerable for endocrine disruption. In the present study, we treated the juvenile air-breathing catfish, *C. gariepinus* (3 days post hatch [dph]) using E_2 analogues, DES and EE_2 and changes in expression pattern of CYP19s were analyzed. Both DES and EE_2 enhanced the expression of both *CYP19A1* and *CYP19A2*, and also the aromatase activity in ovary and brain of 50 dph catfish, but after the withdrawal of treatment, this effect was subsided. Similar changes were observed in the transcript levels of *FOXL2* and *FTZ-F1*. Though the CYP19s expression and activity returned to normalcy in 250 and 350 dph fish, histologically the ovaries were never normal. The ovaries of DES and EE_2 treated catfish showed precocious oocyte maturation and atresia at an early stage. Morphologically, DES treatment showed more deleterious effects like rudimentary or underdeveloped ovaries, but growth retardation was a common phenomenon observed in both DES and EE_2 treated catfish when compared to control. Based on these results, it is possible to suggest that endocrine disruptors possessing estrogenic property can alter ovarian development in teleosts even at low dosage and minimal exposure time.

In conclusion, present study demonstrated possible involvement of *CYP19A2* in catfish ovarian differentiation and development, with sexual dimorphic expression in brain during gonadal differentiation. Marked correlation of expression with enzyme activity was evident during ovarian development and recrudescence. Further, *FTZ-F1* and *FOXL2* showed analogous expression patterns to that of *CYP19A2* in brain which was highest during the critical period of sex differentiation (35-50 dph). These observations together with the results using EMSA and ChIP clearly demonstrate the involvement of *FTZ-F1* and *FOXL2* in the regulation of *CYP19A2*. E_2 anologues, DES and EE_2 exerted remarkable increase in the expression of both forms of CYP19 and the transcription factors, *FTZ-F1* and *FOXL2* in ovary and brain of juvenile catfish during ovarian differentiation, however, the transcript levels restored after treatment withdrawal. Nevertheless, histological examination revealed that the effect of these analogues was either long lasting or deleterious for developing ovaries. Taken together, it is plausible to propose that *CYP19A2* and its related transcription factors, *FOXL2* and *FTZ-F1*, play an important role in 'brain sex differentiation' which might have been organized primarily by gonadal sex differentiation. Further, our data possibly implicate *CYP19A2* as a brain tissue marker for delineating the effect of endocrine disruptors in the central nervous system of teleosts.

Research Publications

Research publications from thesis work

Rasheeda*, M. K., Sridevi*, P. and Senthilkumaran, B. (2010) Cytochrome P450 aromatases: Impact on gonadal development, recrudescence and effect of hCG in the catfish, *Clarias gariepinus*. (2010) General and Comparative Endocrinology, 167, 234-245. (**Equal contribution*).

Sridevi, P., Senthilkumaran, B. (2011) Cloning and differential expression of *FOXL2* during the ovarian development and recrudescence in the catfish, *Clarias gariepinus*. General and Comparative Endocrinology. *(Manuscript under revision).*

Other research publications

Sreenivasulu, G., Sridevi, P., Sahoo, P. K., Swapna, I., Ge, W., Kirubagaran, R., Dutta-Gupta, A., and Senthilkumaran, B. (2009) Cloning and expression of StAR during gonadal cycle and hCG-induced oocyte maturation of air-breathing catfish, *Clarias gariepinus*. Comparative Biochemistry and Physiology, Part B, 154, 6-11.

Chaitanya, R. K., Sridevi, P., Senthilkumaran, B., Aparna-Dutta Gupta. (2011) 20-Hydroxyecdysone regulation of H-fibroin gene in the stored grain pest *Corcyra cephalonica*, during the last instar larval development**.** Steroids, 76, 125–134.

Raghuveer K., Senthilkumaran B., Sudhakumari C. C., Sridevi P., Rajakumar A., Singh R., Murugananthkumar R., and Majumdar K.C. (2011) Dimorphic expression of various transcription factors and steroidogenic enzyme genes during gonadal ontogeny in the air-breathing catfish, *Clarias gariepinus***.** Sexual Development. (*In Press*).

Sreenivasulu, G., Senthilkumaran, B., Sridevi, P., Rajakumar, A., and Rasheeda, M. K. (2011) Expression and immunolocalization of 20β-hydroxysteroid dehydrogenase during testicular cycle and after hCG induction, *in vivo* in the catfish, *Clarias gariepinus*. General and Comparative Endocrinology**.** (*Manuscript under revision*).

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Cytochrome P450 aromatases: Impact on gonadal development, recrudescence and effect of hCG in the catfish, *Clarias gariepinus*

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ABSTRACT

Present study analyzed the importance of two forms of aromatases during ovarian development and recrudescence of north African/air-breathing catfish. We cloned both *CYP19A1* (1941 bp; ovarian form) and *CYP19A2* (1786 bp; brain form), which showed 47% homology between the two forms. Characterization of encoded proteins in non-steroidogenic COS-7 cells illustrated that both isoforms efficiently catalyzed the aromatization reaction by producing estradiol-17β (E_2) from testosterone. Tissue distribution pattern revealed preferential expression of *CYP19A2* in brain while *CYP19A1* predominated in ovary with trace amounts detected in other tissues including brain. Relative real-time PCR analysis revealed high transcript levels of both isoforms in the prespawning phase of ovarian cycle, which is in accordance with serum E_2 level. Aromatase activity in brain was comparatively lower than ovary, indicating the predominant requirement of aromatase in ovary. Ontogeny studies displayed sexual dimorphism, with early expression of *CYP19A1* and *CYP19A2* in ovary and brain, respectively. Phase-dependent rise of expression and enzyme activity of aromatase after hCG treatment revealed the stimulatory role of gonadotropin during preparatory and prespawning phases, preferentially to promote vitellogenesis. Lack of influence of hCG treatment during spawning phase endorses it further. A good correlation of expression, enzyme activity and serum E_2 levels suggests a crucial role of *CYP19A1* during ovarian differentiation and ovarian cycle of catfish. Likewise, *CYP19A2* might also be involved in these processes either indirectly or directly.

1. Introduction

Cytochrome P450 aromatase (P450arom) is the endoplasmic reticular enzyme catalyzing the production of estrogens from androgens. P450arom via production of estradiol-17β (E_2) plays a potent role in preserving the continuity of life in diverse vertebrates including fishes by sustaining important physiological process, reproduction. Its role in oogenesis, sexual behavior, sex change in hermaphrodites and temperature sensitive fishes (Yamamoto, 1969; Donaldson, 1996; Chang et al., 2005) is well characterized. In addition, changes in the expression and/or activity of P450arom along with variations in serum E_2 levels have often been demonstrated during sex change and ovarian differentiation in different fish species (Tanaka et al., 1992; Kwon et al., 2001; Blazquez and Piferrer, 2004; Goto-Kazeto et al., 2004; Barney et al., 2008; Guiguen et al., 2010). Not only in fishes but also in chicken, P450arom vis-à-vis E_2 is required for ovarian development or differentiation (Smith and Sinclair, 2004). This enzyme is encoded by a single gene with multiple tissue specific promoters in both steroidogenic and non-steroidogenic tissues in vertebrates including mammals (Simpson et al., 2002). On the contrary, in teleosts existence of two P450arom (ovarian form, *CYP19A1* and brain form *CYP19A2*) is demonstrated in numerous fish species (Gelinas et al., 1998; Tchoudakova and Callard, 1998; Trant et al., 2001) which is a consequence of genome duplication (Simpson et al., 1994). These two isoforms differ in their sequences, with not more than 60% homology between them (Trant et al., 2001). In addition, both forms differ in their enzyme kinetics and also show preferential expression in brain and gonad during development and reproduction in male and female fish (Zhao et al., 2001; Kobayashi et al., 2004; Sudhakumari et al., 2005). In their physiological roles, while *CYP19A2* is implicated for neuroplasticity and neurogenesis (Forlano et al., 2001), *CYP19A1* is involved in ovarian differentiation (Kwon et al., 2001; Chang et al., 2005; Sudhakumari et al., 2005; Matsuoka et al., 2006; Blazquez et al., 2008; Esterhuyse et al., 2008) and gametogenesis (Ijiri et al., 2003; Kobayashi et al., 2004). Reports also exist from fishes belonging to varied reproductive strata on *CYP19A1*'s specific role during sex determination/differentiation (Nagahama, 2005; Ijiri et al., 2008) and regulation of vitellogenesis during reproductive cycle (Nagahama et al., 1995; Chang et al., 1997; Nakamura et al., 2005). Moreover, there is

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evidence of seasonal variation in transcript levels of *CYP19A2* in channel catfish brain (Kazeto and Trant, 2005), which is relayed via gonadotropins to ovary. However, too little data exist on the interplay of both forms of P450arom during gametogenesis with reference to gonadotropins. Interestingly, in the present study catfish *CYP19A2* expression was found to be brain-specific while in the Nile tilapia, *CYP19A1* transcript was exclusively detected in the ovary (Sudhakumari et al., 2003, 2005; Chang et al., 2005) which poses another diverse situation worthwhile to probe their specificity. Therefore, extensive information is required on both the isoforms, which could be achieved by simultaneously studying their spatiotemporal expression and enzyme activity during reproductive cycle and gonadal differentiation. Such analysis will assist in garnering data whether *CYP19* genes displayed any disparity in their expression pattern vis-à-vis activity during gametogenesis and whether they could be used as candidate marker for ovarian development in the north African/air-breathing catfish. To accomplish this, we initially started with cloning cDNAs of P450arom and studied their expression profiles and enzyme activity during gonadogenesis and ovarian cycle in brain and ovarian tissues, which were collected from the same fish. The analysis was also correlated with serum E_2 levels for seasonal study. We also explored the functional correlation between the two isoforms after *in vivo* induction using human chorionic gonadotropin hormone (hCG) during ovarian recrudescence, using quantitative real-time PCR and radiometric enzyme assay.

2. Materials and methods

2.1. Animals

Male and female juveniles of *Clarias gariepinus* belonging to different age groups were obtained from our laboratory aquaculture facility by breeding and rearing. The hatchlings were kept in the glass water tanks supplied with circulating filtered water under natural photoperiod and ambient temperature. They were fed with live feed, i.e., tubeworms *ad libitum* till they grew to fingerling size (~5–6 mm). Later on they were given goat liver or pelleted fish food till adult. Maintenance of adult fish and its annual reproductive cycle were described earlier (see Sreenivasulu and Senthilkumaran, 2009). In brief, the various phases of female reproductive (annual/seasonal) cycle namely preparatory, prespawning, spawning and resting were closely followed by catfishes (adult) maintained more than a year in outdoor tanks under natural conditions. Gonadal and brain tissues from various age group fish as well as from adult at different phases were collected by sacrificing them (decapitation) after immersing in ice-cold (mild) water. Before sacrificing the adult fish blood was collected by caudal puncture, for estimation of serum E_2 levels by using E_2 Enzyme-linked immuno assay (EIA) kit (Cayman, Ann Arbor, MI, USA) as described later. The tissues and serum samples were stored at −80 °C before being assayed.

2.2. Total RNA isolation and 1st strand cDNA synthesis

Total RNA from the gonad and brain of adult and juvenile catfish was isolated using Sigma TRI-reagent (Sigma, St. Louis, MO, USA). The quality and concentration of total RNA was assessed by using NanoDrop (ND-1000, NanoDrop Technologies, Wilmington, Delaware, USA) spectrophotometer and checked in formaldehyde agarose gel. Then, the total RNA was reverse transcribed to obtain first-strand cDNA template using Superscript III (Invitrogen, Carlsbad, CA, USA) and oligo-dT_{18} primers following the manufacturer's instructions. The efficiency of the transcription was checked by performing a PCR for *β-actin*, a constitutively expressed gene.

2.3. Molecular cloning of catfish CYP19A1 and CYP19A2

A set of degenerate primers were designed from conserved region by aligning the existing sequences of teleost *CYP19* using Lasergene software, (release 3.05; DNASTAR, Madison, WI, USA) which yielded partial cDNA fragments of 268 bp and 264 bp from ovary and brain tissues, respectively. These were then cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and sequence was determined. The identity of amplified partial cDNA was analyzed by NCBI-BLAST.

2.4. Construction of cDNA libraries and screening

Brain and ovarian cDNA libraries of catfish were constructed as per the method described earlier (Senthilkumaran et al., 2002). Briefly, total RNA from brain and ovary of catfish were prepared using Sigma TRI-reagent. Using ~1.5 mg of total RNA, mRNA was prepared with oligotex-mRNA kit (Qiagen, GmbH, Germany). Using 5 μg of mRNA, cDNA synthesis was carried out using a Stratagene cDNA synthesis kit (Stratagene, Cedar Creek, TX, USA). Ends of cDNA were blunted and ligated with EcoRI adaptors using T4 DNA ligase and phosphorylated by polynucleotide kinase. Then the cDNA was size fractionated using Sepharose CL-4B columns (Amersham, Buckinghamshire, England). Fractionated cDNAs were ligated with UNI-ZAP-XR vector and packaged into lambda phage heads using Gigapack II–gold packaging extract kit (Stratagene). Screening of the cDNA libraries (separately for ovary and brain) for *CYP19* was performed using RT-PCR amplified cDNA fragments as probes, homologous to *CYP19A1* and *CYP19A2*, which were radiolabelled with ^{32}P-dCTP using random primer labeling kit (Perkin Elmer, Boston, MA, USA). After three rounds of screening, positive clones were isolated by single clone excision. The pBluescript phagemids obtained after excision were sequenced bi-directionally by ABI prism capillary 316 DNA sequencer and the identity was analyzed by NCBI-BLAST.

2.5. Rapid amplification of cDNA ends (RACE)

RNA-ligase mediated RACE system (Invitrogen, Carlsbad, CA, USA) was used to clone the 5′ and 3′ terminal sequences of *CYP19A1*. Using 5′ and 3′ RACE templates, RT-PCR was performed, using gene-specific primers designed from truncated *CYP19A1* clone obtained from cDNA library screening as per the manufacturer's protocol. The sequences of gene-specific primers used for 5′ and 3′ RACE including the degenerate primers (DF1 and DR1) are listed in Table 1. The RACE products obtained were cloned in pGEM-T easy vector, sequenced bi-directionally and their identities analyzed by NCBI-BLAST search.

2.6. Functional characterization of catfish CYP19A1 and CYP19A2 expressed in COS-7 cells

Functional characterization of *CYP19A1* and *CYP19A2* in COS-7 cells was performed following the method described earlier (Chang et al., 2005). In brief, about 3×10^5 COS-7 cells were plated on 6-cm tissue culture plate holding 4 ml of DMEM with or without (during transfection) 10% (v/v) fetal calf serum and grown at 37 °C in 5% CO_2 until confluent. After confirming the sequence integrity of the *CYP19A1* and *CYP19A2* ORFs in pCDNA3.1$^+$ TOPOV5-His mammalian expression vector (Invitrogen), they were transfected in COS-7 cells using Tfx20 (Promega) reagent. Mock pCDNA3.1 (ORF insert locked in reverse direction) was used as a negative control. After 24 h incubation, 30 nM of testosterone was added as substrate to the cells. Subsequently after 24 h, the culture medium was separated from cells by centrifugation at 500*g*, followed by extraction twice with diethyl ether and evapora-

Table 1
Primers used for cloning and real-time PCR expression analysis of *CYP19A1* and *CYP19A2* in air-breathing catfish.

S. No.	Primer	Sequence 5′–3′	Purpose
1	DF1	5′-TGGWYKGGNATHGGBACDGC-3′	To amplify a fragment
2	DR1	5′-GGVCCDGTBARVGCTTTRG-3′	To amplify a fragment
3	3P	5′-GCACTCTCATCTCCCTCAGCTCCACGTT-3′	3′ primary RACE primer for ovarian form
4	3N	5′-GCATGCGCCGGGCCCTGGATGATGATG-3′	3′ nested RACE primer for ovarian form
5	5P	5′-CTCCTCCCCACAGATCCACACTCGCA-3′	5′ primary RACE primer for ovarian form
6	5N	5′-AGACACTGCAGCCCGACT TTACTC CCG-3′	5′ nested RACE primer for ovarian form
7	RTfo	5′-AGGTCCCTGGTTTTGTCTG-3′	qRT-PCR for ovary
8	RTro	5′-TGCAGATGGCCTGCTGAGG-3′	qRT-PCR for ovary
9	RTfbr	5′-CCA GGT CCA TAC TGG TTA CTG-3′	qRT-PCR for brain
10	RTrbr	5′-CAC AGC AGA TGA CTT GCT TAG-3′	qRT-PCR for brain
11	*β-actin* – Fw	5′-ACCGAAGTCCATCACAATACCAGT-3′	qRT-PCR
12	*β-actin* – Rv	5′-GAGCTGCGTGTTGCCCCTGAG-3′	qRT-PCR

tion in vacuum centrifuge. The steroids were reconstituted in 100 μl EIA buffer supplied in E_2 EIA kit (Cayman, Ann Arbor, MI, USA). The E_2 produced in the culture medium was measured using EIA kit for E_2 (Cayman) according to the manufacturer's protocol. A recovery of 93–95% was obtained by the extraction procedure described in E_2 EIA kit. Cross-reactivity of the E_2 antisera to estradiol-3-glucoronide was (14%), estrone (12%), estradiol-17-glucoronide (10%), estriol (0.3%), 5α-dihydrotestosterone (0.06%), testosterone (<0.01%). The minimal detection threshold was 20 pg/ml for E_2. After measurements, the conversion rates were calculated and the values of cross-reactivity (testosterone) were subtracted. Intra- and inter-assay coefficient of variations were (n = 5; mean ± SEM) 1.36 ± 0.04% and 1.75 ± 0.06%, respectively, for E_2 produced from *CYP19A1* transfected plate and 1.38 ± 0.12% and 1.65 ± 0.05%, respectively, for E_2 produced from *CYP19A2* transfected plate. Results were expressed as mean ± SEM of three replicates each from three independent analysis. Data analysis was carried out using one-way ANOVA followed by Dunnett's test. Significance was accepted at $P < 0.05$.

2.7. Quantitative RT-PCR (qRT-PCR) for CYP19A1 and CYP19A2 during seasonal/reproductive cycle of catfish

Expression of *CYP19A1* and *CYP19A2* was analyzed by qRT-PCR. To study the expression pattern during different phases, total RNA (5 μg) was obtained by dissecting the ovary and brain tissues separately from five fish (n = 5). Gene-specific primers were designed for *CYP19A1* and *CYP19A2* in intron–exon boundaries by aligning the existing sequences. For *CYP19A1*, the forward primer was located in exon II and the reverse primer was located in intron-exon boundary of exon II and III and for *CYP19A2*, forward primer located in intron-exon boundary of exon I and II and reverse primer located in intron-exon boundary of exon II and III. Absence of genomic DNA contamination in the RNA was confirmed by using non-reverse transcribed samples as templates. In addition, absence of DNA in total RNA was ensured by treating with DNAse I before proceeding for 1st strand cDNA synthesis. Primers for *β-actin* used as endogenous control were designed from *β-actin* sequence obtained from catfish which is listed in Table 1. Primer specificity for each primer pair was confirmed by cloning and sequencing the amplicon followed by dissociation curve analysis. Real-time PCR was performed on an ABI Prism® 7500 fast thermal cycler (Applied Biosystems, Foster, CA, USA) using SYBR Green 1 (Applied Biosystems). Each sample was run in triplicate in a final volume of 25 μl containing 0.3 μl of cDNA, 10 pmol of each primer, and 12.5 μl of Power SYBR® Green PCR master mix. During PCR, fluorescence accumulation resulting from DNA amplification was analyzed using the sequence detector software (Applied Biosystems). Comparative C_T method was used to quantify the target genes abundance. RQ Manager 1.2 (Applied Biosystems) was used to compile data from all plates and compare expression levels. Transcript abundance of both the genes were normalized to that of *β-actin* and reported as fold change in abundance relative to the values obtained in preparatory phase using $2^{-\Delta\Delta C_T}$ method. For each PCR run, a no-template control was included to ensure perfect results. Data analysis was carried out using one-way ANOVA followed by Tukey's test. The results were expressed as mean ± SEM of three replicates. Significance was accepted at $P < 0.05$.

2.8. Changes in aromatase activity in brain and ovary at different phases of female seasonal/reproductive cycle

The aromatase activity in brain and ovarian tissues of female catfish collected at different phases of ovarian growth was assayed as per the method described (Tsai et al., 2000) with minor modifications. Briefly, microsomes from the above-mentioned tissues were prepared by homogenizing 2 g of tissue in 3 ml of 100 mM potassium phosphate buffer pH 7.4 (KPO_4 buffer) followed by centrifugation at 9000*g* for 20 min at 4 °C to clear debris, and a final spin at 105,000*g* for 1 h at 4 °C. The microsomal pellet was then rinsed with buffer and resuspended in 500 μl of KPO_4 buffer/0.1 mM EDTA/20% glycerol (v/v). Aromatase activity was measured by incubating 300 μg of microsomal protein and 0.6 μM of 1β-[^{3}H]androstenedione (Amersham) in 500 μl of 100 mM KPO_4 (pH 7.4), 1 mM EDTA, 1 mM β-$NADP^+$, 5 mM glucose-6-phosphate and 10 U of glucose-6-phosphate dehydrogenase at 37 °C for 1 h in a shaker incubator. The reaction was stopped by adding iced 10% trichloroacetic acid containing 20 mg dextran-coated activated charcoal/ml. After centrifugation at 1000*g* for 10 min at 4 °C, the supernatant was collected in scintillation vials containing 3 ml of cocktail-T (SRL Chemicals, Mumbai, Maharashtra, India). The radioactivity was measured using Wallac 1409-liquid scintillation counter (Pegasus Scientific Inc., Rockville, MD, USA). Data analysis was carried out using one-way ANOVA followed by Tukey's test. The entire procedure was repeated thrice and the results expressed as mean ± SEM of three replicates for each tissue sample (brain and ovary) collected individually from five fish (n = 5). Significance was accepted at $P < 0.05$.

2.9. qRT-PCR for CYP19A1 and CYP19A2, and aromatase activity in gonads and brain of developing catfish

Expression pattern of both forms of aromatases were assessed in developing male and female catfish by real-time PCR. The brain and gonadal tissues were dissected out using fine forceps under a stereo-zoom microscope from developing juvenile catfish (Leica, Wetzlar, Germany), after morphological (also histological) identification of testis and ovary at 45, 60, 75, 100 and 150 days post-hatch (dph), under sterile conditions. From histological observation it was evident that the critical period of sex differentiation was around 35–

50 dph when the gonads get morphologically distinguishable in air-breathing catfish (Raghuveer and Senthilkumaran, 2009). Hence, we performed the ontogeny study from 45 dph, which permitted us to unambiguously identify and isolate the ovary and testis from the catfish juveniles for expression and enzyme activity analysis on aromatase. For ontogeny studies (45, 60, 75, 100 and 150 dph), the brain and gonadal tissues from 20 to 25 juveniles were dissected and pooled to have five biological samples (brain/ovary/testis tissue from 4 to 5 juvenile fish constitute one sample, $n = 5$) for total RNA preparation (2.5 μg). For aromatase activity analysis 20–25 (ovaries) and 10–15 (brains) were pooled to have five biological samples (pooling of ovaries from 4 to 5 juvenile fish constitute one biological sample and pooling of brains from 2 to 3 juvenile fish constitute one biological sample $n = 5$) as per their age. The tissue samples were then immediately snap-frozen in liquid N_2 and kept briefly at −80 °C for subsequent analysis.

2.10. RT-PCR and qRT-PCR analysis of tissue distribution pattern of CYP19A1 and CYP19A2

Five micrograms of total RNA was isolated from different tissues and reverse transcribed to first-strand cDNA using Superscript III (Invitrogen) reverse transcriptase. PCR reaction using specific (Sp) primers designed for *CYP19A1* (Sp Fwd: 5′-TTC AAC TCC AAC GTC GCC C-3′, Sp Rev 5′-TAC CTG TGG ATT TTG GAC AC-3′) located in exon III and IV, respectively, and *CYP19A2* (Sp Fwd: 5′-CCA GGT CCA TAC TGG TTA CTG-3′, Sp Rev: 5′-CAC AGC AGA TGA CTT GCT TAG-3′), forward primer located in intron- exon boundary of exon I and II and reverse primer located in intron-exon boundary of exon II and III. The PCR was carried out at 94 °C for 45 s, 60 °C for 30 s, and 72° for 1 min for 30 cycles using a dual-block thermal cycler ABI 9700 (Applied Biosystems, Foster, CA, USA). The tissue distribution pattern was reconfirmed using qRT-PCR as per the method described above using primer sets mentioned in seasonal/reproductive cycle expression analysis of *CYP19A1* and *CYP19A2*.

2.11. hCG-induced in vivo study of expression and activity of cyp19 gene

Fish were collected during different phases, i.e., February (early preparatory), end of May (early prespawning), September (late spawning) and injected with hCG (1000 IU/kg body weight; Pubergen, Uni-Sankyo, Hyderabad, India), intraperitoneally. Ovary and brain tissues from individual fish at every 4 h up to 12 h were collected by sacrificing the fish after exposing it to ice-cold water. The expression and activity analysis of *CYP19* following hCG induction were performed as elucidated above. Present experiment was conducted using different batches of female catfish ($n = 5$) for each time point (4/5 time points using 20/25 fish). All the data were expressed as mean ± SEM. Significance among group was tested by ANOVA followed by Student's–Newman–Keul's test. Difference among groups were considered significant at $P < 0.05$.

3. Results

3.1. Molecular cloning of catfish CYP19A1 and CYP19A2

A set of degenerate primers yielded a partial cDNA of 268 bp (ovary) and 264 bp (brain) whose sequence identity was confirmed by NCBI-BLAST. The ovarian cDNA library screening yielded three truncated clones of *CYP19A1* gene subsequently, to obtain full-length cDNA of *CYP19A1*, 5′, 3′ RACE strategy was employed using gene-specific primers designed from partial cDNA fragments. The full-length *CYP19A1* cDNA obtained from the ovary of catfish was 1941 bp in length with 97 bp 5′untranslated region (UTR), 293 bp 3′ UTR and ATAAA as polyadenylation signal that is 47 bp upstream of poly-A tail. The open reading frame (ORF) is 1551 bp long, encoding a putative enzyme of 517 amino acids (aa). *CYP19A2* full-length clone was obtained from brain cDNA library (6 identical clones) using radiolabelled partial cDNA as probe. The *CYP19A2* cDNA was 1786 bp in length with 1494 bp ORF, 180 bp 5′ UTR, 112 bp 3′ UTR and AATAA as polyadenylation signal present 53 bp before the poly-A tail. The *CYP19A2* cDNA encoded a protein containing 498 aa. ClustalW multiple alignments revealed the presence of conserved signature domains which includes the heme binding region, the I-helix region and the aromatase specific sequence in both isoforms plus a membrane spanning region at the N-terminal side typical of the brain form among teleosts. Both *CYP19* genes of catfish specifically displayed high homology in the signature domain regions with P450arom cloned from other teleosts. The nucleotide sequences of *CYP19A1* and *CYP19A2* were deposited in GenBank (Accession Nos.: *CYP19A1*: GU220075 and *CYP19A2*: GU220076). Phylogenetic analysis by neighbor-joining method revealed that catfish *CYP19A1* has about 73–86% homology with other piscine ovarian form while the *CYP19A2* exhibited 72–88% homology with other teleostean brain aromatase (Fig. 1). In spite of the presence of high consensus in their conserved domains, both forms shared only 47% homology between them.

3.2. Functional characterization in COS-7 cells

The transiently expressed ORFs of *CYP19A1* and *CYP19A2* in COS-7 cells were able to transform the substrate testosterone to E_2 demonstrating that the inserted cDNAs in mammalian expression vector pcDNA3.1 were indeed functional possessing the property to aromatize C19 androgens to C18 estrogens. The catalytic efficiency was expressed as percentage conversion of testosterone to E_2. Mock-transfected cells of both forms (obtained by reverse orientation of the ORF in the expression vector) showed no significant production of E_2 from testosterone. The percentage conversion of the substrate testosterone to E_2 was 42% and 38% by *CYP19A1* and *CYP19A2*, respectively (Fig. 2).

3.3. Phase-dependent (seasonal/reproductive cycle) expression and enzyme activity of CYP19A1 and CYP19A2 in ovary and brain

The ovarian form, *CYP19A1* transcript was abundantly expressed in the prespawning phase followed by a sharp decline in the spawning and regressed phases (Fig. 3A). Analysis of ovarian aromatase activity during different phases of ovarian cycle was in accordance with the mRNA levels (Fig. 3B). The brain form, *CYP19A2* also exhibited maximum transcript levels in the prespawning phase when compared to preparatory, spawning and regressed phases (Fig. 3A). Aromatase activity in brain tissue corroborated well with the *CYP19A2* transcript levels (Fig. 3B). Enzyme activity in brain was relatively low when compared to ovarian samples though the transcript level of *CYP19A2* was higher than that of *CYP19A1* in the prespawning phase. Serum E_2 levels followed seasonal variation peaking in the prespawning phase (Fig. 3C), further supporting the results of *CYP19* transcripts and aromatase activity during different phases of ovarian cycle.

3.4. Ontogeny of aromatase expression and activity

Expression of *CYP19A1* was prominent in ovary and female brain (Fig. 4A). In male brain and testis, relatively low levels of *CYP19A1* were detected from 60 dph. Real-time PCR analysis at 45, 60, 75,100 and 150 dph in juvenile catfish revealed early onset of *CYP19A2* transcript in female brain from 45 dph (Fig. 4B) with levels gradually increasing. Transcript of *CYP19A2* in male brain was detected only from 100 dph. On the other hand, no expression

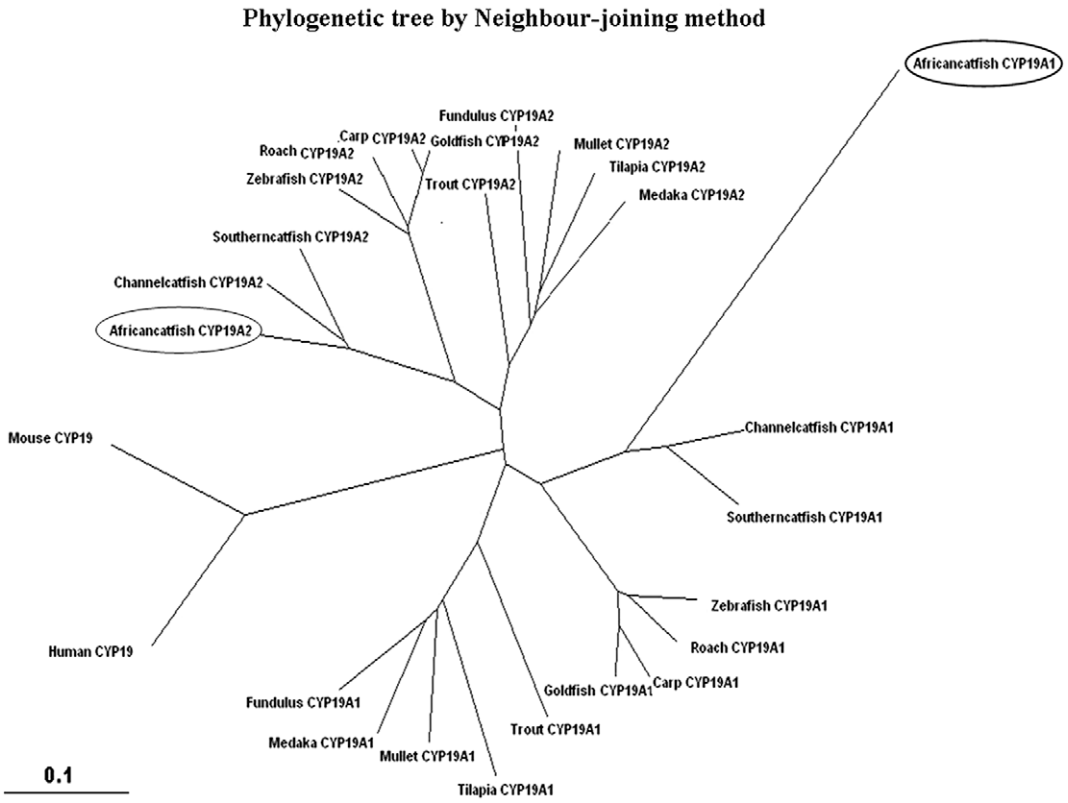

Fig. 1. Phylogenetic tree showing the evolutionary relationship of catfish *CYP19A1* (GU220075) and *CYP19A2* (GU220076). Bootstrap analysis with 1000 replicates was used to assess the strength of nodes. Phylogenetic analysis was done using TreeView software package version 1.4. *CYP19A1* accession numbers: African catfish GU220075, Channel catfish S75715, Southern catfish AY325908, Zebrafish AF226620, Roach AB190291, Mullet AY859425, Medaka D82968, Tilapia U72071, Fundulus AY428665, Trout AB210815, Carp EU375455, Goldfish AB009336. *CYP19A2* accession numbers: African catfish GU220076, Channel catfish AF417239, Southern catfish AY325907, Zebrafish AF226619, Roach AB190292, Mullet AY859423, Medaka AY319970, Tilapia AF472621, Fundulus AY428666, Trout AJ311938, Carp EU375456, Goldfish U18974, Human CYP19 NM000103, Mouse CYP19 NP031836.

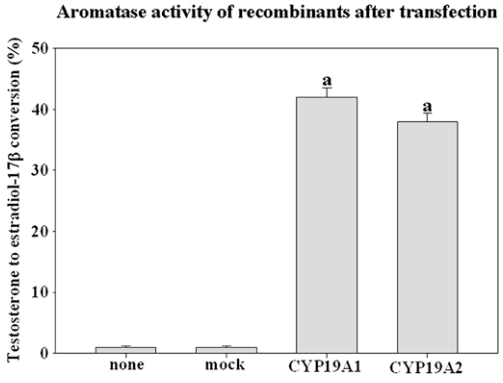

Fig. 2. Representative histogram showing the % conversion of testosterone to estradiol-17β by CYP19A1 and CYP19A2 recombinant protein. Common letters indicate means that are not significantly different ($P < 0.05$).

of *CYP19A2* was detected in the ovary however faint expression was spotted in testis from 60 dph. The enzyme activity analysis demonstrated elevation in ovarian aromatase activity in age-dependent manner (Fig. 4C). In female brain, the aromatase activity was high (Fig. 4D) when compared to male brain during early development in catfish. It also showed an increase in age-dependent manner.

3.5. Tissue distribution of CYP19A1 and CYP19A2

Using RT-PCR analysis, *CYP19A1* expression was detected in several tissues other than ovary including brain, spleen, liver, intestine, kidney, heart and muscle. The expression was however predominant in ovary, (Fig. 5A1). Expression of *CYP19A2* was detected only in brain (Fig. 5A2). The tissue distribution pattern using qRT-PCR analysis (Fig. 5B1 and B2) further endorsed the specific distribution of *CYP19A1* (ovary and other tissues including brain) and *CYP19A2* (brain-specific).

3.6. Expression and activity of CYP19A1 and CYP19A2 in female catfish during preparatory, prespawning and spawning phases after in vivo induction using hCG

In vivo hCG treatment during the preparatory phase resulted in significant increase of *CYP19A1* transcripts and enzyme activity from 8 h with subsequent rise measured up till 24 h, when compared to control ovarian samples (Fig. 6A and B). In the prespawning phase, induction with hCG demonstrated a rapid increase in *CYP19A1* transcript levels and aromatase activity right from 4 h reaching a maximum value at 8 h followed by drop at later time points (Fig. 7A and B). The hCG treatment up-regulated *CYP19A2* expression and aromatase activity in the brain of preparatory phase catfish from 8 h up to 24 h (Fig. 6A and B) while in the prespawning phase alike ovarian form, the brain form demonstrated a peak in transcript level at 8 h, surpassing the *CYP19A1* expression measured in the prespawning ovary (Fig. 7A). However, the enzyme activity analysis in the prespawning phase demonstrated considerably higher activity in ovarian tissue than brain (Fig. 7B) contrary to the high *CYP19A2* transcript level observed at different time points relative to the ovarian form during prespawning phase (Fig. 7A). There was no significant change in the transcripts of both

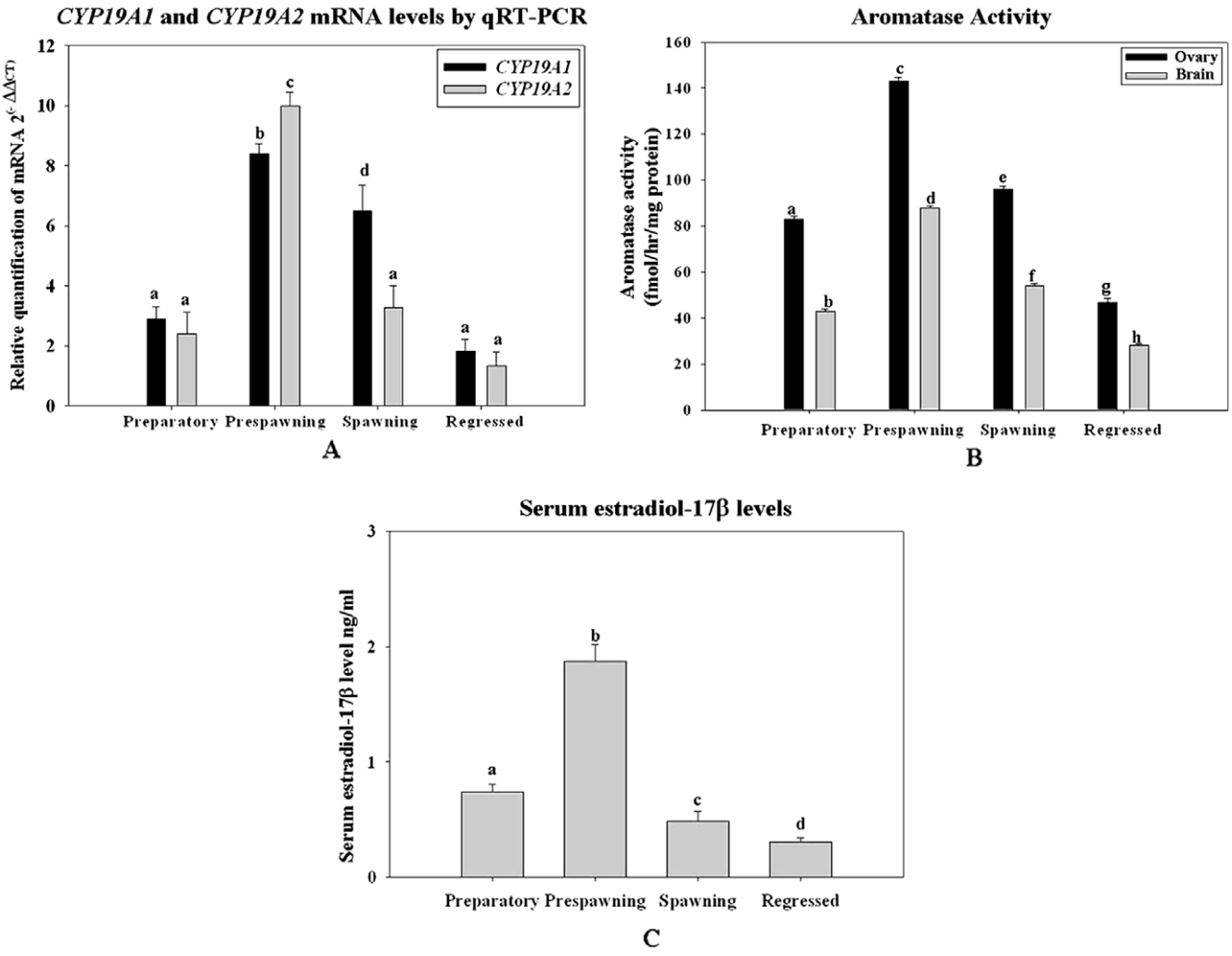

Fig. 3. (A) Changes in transcript levels of *CYP19A1* (from ovary) and *CYP19A2* (from female brain) by real-time PCR (qRT-PCR) and (B) aromatase activity in ovary and brain, respectively, and (c) serum estradiol-17β at different phases of ovarian cycle, preparatory, prespawning, spawning, and regressed. Means with different letters differ significantly and compared group-wise ($P < 0.05$).

isoforms and enzyme activity after hCG induction in the spawning phase (Fig. 8A and B).

4. Discussion

Various approaches have been attempted to validate the significance of E_2 and *CYP19A1* during ovarian differentiation and oogenesis across vertebrate phyla including teleosts. There are also increasing evidences from different fish species supporting the involvement of the brain form, *CYP19A2* in neuronal differentiation, synaptogenesis and sexual behavior (see Diotel et al., 2010). Previous independent studies, from diverse groups of teleosts that displayed varying reproductive strategies, i.e., gonochoristic, sequential hermaphrodites and true hermaphrodite mostly marine, have furnished information about the promoters/structure of *CYP19* genes, their transcript levels and/or their enzyme activity in the ovary and brain during embryogenesis, gonadal development and female reproductive cycle to discern their specific contribution (see Piferrer and Blazquez, 2005). Nevertheless, a comprehensive approach on both the isoforms of *CYP19* has not been attempted so far in a teleostean fresh water annual breeder that exhibits distinct seasonal/reproductive cycle. Furthermore, radiometric assay adopted in the present study for measuring aromatase activity is better when compared to the method adopted by Chourasia and Joy (2008), where the limitation of assay was end product E_2 measurement which may not take into account the loss of E_2 conversion if any, by E_2 converting enzymes (estrogen-2/4-hydroxylases). In the present study, we focused on elucidating the contributions of the brain and ovarian form of aromatase during ovarian differentiation and gametogenesis. This was accomplished by initially analyzing the transcript and/or activity of *CYP19* isoforms in brain, ovary and testis of juvenile catfish from 45 dph up till the catfish reached 150 dph, followed by monitoring, the transcript levels (by qRT-PCR), the aromatase activity in the brain and ovarian tissues in relation to the pattern of serum E_2 levels during different phases. Lastly, we also tried to study the responsiveness of the *CYP19A1* and *CYP19A2* transcript levels and enzyme activity after hCG (non-homologous gonadotropin) induction at different phases of ovarian cycle, to substantiate our findings that the production of E_2 might be under the control of gonadotropins. On the whole, the present work virtually tried to deal with the various parameters that contributed in the maintenance of female reproductive cycle (mRNA, terminal enzyme (aromatase) activity and its modulation by gonadotropins) leading to the production of E_2 thereby demonstrating the possible contribution of *CYP19s* either directly or indirectly in ovarian growth and folliculogenesis.

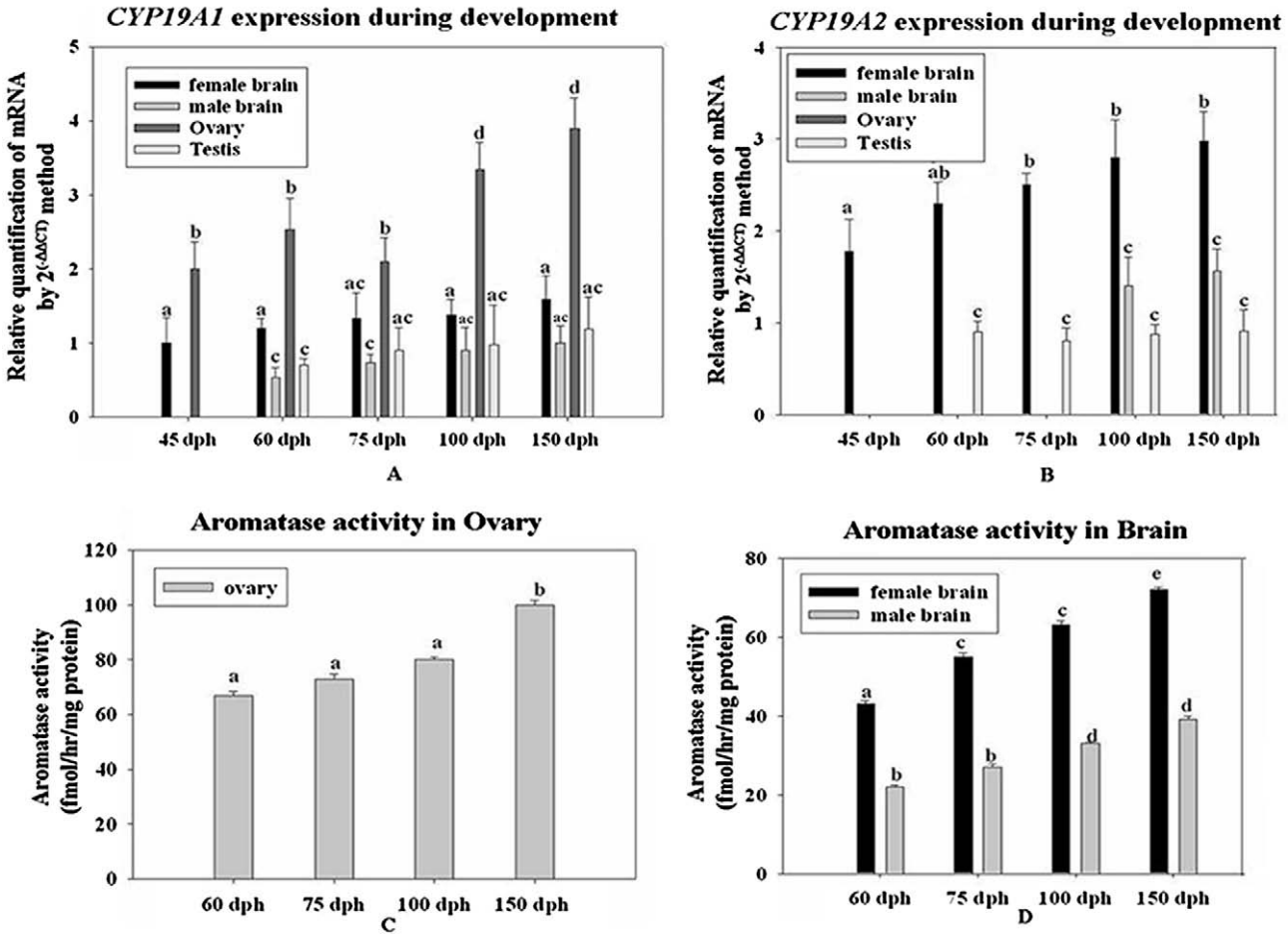

Fig. 4. (A–B) Real-time PCR analysis of temporal expression pattern of *CYP19A1* and *CYP19A2* in juveniles at 45, 60, 75, 100 and 150 dph in gonad and brain of male and female catfish and (C–D) enzyme activity of aromatase at 60, 75,100 and 150 dph in ovarian and brain tissues, respectively. Means with different letters differ significantly ($P < 0.05$) and compared group-wise. Common letters indicate means that are not significantly different. Note that the expression of *CYP19A2* is detected in the juvenile testis in addition to brain during development.

4.1. Cloning, phylogenetic analysis and functional characterization of cloned cDNAs of CYP19s

The inception of the study was by cloning the cDNAs of both the forms of aromatase and the results obtained revealed that unlike many teleosts, i.e., goldfish (Tchoudakova and Callard, 1998), zebrafish (Kishida and Callard, 2001) and channel catfish (Trant, 1994; Kazeto and Trant, 2005), in air-breathing catfish (present study), the transcript length of brain form (*CYP19A2*: 1786 bp) is shorter than the ovarian form (*CYP19A1*: 1941 bp) although the protein encoded by the brain form followed the usual norm of being 18 aa lesser than the ovarian form. Phylogenetic analysis revealed that these two paralogous genes share 47% homology, indicating that they have evolved separately after genome duplication, albeit preserving a greater identity in their functional domains thus reinforcing the essential role of these regions. The ovarian form (*CYP19A1*) exhibits a separate branch within the siluriforms clade in the distance tree. Similarly the brain form (*CYP19A2*) also shares greater identity with brain form of siluriforms. Transiently expressed CYP19 protein in non-steroidogenic mammalian COS-7 cells catalyzed the aromatization of testosterone to E_2 indicating that the cloned cDNAs indeed code for P450arom. Interestingly, the efficiency of ovarian form (*CYP19A1*) was higher than the brain form (*CYP19A2*), which is in accordance with P450arom cloned from the Nile tilapia (see Chang et al., 2005).

4.2. Phase-dependent (seasonal/reproductive cycle) expression and enzyme activity in ovary and brain in adults

Expression of both brain and ovarian aromatases correlated well with the activity and the plasma E_2 levels in, *C. gariepinus*. The *CYP19A1* expression in channel catfish also correlated with plasma E_2 levels (Kumar et al., 2000). The transcript levels and enzyme activity precipitously increased during ovarian recrudescence, i.e., from preparatory phase to prespawning phase, a phase when the ovaries were filled mostly with vitellogenic and early post-vitellogenic oocytes, in the synchronous spawning catfish, followed by a steep decline in the transcripts and activity of aromatase as the oocyte ensued maturation. A similar result of seasonal variation in *CYP19A1* transcript peaking just prior to oocyte maturation was observed in zebrafish (Goto-Kazeto et al., 2004). With respect to *CYP19A2*, the transcript levels and enzyme activity exhibited seasonal variation peaking at prespawning phase, although its activity was low when compared with the aromatase activity in ovary. Similar seasonal pattern of *CYP19A2* transcript levels was reported in goldfish and channel catfish with mRNA levels peaking prior to the spawning phase (Gelinas et al., 1998; Kazeto and Trant, 2005). We substantiate our findings on synchronous seasonal pattern of *CYP19A1* and *CYP19A2* with the previous report (Kazeto and Trant, 2005) in channel catfish that demonstrates the significance of *CYP19A2* in regulating the gonadotropin levels di-

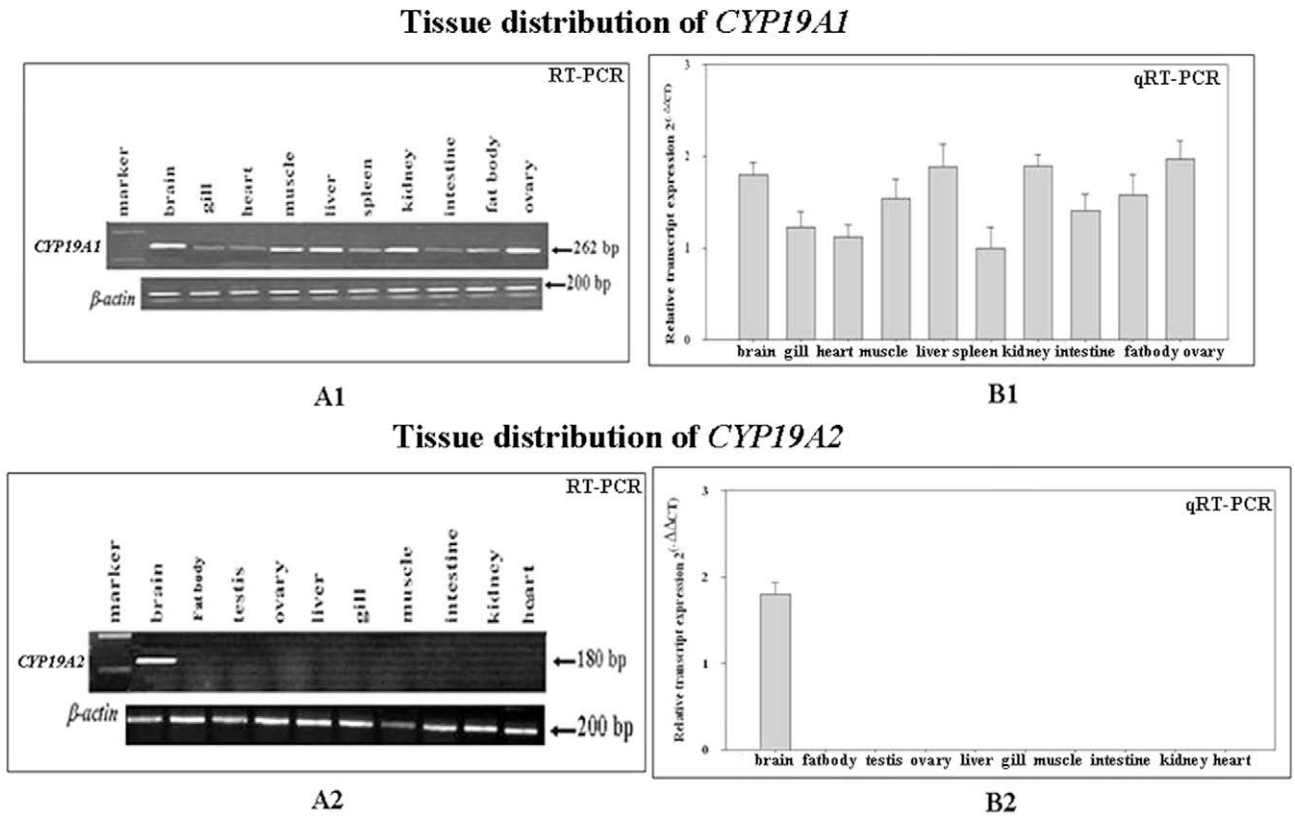

Fig. 5. Tissue distribution pattern by RT-PCR and qRT-PCR analysis was done for *CYP19A1* (A1 and B1, respectively) and *CYP19A2* (A2 and B2, respectively) in different tissues.

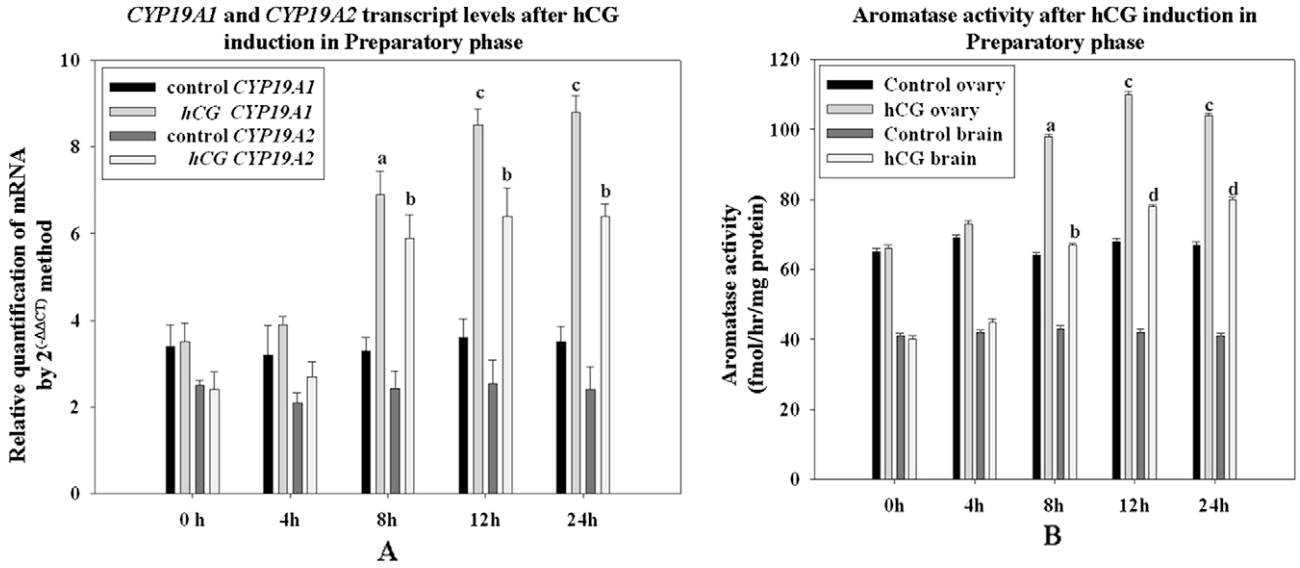

Fig. 6. (A) Real-time PCR analysis of *CYP19A1* and *CYP19A2* expression. (B) Enzyme activity of aromatase at preparatory phase after hCG induction. Means with different letters differ significantly ($P < 0.05$; group-wise comparison) and others are not significant.

rectly or indirectly by local production of estrogen in brain, thus implying a plausible role of brain aromatase in the brain–pituitary–gonadal axis. On the contrary in goby, significant correlations between isoforms-specific transcript levels and aromatase activity were observed in ovary but not in brain (Kobayashi et al., 2004). Further, there are also evidences supporting the low aromatase activity observed in catfish brain when compared to ovary reported in few other fish species viz., *Chasmichthys dolichognathus* (Agohaze), *Leucopsarion petersii* (Shiro-yu), yellowfin goby, rainbow trout and coho salmon (Sasaki and Asahina, 2006). Alternatively high catalytic activity of aromatase in fish brain compared to ovarian tissue is also demonstrated in goldfish (Pasmanik and Callard, 1988; Zhao et al., 2001). Further the fall in enzyme activity of aromatase in *C. gariepinus* ovary during the regressed phase was less pronounced than their northern counterparts (*Heteropneustes fossilis*). Same is true for serum E_2 level. This may be essentially due to

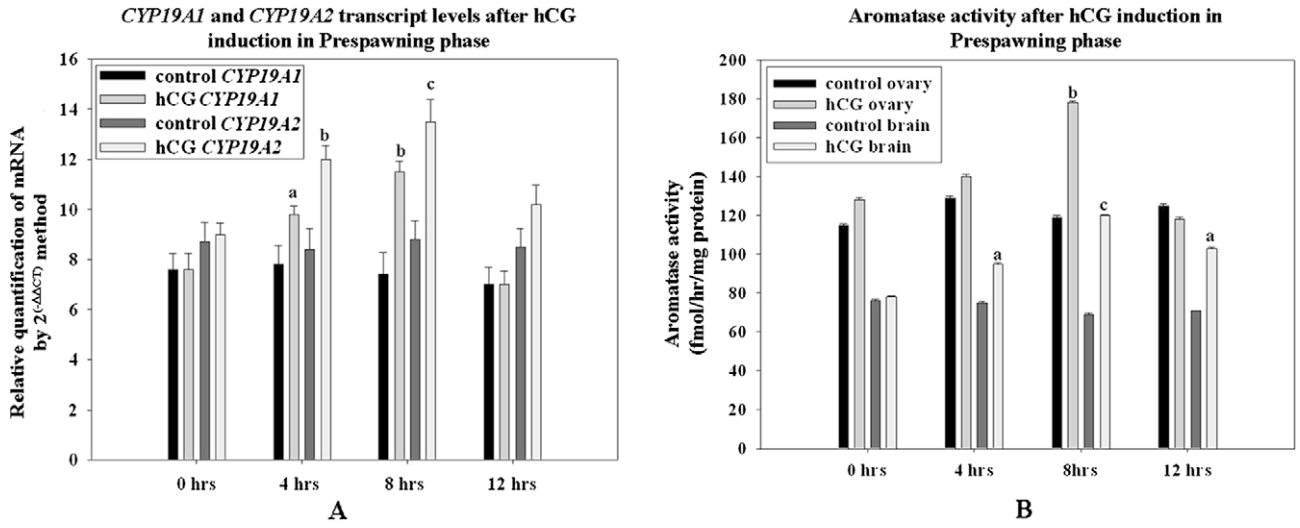

Fig. 7. (A) Real-time PCR analysis of *CYP19A1* and *CYP19A2* expression. (B) Enzyme activity of aromatase at prespawning phase after hCG induction. Other details are as in Fig. 6.

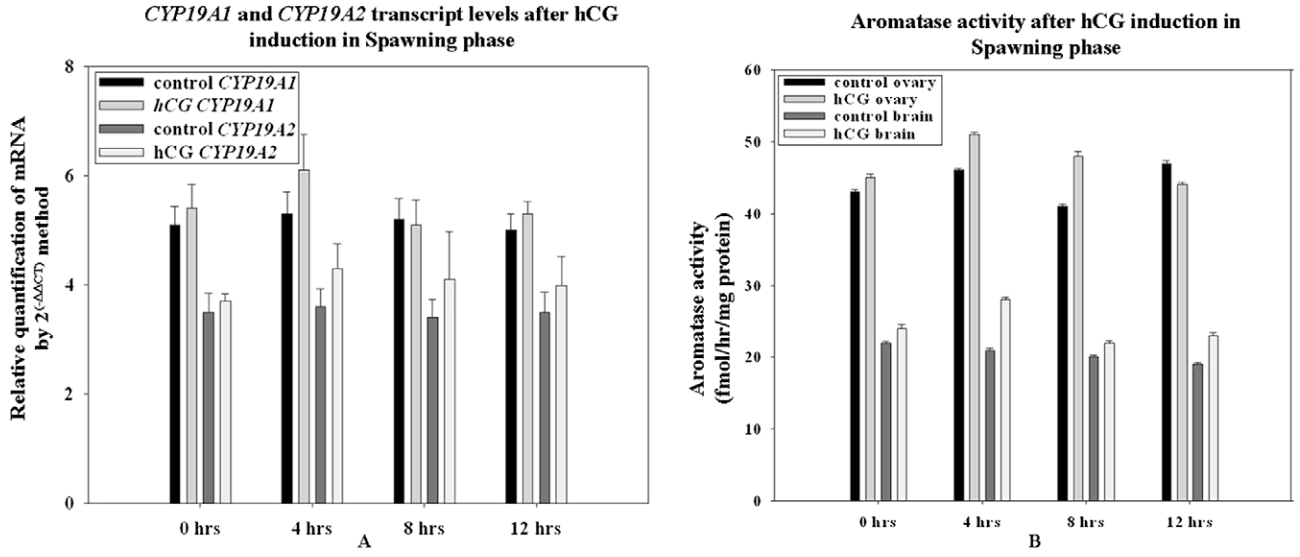

Fig. 8. (A) Real-time PCR analysis of *CYP19A1* and *CYP19A2* expression. (B) Enzyme activity of aromatase at spawning phase after hCG induction.

extended spawning phase in *C. gariepinus* followed by a short resting phase when compared to other catfish species which inhabits northern part of India. Changes in the months of seasonal phases (spawning–postspawning and resting) of catfish reproductive cycle inhabiting northern and southern parts of India may be attributed to explain this phenomenon broadly.

4.3. Ontogeny of aromatases

The relative change in aromatase transcript levels were studied after the phenotypic sex differentiation in *C. gariepinus* beginning at 45 dph followed by enzymatic activity at 60 dph. Present study revealed dimorphic expression pattern of both the forms of aromatase from 45 dph with early expression of brain and gonadal form in female juveniles suggesting a crucial role of estrogen in maintenance of ovarian differentiation and development. Furthermore, detection of relatively low levels of *CYP19A1* expression in the testis and late onset of *CYP19A2* expression in male brain implied that estrogens may not be required during early testicular differentiation, reinforcing the validity of the gynoinductor hypothesis of E_2 (Yamamoto, 1969). The findings in catfish are in accordance with juvenile sea bass (Blazquez and Piferrer, 2004) which exhibited higher *CYP19A2* expression in female brain compared to male brain at 20 days post-fertilization suggesting *CYP19A2* role in sea bass sex differentiation and also concurrent with reports from zebrafish and the Atlantic halibut where high expression of *CYP19A2* prior to and after gonadal differentiation clearly indicated the role of brain aromatase as a key determinant of phenotypic sex in fish (Trant et al., 2001; Matsuoka et al., 2006). However, Kallivretaki et al. (2007) have ruled out the possibility of *CYP19A2*'s role during sex differentiation by detecting similar pattern of *CYP19A2* expression and localization in zebrafish brain from both the sexes, concomitant to the findings in carp where brain form showed no sexual dimorphism (Barney et al., 2008). Nevertheless, in zebrafish embryos *CYP19A2* expression occurred sooner and reached higher levels compared to *CYP19A1*, and was estrogen inducible (Sawyer et al., 2006). The abundance of ovarian form in juvenile female catfish ovary is concurrent to earlier reports from the Nile tilapia (Kwon et al., 2001) and zebrafish (Kishida and Callard, 2001) where high expression of *CYP19A1* in the ovary, is associated with its cru-

cial role at the time of ovarian differentiation and oocyte growth. Furthermore, Chiang et al. (2001) emphasized that *CYP19A1* but not *CYP19A2* is a strong candidate involved in ovarian differentiation by the existence of binding regions for certain transcription factors in the 5′ flanking region of the Nile tilapia, zebrafish and goldfish *CYP19A1* gene. The sexual dimorphic expression pattern displayed by catfish juveniles similar to roach (Lange et al., 2008) indicates that the *CYP19A1* and *CYP19A2* may contribute to ovarian differentiation and sexual development. The contribution of the former might be greater than the latter. The significance of faint expression of *CYP19A2* during catfish testicular differentiation which is in agreement with reports from the Nile tilapia, the Japanese medaka and rainbow trout (Sudhakumari et al., 2003, 2005; Chang et al., 2005; Patil and Gunasekera, 2008; Dalla valle et al., 2002) is yet to be resolved.

4.4. Tissue distribution pattern of CYP19A1 and CYP19A2

Similar to channel catfish (Trant, 1994; Kazeto and Trant, 2005), in the catfish, *C. gariepinus* aromatases are preferentially expressed in brain (*CYP19A2* > *CYP19A1)* and ovary (*CYP19A1)* contradictory to the reports from goldfish, the Nile tilapia, zebrafish and southern catfish where *CYP19A1* was exclusively found in ovary while *CYP19A2* was detected both in ovary and brain (Tchoudakova and Callard, 1998; Trant et al., 2001; Chiang et al., 2001; Kwon et al., 2001; Sudhakumari et al., 2003, 2005; Chang et al., 2005; Zhihao et al., 2007). But the exact role of this overlapping expression is not yet clear. Further investigation, using antibodies specific to the isoforms are required, to study the distribution of these co-expressed genes in different regions of adult and juvenile catfish brain in order to assign a specific and definite function to *CYP19A1* and *CYP19A2*. On the other hand, the catfish brain form was confined to the adult brain nevertheless ontogeny study detected faint testicular expression of *CYP19A2* in testis of 60 dph catfish. Tissue specific expression revealed presence of *CYP19A1* isoforms in almost all tissues studied with faint expression in fat body (Patil and Gunasekera, 2008). Presence of *CYP19A1* in these non-steroidogenic tissues, muscles, liver (Piferrer and Blazquez, 2005; Barney et al., 2008) intestine, spleen, heart and gill are consistent with previous report from the Nile tilapia (Chang et al., 2005). Expression of *CYP19A1* was also detected in kidney but at lower levels concomitant to the report from rainbow trout and the Nile tilapia, (Dalla Valle et al., 2002; see Chang et al., 2005). However, unlike the Nile tilapia (Sudhakumari et al., 2003, 2005; Chang et al., 2005) *CYP19A2* transcript was detected exclusively in the brain of *C. gariepinus* as that of channel catfish (Kazeto and Trant, 2005). Discrepancy among fishes in the spatial expression of the two isoforms in steroidogenic and non-steroidogenic tissues may be explained as a consequence of species specific gene specialization that befits their mode of reproduction, sexual and courtship behavior and their habitat. However, to implicate a biological role to these isoforms one has to measure the protein in those tissues independently.

4.5. Phase-dependent (seasonal/reproductive cycle) expression and activity of aromatases after in vivo hCG treatment

Earlier report from the north African catfish have demonstrated the presence of LH receptor in ovary and brain and *in vitro* studies showed positive response of LH receptor by catfish LH, salmon LH, human LH, hCG, and human FSH (Vischer and Bogerd, 2003) which made possible the use of non-homologous gonadotropin, i.e., hCG to study the modulation, if any, in the transcript and activity level of aromatase isoforms at different reproductive phases of adult female catfish. Our findings revealed a positive response in the activity and transcript levels of aromatase after hCG induction in the preparatory and prespawning phases while no response was seen in the spawning phase. Increase in the aromatase activity in the isolated ovarian follicles after the treatment of gonadotropins or agents that raise the intracellular cAMP levels has been demonstrated in several fish species (Nagahama et al., 1991; Kagawa et al., 2003; Senthilkumaran et al., 2004). However, gonadotropins had no influence on ovarian P450arom that were under going final oocyte maturation (Yoshiura et al., 2003; Senthilkumaran et al., 2004). Induction by hCG in the preparatory and prespawning phases but not in the late spawning phase could be explained by the reported shift in the steroidogenesis which occurs in full-grown immature oocytes undergoing meiotic maturation (Senthilkumaran et al., 2004; Sreenivasulu and Senthilkumaran, 2009). Administration of non-homologous gonadotropin, hCG during preparatory and prespawning phases elevated *CYP19A1* for the production of E_2 which in turn might have caused an increase in *CYP19A2*. On the other hand, direct effects if any on *CYP19A2* needs to be ascertained based on the reports on *de novo* synthesis of aromatizable androgen in teleost brain (see Diotel et al., 2010) and the neuroestrogen thus synthesized might govern, the gonadotropin secretion by pituitary, leading to folliculogenesis and ovarian recrudescence. In the present study, administration of hCG did not induce *CYP19A1* and *CYP19A2* or selectively *CYP19A2* during spawning phase when E_2 levels were very low (Joy et al., 1998). This perhaps indicates that gonadotropin regulates gonadal recrudescence primarily via *CYP19A1* and *CYP19A2* might contribute secondarily via E_2 production either from ovary or neuroestrogens. In accordance to the results on transcripts, the aromatase activity in brain and ovary was inducible by hCG treatment during preparatory and prespawning phases but not in spawning. This may further substantiate the effect of gonadotropin (hCG) on brain and ovarian aromatase. To our knowledge, present study is first of its kind to simultaneously correlate transcript and enzyme activity changes in both brain and ovarian tissues after hCG treatment. Judging from the similar response of both isoforms after gonadotropin treatment, it may be interesting to understand the regulation of aromatases in catfish.

5. Conclusion

In summary, full-length cDNAs of *CYP19A1* and *CYP19A2* were cloned from ovary and brain of catfish, respectively, which exhibited high homology with other siluriforms. Purified recombinant proteins catalyzed the aromatization of testosterone to E_2. Present study demonstrated the phase-dependent expression and activity of *CYP19* simultaneously in brain and ovarian tissues during ovarian cycle. Based on our results, we report specific role for *CYP19A1* during ovarian differentiation and recrudescence. Since *CYP19A2* showed similar pattern of changes as that of *CYP19A1*, it might also play an important role in these processes either directly or indirectly. Interestingly, the responsiveness of *CYP19A1* and *CYP19A2* to hCG was also comparable during different phases of reproductive cycle. These results also warrant different pattern of gene regulation for these isoforms.

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Cloning and expression of *StAR* during gonadal cycle and hCG-induced oocyte maturation of air-breathing catfish, *Clarias gariepinus*☆

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ABSTRACT

Complementary DNAs encoding steroidogenic acute regulatory protein (*StAR*) have been isolated from different fish species, yet the relevance of *StAR* during gonadal cycle and more importantly in final oocyte maturation has not been assessed so far. A cDNA encoding *StAR* was isolated from the ovarian follicles of air-breathing catfish, *Clarias gariepinus*. Catfish *StAR* exhibited 55 to 72% identity at nucleotide level with other vertebrate orthologs. RT-PCR analysis of tissue distribution pattern demonstrated the presence of *StAR* mRNA in various tissues including gonads, kidney, liver, brain and intestine of catfish. Real-time RT-PCR analysis revealed high expression of *StAR* mRNA in the pre-spawning phase of ovary while it was low in preparatory, spawning and regressed phases. In testis, maximum expression was noticed during the preparatory phase. During human chorionic gonadotropin (hCG)-induced oocyte maturation, both *in vitro* and *in vivo*, *StAR* mRNA levels were augmented by 2 h and then declined gradually to reach basal levels by 12 h as that of saline-treated controls. Taken together, high level of expression during hCG-induced oocyte maturation vis-à-vis in spawning suggests a role for *StAR*, in addition to the steroidogenic enzyme genes in final oocyte maturation.

1. Introduction

Steroid hormones play a crucial role in the regulation of growth, development, differentiation, reproduction and several other functions in vertebrates. Production of different classes of steroids occurs from a common precursor, cholesterol and involves a battery of oxidative enzymes (Payne and Hales, 2004). The first committed step in steroid hormone biosynthesis is the conversion of cholesterol to pregnenolone, which occurs in mitochondria by the action of P450 side chain cleavage enzyme (P450scc). However, cholesterol cannot cross the mitochondria from cytoplasm and is delivered by a sterol transfer protein, steroidogenic acute regulatory protein (StAR; Stocco, 2000). Now, it has been accepted that the true rate-limiting step in steroidogenesis is the delivery of cholesterol across mitochondrial membrane. This is an important target for acute steroidogenesis by tropic hormones (Stocco, 2001), few other mediators and some endocrine disruptors (Walsh and Stocco, 2000) as well. Therefore, StAR is indispensable for mediating cholesterol transfer vis-à-vis steroidogenesis. Perhaps the most compelling evidence came from the identification of mutations in *StAR* gene during congenital adrenal hyperplasia (CAH), a condition in which cholesterol and cholesterol esters accumulate and the newborn fails to synthesize adequate levels of steroid hormones (Lin et al., 1995). This is further evidenced by *StAR* knockout mice which showed phenotypic mirrors of human lipoid CAH (Caron et al., 1997).

A cDNA encoding a 30 kDa mouse *StAR* was first characterized by Clark et al. (1994). StAR is believed to transfer cholesterol across mitochondria either by forming a transport tunnel (Tsujishita and Hurley, 2000) or by a cavity (Mathieu et al., 2003). It is plausible that StAR interacts with contact sites where the inner and outer mitochondrial membranes are in close proximity (Thomson, 2003). Further, studies have shown that StAR is rapidly synthesized in response to stimulation of several hormones such as luteinizing hormone (LH), adrenocorticotropic hormone (ACTH; Clark et al., 1995) typically with activation of the cAMP second messenger system. Although StAR appears to be critical for steroidogenesis in the adrenal and gonads, some of the tissues that do not express StAR, including placenta, synthesize large amounts of pregnenolone suggesting for the

☆ Catfish ovarian StAR nucleotide sequence has been submitted to GenBank under the accession no. FJ793811.

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existence of StAR-independent mechanisms for movement of cholesterol to P450scc enzyme (Stocco, 2001).

Complementary DNA-encoding proteins with high homology to StAR of mammals were cloned from zebrafish, rainbow trout, eel, cod and stingray (Bauer et al., 2000; Kusakabe et al., 2002; Li et al., 2003; Goetz et al., 2004; Nunez et al., 2005). In teleosts, besides correlating increase in *StAR* mRNA to acute interrenal or gonadal steroid production, very little is known about the dynamics of *StAR* transcripts in relation to gonadal cycle and more importantly final oocyte maturation. A shift in steroidogenesis from estradiol-17β (E_2) to 17α,20β-dihdroxy-4-pregnen-3-one (17α,20β-DP) is important for final oocyte maturation that is associated with pre-ovulatory LH surge (Nagahama, 1997; Senthilkumaran et al., 2004; Nagahama and Yamashita, 2008). This steroidogenic shift is governed by the down regulation of ovarian P450 aromatase and up-regulation of 20β-hydroxysteroid dehydrogenase, the enzymes that produce E_2 and 17α,20β-DP, respectively (Yoshiura et al., 2003; Senthilkumaran et al., 2004; Sreenivasulu et al., 2005, Sreenivasulu and Senthilkumaran, 2009a). However, involvement of *StAR* during shift in steroidogenesis would be possible, owing to the reason that *StAR* is rapidly synthesized in response to trophic hormone stimulation. In the present study, a cDNA encoding *StAR* was isolated from ovarian follicles of the air-breathing catfish, *Clarias gariepinus*. We then analyzed *StAR* transcript abundance during hCG-induced oocyte maturation, *in vitro* and *in vivo* by real-time RT-PCR. To complement our results, expression of *StAR* was also analyzed during different stages of gonadal cycle.

2. Materials and methods

2.1. Animals and treatments

Adult catfish, *Clarias gariepinus* (Actinopterygii, Siluriformes), of about 400–500 g were purchased live from local fish markets (Hyderabad, India) and acclimated for 2–3 weeks in aquaria filled with filtered tap water and maintained at normal photoperiod and ambient temperature conditions. Fish were fed *ad libitum* with minced goat liver/commercial food pellets during acclimation and experimentation. Catfish gonad undergoes four broadly distinguishable phases including an extensive preparatory phase followed by pre-spawning, spawning and regressed/resting phases (Swapna et al., 2006). For seasonal cycle analysis, gonads at different phases of reproductive cycle were collected, snap frozen in liquid nitrogen and stored at −80 °C. For *in vitro* oocyte maturation studies, animals were killed by decapitation and oocytes with centrally located germinal vesicles were collected. About 100 oocytes were then incubated (separately for each time point) in triplicate in catfish oocyte incubation medium (Senthilkumaran and Joy, 2001) with 100 IU/ml of human chorionic gonadotropin (hCG; Pubergen, Uni-Sankyo Pvt. Ltd., India). Controls were treated with saline. Follicles were collected at different time points and used for real-time RT-PCR. For *in vivo* oocyte maturation studies, fish were injected intra-peritoneally with hCG (1000 IU/kg body mass) and about 100 follicles at different time points were collected by gently stripping from ovipore and utilized for real-time RT-PCR. Controls were maintained similarly except for injecting with physiological saline. Both *in vitro* and *in vivo* studies are repeated thrice ($n=3$–5) with different batch of female fish in two consecutive years. Dosages of hCG for *in vitro* and *in vivo* induction were chosen based on standardization done earlier (Sreenivasulu and Senthilkumaran, 2009a).

2.2. Cloning of catfish StAR cDNA and sequence analysis

Total RNA was prepared from 100 mg of catfish ovarian follicles using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) as per manufacturer's instructions. One μg of total RNA was reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA, USA) and cDNA was used as template in PCR with a set of degenerate primers (listed in Table 1) designed by aligning existing fish *StAR* cDNA sequences. RT-PCR products were separated on agarose gel, a band corresponding to *StAR* was sub-cloned into pGEM-T Easy (Promega, Madison, WI, USA) vector and subsequently sequence was determined from both the ends. The identity of cDNA clone was confirmed by BLAST analysis.

5′ and 3′ rapid amplification of cDNA ends (RACE) were subsequently performed using RNA-ligase mediated RACE kit (Invitrogen) as per the manufacturer's protocol. The gene specific primers used in RACE were given in Table 1 and were designed from partial cDNA sequence. The PCR products were sub-cloned and sequenced as mentioned above. The full-length cDNA of catfish ovarian *StAR* was obtained by aligning the overlapping sequences of 5′, 3′ RACE and partial cDNA sequences. Finally, the open reading frame (ORF) was amplified using gene specific primers and the sequences were confirmed bi-directionally. Phylogenetic analysis was performed with MEGA (4.1) software.

2.3. Northern blot analysis

Twenty five μg of total RNA from ovary and testis were separated on a 1% denaturing formaldehyde-agarose gel and transferred on to a positively charged nylon membrane (Amersham, Buckinghamshire, England) by capillary transfer. Gel was stained with ethidium bromide and RNA ladder was used to track the size of transcripts. The membrane was hybridized under high stringency conditions overnight with a catfish *StAR* ORF labeled with ^{32}P-dCTP by random primer labeling kit (Perkin Elmer, Boston, MA, USA). Following hybridization, membranes were washed and signals were detected by Phosphor-imager (Typhoon, GE Healthcare). Northern blot analysis was repeated thrice independently.

2.4. RT-PCR analysis of tissue distribution

To analyze tissue distribution pattern of *StAR* in catfish tissues, 1 μg of total RNA obtained from different tissues was reverse transcribed and RT-PCR was performed as mentioned above using catfish *StAR* ORF primers.

2.5. Real-time RT-PCR

Transcript abundance of *StAR* was quantified by real-time RT-PCR of total RNA isolated from the ovarian follicles collected at different durations of hCG-induced oocyte maturation, *in vitro* and *in vivo* as well as ovary and testis at different stages of reproductive cycle. One μg of total RNA was reverse transcribed using random hexamers and MMLV-reverse transcriptase (Invitrogen). Gene-specific primers (Table 1) for PCR amplification were designed according to the requirements set forth by Primer Express software and the developer

Table 1
Primers used for cloning and expression analysis of *StAR*.

Primer[§]	Sequence 5′–3′	Annealing position[*]	Purpose
DF1	TGTGYGCTGGCATYTCYTAC	23–42	Degenerate RT-PCR
DR1	GGTGRTTKRCRAARTCCACCT	785–805	Degenerate RT-PCR
GSP-F1	CCAACATCCTAGCATGCCTGACCA	606–629	5′ RACE
GSP-F2	GACCAAGTTCACCTGGTTACTCAG	702–725	3′ RACE
GSP-R1	GGATAGCTTGCTCAGCTCGTGGTG	94–117	3′ RACE
GSP-R2	ATGGCCATCATGGCGTTCCTCCTC	69–92	3′ RACE
ORF-F	ATGCTACCTGCAACTTTTAAG	1–21	ORF cloning & tissue distribution
ORF-R	GCAGGCCATTGCCTCCTCCA	836–855	ORF cloning & tissue distribution
qRT-F	TGGCCATCCACCACGAGCTG	86–105	Real-time RT-PCR
qRT-R	CAATCTCAGTTTGCCAGCCATC	274–295	Real-time RT-PCR

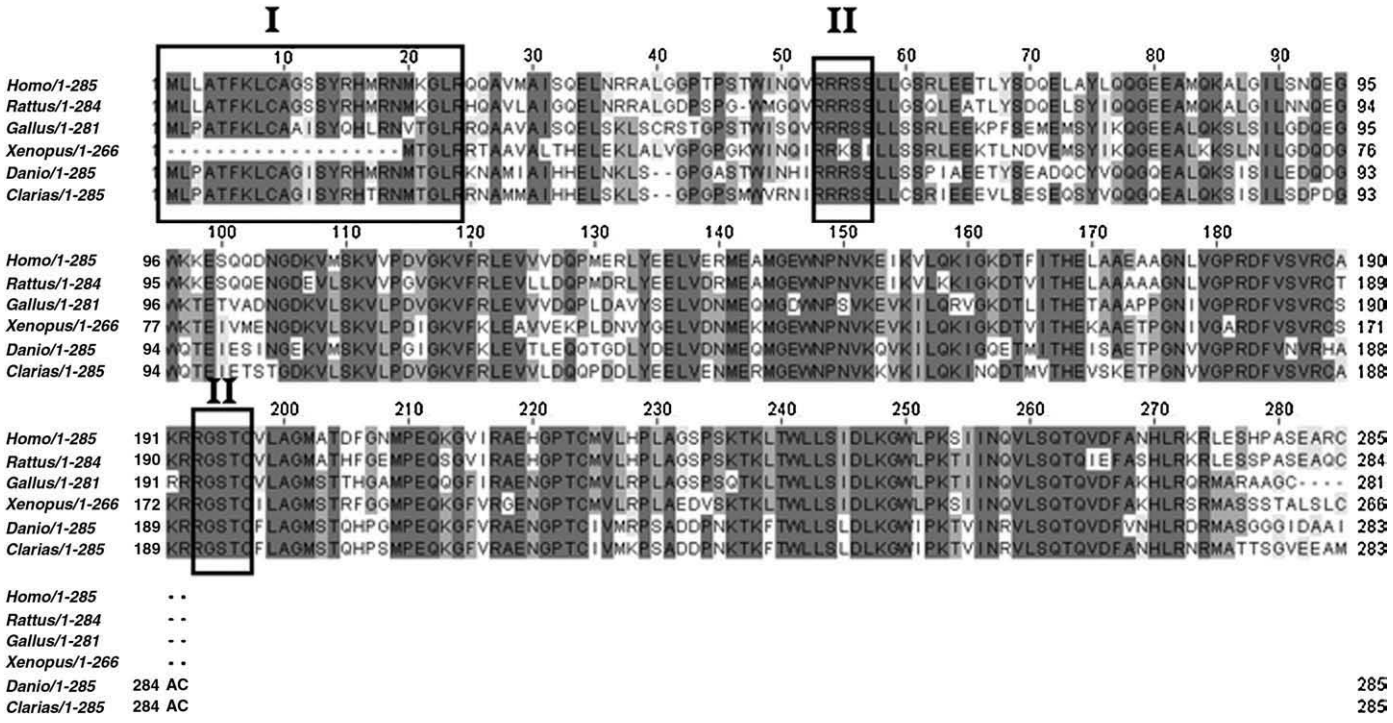

Fig. 1. ClustalW multiple alignment of deduced amino acid sequence of catfish ovarian *StAR* with other vertebrates. Shaded region represents conserved amino acids and signature domains are shown in rectangles (I: Mitochondrial targeting sequence, II: Protein kinase A phosphorylation site).

of the ABI Prism Sequence Detector. The cDNA corresponding to 1 μg of reverse-transcribed RNA served as templates for each of triplicate 25 μL PCR reactions using power SYBRGreen master mix. The PCR amplifications and fluorescence detection were performed with the ABI Prism Sequence Detector 7500 under the manufacturer's universal thermal cycling conditions. *StAR*, including *β-actin* (internal control), was amplified in separate reactions using same pool of cDNA templates. Transcript abundance of *StAR* was normalized to that of *β-actin* and reported as fold change in abundance relative to the values obtained for spawning phase gonad using the formula $2^{-\Delta\Delta CT}$. All real-time RT-PCR data were expressed as mean ± SEM. Data were $\log_{10}$ transformed and plots were drawn on back transformed data. Significance between groups was tested by ANOVA followed by Student–Newman–Keuls' test using Sigmastat3.1 software. Differences among groups were considered significant at $P<0.05$.

3. Results

3.1. Cloning of catfish ovarian StAR

Using a degenerate primer pair, a partial cDNA of 750 bp was amplified by RT-PCR and sequence was determined, subsequently sequence identity was confirmed by BLAST analysis. Further, full-length cDNA was obtained by 5′ and 3′ RACE approaches. Catfish ovarian *StAR* cDNA was 1.150 kb long with an ORF of 858 bp encoding a putative protein of 285 amino acids. 5′ untranslated region (UTR) was 64 bp while 3′ UTR was 248 bp long with a potential polyadenylation signal.

ClustalW multiple alignment of deduced amino acid sequence of catfish *StAR* with other vertebrate orthologs demonstrated that the signature domains such as, mitochondrial targeting sequence, cholesterol recognition site and protein kinase A phosphorylation site are highly conserved among vertebrates (Fig. 1). Phylogenetic analysis revealed that catfish ovarian *StAR* is highly homologous to its fish counterparts with 55–72% identity at nucleotide level where as it exhibited only 55–60% with higher vertebrates (Fig. 2).

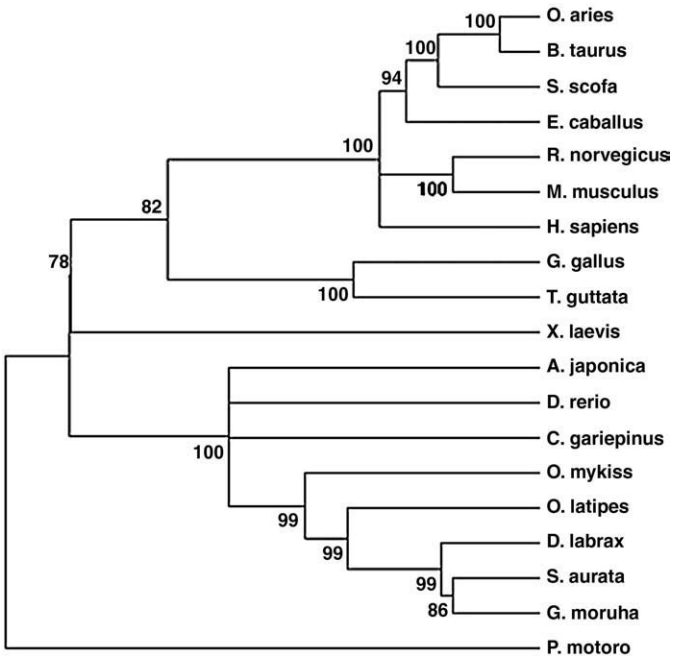

Fig. 2. Phylogenetic analysis of catfish *StAR* showing evolutionary relationship. (Accession No: *Homo sapiens* variant 1, ***NM_000349***; *Homo sapiens* variant 2, ***NM_001007243***; *Rattus norvegicus*, ***NM_031558***; *Mus musculus*, ***NM_011485***; *Sus scrofa*, ***NM_213755***; *Ovis aries*, ***NM_001009243***; *Bos taurus*, ***NM_174189***, *Equus caballus*, ***NM_001081800***; *Taeniopygia guttata*, ***NM_001076686***; *Gallus gallus*, ***NM_204686***; *Xenopus laevis*, ***AF220437***; *Oncorhynchus mykiss*, ***NM_001124202***; *Danio rerio*, ***NM_131663***; *Sparus aurata*, ***EF640987***; *Oryzias latipes*, ***NM_001104910***; *Dicentrarchus labrax*, DQ166820; *Godus moruha*, DQ646787; *Anguilla japonica*, AB095110; *Clarias gariepinus* FJ793811; *Potamotrygon motoro*, AY553723).

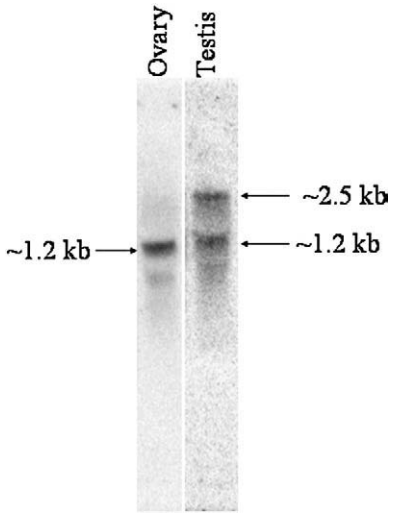

Fig. 3. Northern blot (representative, $n=3$) analysis of 25 µg catfish ovary and testis total RNA probed with *StAR* ORF. 1 kb RNA ladder was used to track the size of transcript.

3.2. Transcript size and sites of expression

Northern blot analysis in ovary identified a single band of 1.2 kb in ovary where as in testis a larger transcript of 2.5 kb was also detected in addition to 1.2 kb transcript (Fig. 3). RT-PCR analysis of tissue distribution pattern of *StAR* showed predominant expression in gonads and liver. In addition, transcript was also found in kidney, brain and intestine (Fig. 4).

3.3. Expression in different stages of gonads

Real-time RT-PCR analysis of *StAR* mRNA abundance at different stages of gonadal cycle demonstrated high expression in the pre-spawning phase of ovary while it was low at preparatory, spawning and regressed phases (Fig. 5A). On the other hand, maximum expression was noticed in the preparatory phase of testis. *StAR* expression was minimal during pre-spawning and regressed phases of testis (Fig. 5B).

3.4. Expression during hCG-induced oocyte maturation, in vitro and in vivo

In hCG-induced oocyte maturation, *in vitro*, *StAR* mRNA expression showed a remarkable increase by 2 h followed by a gradual decrease (Fig. 6A). A similar trend was also observed in hCG-induced oocyte maturation, *in vivo* (Fig. 6B).

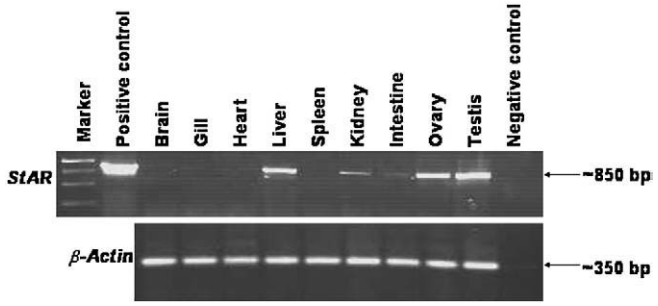

Fig. 4. Spatial expression pattern of *StAR* (upper panel) in various tissues of catfish. *β-actin* (internal control) is shown in lower panel. Plasmid clone of catfish *StAR* was used as positive control and negative control contains no cDNA template.

4. Discussion

Primary goal of this study is to delineate the role of *StAR* during final oocyte maturation. In addition to this, we also assessed changes in *StAR* transcripts during gonadal cycle. Since *StAR* is highly conserved among vertebrates (Bauer et al., 2000), we could have directly proceeded for gene expression analysis following hCG-treatment, but the presence of multiple forms (including splice variants and UTR variants are known) necessitates isolation of *StAR* cDNA from catfish. Therefore, we cloned a *StAR* cDNA from ovary of catfish. The catfish *StAR* cDNA encodes a protein of 285 amino acids that is similar in size with other teleosts and higher vertebrate *StAR*s identified so far. Phosphorylation of *StAR* at serine195 is shown to increase the steroidogenic activity (Arakane et al., 1997) and in comparison with *StAR*s of other vertebrates, the putative protein phosphorylation site is also conserved in catfish *StAR*. StAR is synthesized in cytosol and targeted to mitochondria (Stocco, 2000). The N-terminal region of catfish StAR, composed of basic and hydrophobic amino acids, has characteristic mitochondrial targeting sequence and also showed more than 90% identity with other teleosts. The C-terminal domain with hydrophobic amino acids forms a tunnel structure that is believed to transport cholesterol to mitochondria is also highly conserved. Our data further supports that the primary structure of StAR, particularly in the functional domain is highly conserved throughout vertebrates. Northern blot analysis identified a single transcript of about 1.2 kb in catfish ovary. Though multiple transcripts have been identified in humans (Sugawara et al., 1995) and trout (Kusakabe et al., 2002), the size of StAR protein seems to be more or less similar in all the vertebrates except for human splice variant.

We identified a single transcript in ovary and multiple transcripts in testis. Occurrence of multiple transcripts in testis could be either due to the presence of different UTR variants or un- processed mRNAs as described in earlier reports (Kusakabe et al., 2002; Li et al., 2003). We could isolate a single cDNA of *StAR* from catfish, though we could

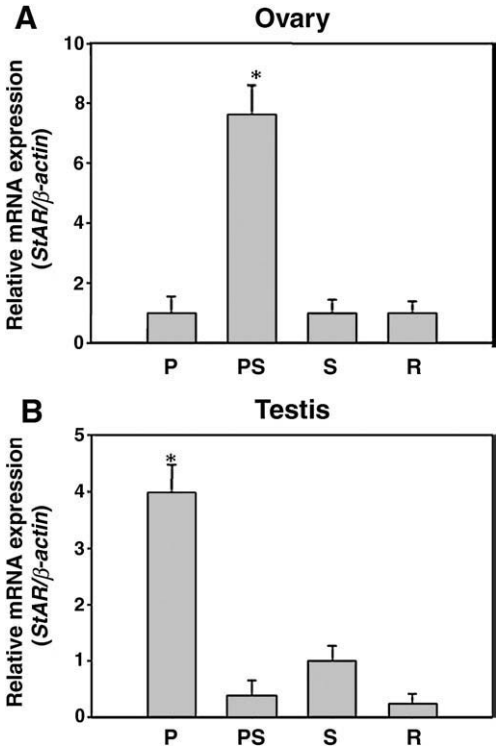

Fig. 5. Stage-dependent expression of *StAR* in catfish ovary (A) and testis (B) by real-time RT-PCR. *StAR* expression is reported as fold change compared to spawning phase. *Indicates significance (ANOVA, $n=3$, $P<0.05$). P, preparatory; PS, pre-spawning; S, spawning; R, regressed.

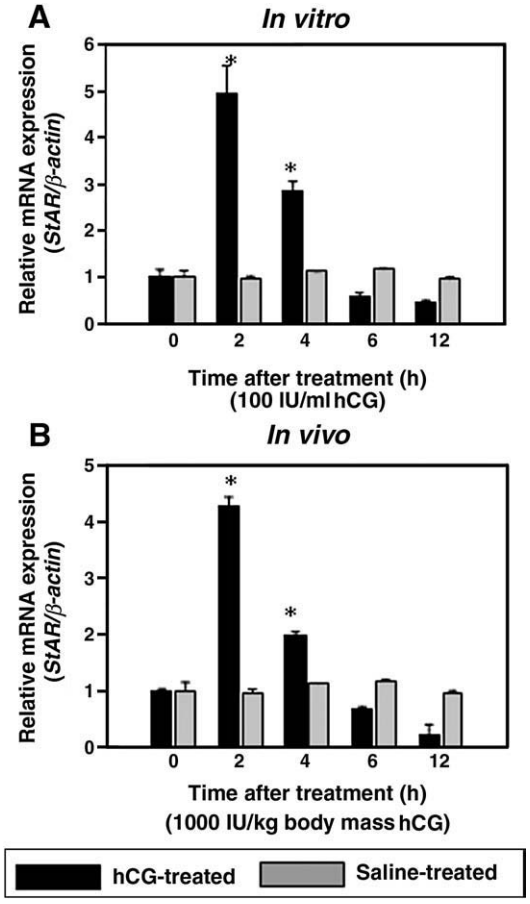

Fig. 6. Real-time RT-PCR analysis showing the expression of *StAR* during hCG-induced oocyte maturation, *in vitro* (A) and *in vivo* (B). *StAR* expression is reported as fold change is reported as fold change compared to 0 h after treatment with hCG. * Indicates significance (ANOVA, $n=5$, $P<0.05$).

not look for the additional forms, presence of multiple forms in catfish cannot be ruled out as some teleosts have undergone fish specific genome duplication (Mayer and Van de Peer, 2005). Consistent with previous studies, using RT-PCR, *StAR* transcripts were found both in classical and non-classical steroidogenic tissues including ovary, testis, head kidney, liver and a faint signal was also observed in intestine and brain. Presence of high level of *StAR* transcripts in gonads and kidney is in agreement with the previous reports (Sugawara et al., 1995; Kusakabe et al., 2002). A low level of *StAR* transcript was also found in catfish brain. However, presence of *StAR* in brain was already reported in rat and marmoset (Furukawa et al., 1998; Bauer et al., 2000). Co-localization of *StAR* with P450scc and 3β-hydroxysteroid dehydrogenase in brain indicates a role for *StAR* in neurosteroid synthesis (Furukawa et al., 1998). In addition to classical steroidogenic tissues, teleost *StAR* was also found to express in a wide range of tissues including spleen, intestine, gill, skin (Kusakabe et al., 2002; Bauer et al., 2000; Li et al., 2003; Goetz et al., 2004) and muscle though the function of *StAR* in these tissues is yet to be established. However, in mammals, *StAR* expression is largely restricted to tissues capable of *de novo* steroid synthesis (Sugawara et al., 1995).

In the present study, using real-time RT-PCR, we found maximum expression in late vitellogenic stage of ovary. Previous studies using Northern blot hybridization documented similar kind of results in cod (Goetz et al., 2004) and trout (Kusakabe et al., 2002; Nakamura et al., 2005). Our results are even in accordance with mammalian species. In dairy cows, ovarian follicles of <1 mm had no *StAR* expression while its expression decreased with the progression of atresia (Braw-Tal and Roth, 2005). Similarly *StAR* expression was found to be co-ordinatedly regulated during follicular development of other mammalian species such as pig (LaVoie et al., 1997) and rat (Ronen-Fuhrmann et al., 1998) and also birds (Jhonson et al., 2002). In mammals, corpus luteum also produces progesterone and *StAR* expression was noticed in luteal phase (Jwngel et al., 1995; LaVoie et al., 1997). *StAR* expression in post-spawning/regressed phase of catfish was minimal and similarly in contrast to mammals, *StAR* expression in post-ovulatory ovary of birds was low because of the absence of corpus lutuem (Jhonson et al., 2002). In contrast to mammals and other teleosts studied, *StAR* expression in zebrafish decreased with increase in follicle growth to maturation (Ings and Van Der Kraak, 2006). Though we could not sample at many durations of different stages, a gradual increase in *StAR* transcript from early vitellogenesis to late vitellogenesis is in accordance with circulating gonadotropins and presumed to be important for the production of estrogens/androgens at those times. In mammals, where spermatogenesis is continuous, *StAR* expression in testis is persistent and is known to be escalated by gonadotropins (Tsuchiya et al., 2003). *StAR* expression during spermatogonial proliferation was shown to be up-regulated in Atlantic salmon (Maugars and Schmitz, 2008). In catfish testis, we noticed high expression in preparatory phase while it was low in spawning phase. On the contrary, a 40 fold increase in *StAR* transcripts over the course of spermatogenesis, with peak values around late spermatogenesis and spermiation, was reported in trout (Kusakabe et al., 2002; Nakamura et al., 2005). The observed discrepancy of *StAR* expression among fish species could be attributed to either sensitivity of the techniques used or to varied sampling patterns. However, high expression in the preparatory phase of catfish gonads that reflects active stage of spermatogenesis/vitellogenesis shows a positive correlation with gonadal cycle as of other steroidogeneic enzyme genes in catfish (Kumar et al., 2000; Sreenivasulu and Senthilkumaran, 2009a,b). In spite of the variations in expression pattern of *StAR* in different species, change in the levels of *StAR* mRNA seems to be in harmony with the circulating gonadotropin levels indicating that *StAR* expression is under gonadotropin control.

Though there are reports on *StAR* expression following acute disturbance (Kusakabe et al., 2002), ACTH injection and change in salinity (Kim et al., 1997; Stocco, 2001; Li et al., 2003), reports on induction of *StAR* mRNA by gonadotropins in non-mammalian vertebrates are limited. In the present study, we demonstrate the induction of *StAR* mRNA by hCG both *in vitro* and *in vivo* during final oocyte maturation by real-time RT-PCR. Our observation shares similarity with that of zebrafish (Ings and Van Der Kraak, 2006) and humans (Kiriakidou et al., 1996), wherein human *StAR* gene transcription was shown to be inducible by cAMP that is produced by LH-surge. Our observation of biphasic response of *StAR* expression, a maximum expression during follicle development and an induction by an ovulatory dose of hCG, seems to be conserved as was noticed in other fishes like zebrafish (Ings and Van Der Kraak, 2006) and mammals (Kiriakidou et al., 1996; Ronen-Fuhrmann et al., 1998). On the contrary, ovarian follicles sampled at different time period of naturally ovulating trout showed minute expression before the onset of germinal vesicle breakdown (GVBD) and after GVBD while a several fold increment was observed at the time of ovulation and post ovulation (Kusakabe et al., 2002; Nakamura et al., 2005). In the case of Atlantic croaker, treatment of ovarian follicles with hCG did not alter *StAR* mRNA expression till 6 h and a 17 fold increase was noticed at 24 h (Nunez and Evans, 2007). Extensive sampling following hCG-treatment, *in vitro* and *in vivo* in catfish demonstrated the induction of *StAR* by gonadotropin in teleosts. Thus induction of *StAR* might be playing a crucial role during gonadal recrudescence and final oocyte maturation.

In conclusion, we isolated a cDNA encoding *StAR* from ovary of catfish which exhibited high level of sequence identity to other vertebrate *StAR* cDNAs. Spatial expression pattern together with expression in different stages of gonadal cycle are presented here.

Elevation of *StAR* during hCG-induced oocyte maturation, *in vitro* and *in vivo* indicated a role for *StAR* in shift in steroidogenesis occurring in catfish ovarian follicles prior to oocyte maturation or during final oocyte maturation. Based on our results, it is important to analyze the contribution of *StAR* in addition to sterodogenic enzyme genes or related transcription factors during meiotic maturation in teleosts.

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20-Hydroxyecdysone regulation of H-fibroin gene in the stored grain pest *Corcyra cephalonica*, during the last instar larval development

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ABSTRACT

20-Hydroxyecdysone (20E) controls molting, metamorphosis and reproduction of insects. It binds to a heterodimeric complex of ecdysone receptor (EcR) and ultraspiracle (USP), and regulates the transcription of genes containing ecdysone response elements (EcREs). However, the 20E regulation of silk fibroin genes is largely unexplored. In most lepidopteran larvae, the silk fibroin primarily consists of a large protein, heavy chain fibroin (H-fibroin) that is associated with two small proteins, L-chain fibroin and P25. In the present study, we demonstrate that 20E regulates the expression of H-fibroin gene in *Corcyra cephalonica*, in a dose-dependent manner during the last instar larval development. Semi-quantitative and real-time PCR studies reveal that physiological doses of 20E do not alter the normal expression, whereas higher doses cause a significant decline in the expression. Luciferase activity assays and gel shift experiments further confirm the presence of a functional EcRE in the upstream region of H-fibroin which regulates the ecdysteroid dependent transcriptional activity of fibroin gene through EcR.

In vitro treatment with 20E mimicking insecticides, RH-5849 and RH-5992 decreases the expression of H-fibroin in isolated salivary glands. Insects fed with similar concentrations of these insecticides, metamorphose abnormally. Differences are also observed in the ultrastructure of the silk fibers of control and insecticide fed insects providing additional insight into the disruptive effects of these non-steroidal ecdysteroid agonists.

1. Introduction

Most lepidopteran larvae produce silk from a pair of modified salivary glands, each of which consists of silk-secreting posterior, middle and anterior regions [1]. The fibrous silk core is produced by the posterior silk gland (PSG), that is generally constituted of a complex of three proteins namely, H-chain fibroin (H-fibroin), L-chain fibroin (L-fibroin) and glycoprotein P25 (also called fibrohexamerin) [2,3]. Both H-fibroin and L-fibroin are disulphide linked and this linkage is indispensable for the secretion of these proteins [4,5]. Non-covalent interaction of P25 with the H-fibroin N-terminus facilitates transport and secretion of the H-fibroin/L-fibroin heterodimers. Simultaneously, L-fibroin plays a protective role in P25 glycosylation [6]. The middle silk gland (MSG) secretes several sericins (ranging from 65 to 400 kDa) that provide a sticky coating of the fiber and also add several low molecular weight components with presumably protective functions to the silk [7,8]. Although synthesis and secretion of silk fibroin has been studied in detail in insects like *Bombyx mori* [9] and *Galleria mellonella* [10], the mechanism by which it is hormonally regulated is not well documented.

In the present study, we made an attempt to understand the 20-hydroxyecdysone (20E) regulation of H-fibroin gene in the stored grain pest, *Corcyra cephalonica*, during the last instar larval development. H-fibroin makes up the bulk of the lepidopteran silk [10] hence, we chose to work on it. The principal biologically active steroid hormone in insects is 20E [11]. The action of 20E is mediated by a heterodimer of two nuclear receptors, ecdysone receptor (EcR) and ultraspiracle (USP). Ecdysteroids may specifically promote EcR association with USP, the insect homologue of retinoid X receptor (RXR), and control transcription of genes containing EcRE [12]. Both *in vitro* and *in vivo* analyses have led to the identification of various EcREs, which are invariantly composed of direct or inverted repeats of the consensus sequence (G/T)NTCANTNN(A/C)(A/C) [13–16].

Although the mechanism of 20E action on various genes, mediated through EcR/USP heterodimer, is well known [13,17], however, the presence of EcR in insect salivary glands was recently reported [18]. It was identified and characterized in *Drosophila melanogaster* by photo affinity labeling studies which show that 20E gets covalently linked to the receptor protein. The EcR gene of *D. melanogaster* encodes three protein isoforms namely EcRA, EcRB1 and EcRB2, which differ only in their transactivation domain and exhibit differential transcriptional capabilities [19]. The findings in

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B. mori suggest that the anterior silk gland (ASG) has high levels of EcR. Isoform EcRA was present during the intermolt period, while EcRB1 appeared at the time of the molts. Ecdysone was shown to induce the expression of the EcR gene in cultured silk glands [20]. Further, it was demonstrated that *B. mori* ASG undergoes degeneration through programmed cell death in response to a metamorphic increase in hemolymphatic ecdysteroid at the end of last larval instar [21]. These reports indicate that the development as well as function of salivary gland in insects is 20E regulated. Most hormonal studies on H-fibroin are limited to *B. mori*, being stimulated by the interests of commercial sericulture [22]. However, these studies, in particular, do not analyze the regulation of 20E on H-fibroin expressed in the salivary glands. Furthermore, till date no report shows that the transcriptional regulation of H-fibroin gene by 20E is mediated through EcR.

We chose *C. cephalonica*, which is a serious lepidopteran pest of stored cereals such as wheat, rice, sorghum, maize, and millet, in tropical and sub-tropical regions of the world [23], to study the mechanism of 20E regulation of H-fibroin gene. Insect growth regulators that mimic ecdysone and interfere with the molting and metamorphosis are in use as potential insecticides currently. These compounds are selectively toxic against lepidopteran pests and are environmentally benign [24]. Hence, an understanding of the mechanism of 20E regulation of H-fibroin gene of *C. cephalonica* would predictably provide an insight to efficiently target the larval forms of this pest using 20E mimicking analogs.

In the present study, we cloned 5′ region of H-fibroin gene to demonstrate the effect of 20E on its expression. The presence of a functional EcRE in the upstream region suggests the involvement of EcR complex in 20E regulation of this gene at transcriptional level. The decreased H-fibroin expression, abnormal metamorphosis and the differences in the ultrastructure of silk fibers observed with nonsteroidal agonist treatment further confirm the role played by 20E in its regulation.

2. Materials and methods

2.1. Experimental insects

C. cephalonica is commonly known as rice moth and belongs to the order Lepidoptera and family Galleridae. The larval forms were reared in culture troughs that contained coarsely crushed sorghum seeds. The cultures were maintained at 26 ± 1 °C, 60 ± 5% relative humidity (RH) and 14:10 h light:dark (LD) photoperiod. The larval development proceeds through five instars and is completed in about 45–50 days. The final (5th) larval instar was further classified based on their body weight and head capsule size into early-last (ELI), mid-last (MLI) and late-last instar (LLI) followed by the non-feeding prepupal (PP) stage [25], that was conveniently divided into wandering or early-prepupal (EPP) and late-prepupal (LPP) stages.

2.2. Cloning of 5′ fragment of H-fibroin

Total RNA was isolated from the larval salivary glands using Trizol reagent (Sigma Chem. Co., USA). Five micrograms of total RNA was used for cDNA preparation using Superscript™ III first strand synthesis system (Invitrogen, USA). Primers were designed based on the existing H-fibroin sequences of various insects, using GeneRunner software (version 3.05). Multiple alignment with H-fibroins of other known insect species was performed using ClustalW program. A partial clone of H-fibroin was amplified using, F1 [5′-ATG AGA GTC ACA ACC TTC-3′] as the forward primer and R1 [5′-TAT AAC TAC GTC TTC TTC GTA-3′] as the reverse primer. The cloned fragment was sequenced and submitted to GenBank under the accession no. GQ901977.

2.3. Chemicals and experimental treatments

20E and RH-5992 (3,5-dimethylbenzoic acid 1-(1,1dimethylethyl)-2-(4-ethylbenzoyl) hydrazide) were commercially procured from Sigma Chemical Company (USA). RH-5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) was purchased from Rohm and Haas Company (USA). RH-5849 and RH-5992 are bisacylhydrazine ecdysone agonists that mimic natural insect moulting hormone (principally 20-hydroxyecdysone) by binding competitively to ecdysteroid receptors in insect cells. The actions of these ecdysone agonists are specific to lepidopteran larvae. The chemical structures of 20E, RH-5849 and RH-5992 are given in AppendixBSupplementary Fig. 1. All the stock solutions were prepared in ethanol at a concentration of 1 mg/ml and diluted as per the requirement. The heterologous anti-Chironomus ecdysone receptor polyclonal antibody rose against a conserved synthetic peptide sequence of 15 amino acid residues was supplied by Pierce Protein Research Products (USA). This antibody was reactive against ecdysone receptor of various insect species.

For *in vitro* studies, salivary glands dissected under sterile condition in ice cold insect Ringer (130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 1 mM PMSF) were rinsed in 100 μl of TC-100 insect culture medium (Sigma Chem. Co., USA) supplemented with streptomycin sulfate (50 μg/ml). This was followed by transfer to fresh 500 μl medium for 1 h for preconditioning prior to the required experimental set up. Based on the experiment, the tissue was incubated in the culture medium along with different concentrations of 20E/RH-5849/RH-5992 for varying time periods in the incubator (95% relative humidity, 5% CO_2, and 25 °C). Solvent treated (0.05% ethanol) salivary glands were used as control. After incubation, the tissue was rinsed in ice cold Ringer and processed for RNA isolation. For *in vivo* studies with RH-5849 and RH-5992, concentrations similar to that used in *in vitro* experiments were given along with the feed. Control larvae received 0.05% ethanol treated feed.

2.4. Semi-quantitative and real-time PCR

Total RNA was isolated from control as well as treated tissue. The purity and quantity of RNA was checked by using Nanodrop (ND-1000) spectrophotometer. PCR reactions (20 μl) were carried out using Power SYBR Green Master mix (Applied Biosystems, USA). Reaction was set up using primer sets for H-fibroin (sense 5′-CCT TCG TGA TCT TGT GCT GTG CC-3′ and antisense 5′-CTG ACG ATC TTG TCC TCA CCG GA-3′) and β-actin gene (sense 5′-GGT AGT AGA CAA TGG CTC CGG-3′ and antisense 5′-CCC AGT TAG TGA CGA TTC CGT G-3′). Semi-quantitative PCR products were visualized on 1% agarose gel. For real-time PCR, reactions were measured using Fast 7500 Real time PCR system (Applied Biosystems, USA). Dissociation curve analysis was performed after the last cycle to confirm amplification of a single product. The real-time results are presented as change in expression relative to control using target gene Ct values normalized to that of β-actin gene Ct values based on the $2^{-(\Delta\Delta C(T))}$ method [26].

2.5. Construction of genome walking library and isolation of 5′-upstream region

Genomic DNA was isolated from the larval salivary glands. Genome walking library was constructed using Universal Genome Walker kit (Clontech, USA) following manufacturer's protocol. Twenty-five micrograms of genomic DNA was digested overnight either with EcoRV or PvuII or DraI or StuI. Digested DNA was purified using phenol–chloroform extraction method. Genomic DNA digested with each restriction enzyme was ligated to adaptors separately. H-fibroin upstream region was isolated using gene specific primer 1 (5′-GCA AGG CAC AGC ACA AGA TCA CGA AGG-3′) and

the adaptor primer 1. A nested PCR was carried out using gene specific primer 2 (5′-GCA CAA GAT CAC GAA GGT TGT GAC TCT C-3′) and adaptor primer 2. All the amplicons were cloned into pGEM-T Easy vector (Promega, USA) and sequence was determined bi-directionally. Putative transcription factor binding sites were predicted using the database of MatInspector and TESS program.

2.6. Cloning of PCR based progressive deletion constructs

Progressive 5′ deletion constructs were amplified by PCR using primers listed below. Resultant constructs were cloned in KpnI and HindIII sites of pGL2 basic firefly luciferase vector (Promega, USA). The identity of each construct was verified by double digestion and the absence of cloning artifacts was determined by nucleotide sequencing. The forward primers used were (i) 549/+41 H-fibroin: 5′-CCTATGTCCAGCAGTGGACTG-3′, (ii) −86/+41 H-fibroin: 5′-CGCGTCAACGAAATAGTGGA-3′, (iii) −63/+41 H-fibroin: 5′-TCAGTATAAAAAGCCTTG-3′ and (iv) −86/+41 mutated EcRE: 5′-CGCGTCAACGAAATAGTGGATTTGTGGT-3′, while the reverse primer was +41 H-fibroin: 5′-TCGATAGCATCTGCAGTGACATAC-3′.

2.7. Transient transfection and luciferase activity assays

EcR-293 cell lines (derived from HEK-293 cells by incorporation of the regulatory vector pVgRXR which encodes both the RXR and EcR receptor proteins) were obtained (Invitrogen, USA) and cultured using DMEM (Dulbecco's modified Eagle medium) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 30 μg/ml Bleocin and 300 μg/ml of Geneticin. All the cell culture reagents were purchased from GIBCO/BRL Life Technologies Inc. (USA). Cultures were grown in an incubator at 37 °C, 5% CO_2 and 95% relative humidity. Cultures were maintained at low passage number ($n < 10$). Cells were plated 48 h before transfection in 24 well plates. Reporter gene constructs (Firefly luciferase) and the pRL-TK plasmids (Renilla luciferase) were co-transfected into 80–90% confluent cell cultures. Promoter activation leads to expression of secreted luciferase protein. Hence, 48 h after co-transfection, cells were collected and washed with phosphate buffered saline solution. The cells were lysed by the addition of 100 μl passive lysis buffer per well. Luciferase reporter activity was determined with the dual-luciferase reporter assay system (Promega, USA) according to the manufacturer's instructions. Light intensity was measured using Turner Design 20/20 luminometer and the promoter activity was expressed as relative luciferase units. The functional activity of the promoter constructs was measured both in the presence as well as in the absence of 20E. 5.7 pg/μl of 20E was used for induction of cells. Light intensity values from cell cultures transfected with the promoterless (pGL2) vector were used to correct background. Corrected firefly luciferase activities were normalized to *Renilla reniformis* luciferase activities and expressed as fold increase with respect to the values obtained with pGL-2 empty vector. Non-transfected cells were shown as negative control. In all cases, three independent experiments were performed to evaluate reproducibility.

2.8. Nuclear extract preparation

Silk glands were excised and homogenized gently in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4). The homogenate was incubated in 0.4 ml of hypomolar HEPES buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml Leupeptin, 2 μg/ml Aprotinin and 0.5 mg/ml Benzamidine) for 15 min in ice. 12.5 μl of 10% NP-40 was then added for lysis of the cells. The sample was vortexed vigorously for 10 s and was centrifuged for 30 s at 2000 rpm. The supernatant (cytoplasmic extract) was collected and stored at −70 °C. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml Leupeptin, 2 μg/ml Aprotinin and 0.5 mg/ml Benzamidine) and the tubes were incubated on ice for 30 min. This nuclear extract was then centrifuged for 5 min at 2000 rpm and was stored at −70 °C for further use.

2.9. Immunoblotting and gel shift assay

Cytoplasmic and nuclear extracts (40 μg) were separated by 10% SDS-PAGE, blotted onto nitrocellulose membrane, probed either with sheep anti-*Chironomus* ecdysone receptor or with anti-β-actin antibodies, detected by goat anti-rabbit IgG-HRP developed using chemiluminiscence kit (Pierce, USA). Gel shift assay was performed by incubating 5 μg of nuclear extract with 15 fmol of [^{32}P] end labelled double stranded wild or mutated EcRE oligonucleotide (wild type: 5′ACG AAA TAG TGG ATT TTC AGT ATA AAA AGC CTT GGA AAT CTG G3′ and mutated: 5′ACG AAA TAG TGG ATT TGT GGT ATA AAA AGC CTT GGA AAT CTG G3′) in the presence of 0.5 μg of poly dI:dC in a binding buffer (20 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.4 mM DTT and 5% glycerol) for 15 min at 37 °C. The DNA–protein complex formed was separated from free oligonucleotide on a 6% native polyacrylamide gel. 5.7 pg/μl of 20E was used for induction of samples prior to loading. For supershift, 1 μg of anti-ecdysone receptor antibody was incubated with the nuclear extract at room temperature for 10 min prior to addition of radiolabelled probe.

2.10. Scanning electron microscopic studies

Silk synthesized by control as well as non-steroidal agonist treated insects was collected manually and mounted onto the stubs containing carbon tape, in a sterile environment. These were then loaded onto the specimen stub and were made electrically conductive using gold metal coating. The surface of the silk fibers was scanned and analyzed.

2.11. Statistical analysis

Data is expressed as mean ± SEM ($n = 3$). Significance between groups was analyzed by ANOVA followed by the Student–Newman–Keuls test using Sigmastat. Values are considered significant at $P < 0.05$.

3. Results and discussion

3.1. Cloning of 5′ end of H-fibroin

A 294 nucleotide 5′-fragment of H-fibroin was cloned. The fragment encoded 98 amino acid residues. The alignment of the deduced peptide with the N-terminus of known lepidopteran H-fibroins showed clear homology in the putative signal peptide region which included 18 residues. The non-repetitive sequence that follows was characterized by frequent grouping of two non-polar residues (Ile, Leu, Val, Phe, and Pro) flanked by one or two charged residues (Asp, Glu, Lys, and Arg). The two conserved motifs, RTFVIETD and YEEDVVI, the overall negative charge and the distribution of charged residues resembled to that of the N-terminus of H-fibroins of *G. mellonella*, *B. mori* and *Y. evonymella* H-fibroins (Fig. 1).

The presence of signal peptide cleavage site in the deduced amino acid sequence indicates the secretory nature of this protein. The high degree of homology of the H-fibroin N-terminus of *C. cephalonica* even with that of a distant superfamily Yponomeutoidea, indicates that all known H-fibroins have conserved amino-terminus [27]. The striking feature of this region is the spacing of charged residues, especially the distribution of glutamate,

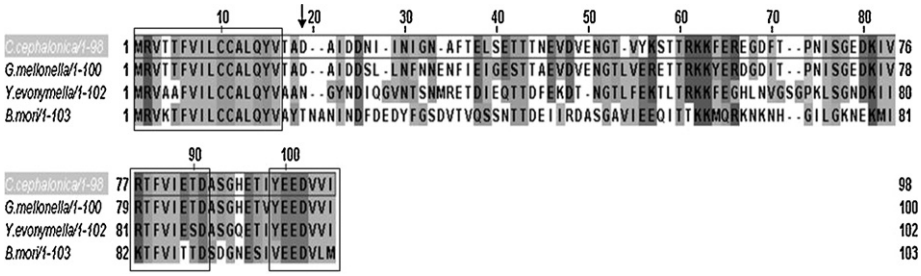

Fig. 1. Comparison of 5′ region of H-fibroin gene of *C. cephalonica* with other insect species. Sequence alignment of the deduced amino acid sequence of 5′-N-terminal region of *Corcyra cephalonica*, *Galleria mellonella*, *Yponomeuta evonymella* and *Bombyx mori* (GenBank accession nos. GQ901977, AF095239, AB195979 and AF226688 respectively). Conserved motifs are represented in rectangles. Arrow (↓) shows the putative signal peptide cleavage site.

each of them close to a valine or a similar residue. Conservation of such a pattern in all the compared insect species indicates their functional importance [2].

3.2. Effect of 20E on H-fibroin gene expression

Earlier studies in our laboratory showed that the salivary glands were viable in the culture medium till 24 h and hence the time points from 0.5 h to 24 h were chosen for various treatments [28]. The hemolymph levels of 20E during ELI, LLI larval and EPP stages were estimated by enzyme immunoassay and were found to be 0.008 pg/μl, 0.4 pg/μl and 5.7 pg/μl respectively [28]. These concentrations of 20E were considered as physiological levels of the respective stage. We examined the effect of physiological dose of a particular stage as well as the subsequent stage on the expression of H-fibroin gene using semi-quantitative (Figs. 2A and 3A) and quantitative PCR (Figs. 2B and 3B). The H-fibroin transcript levels of ethanol (0.05%) treated glands that were cultured through the same time points were used as external control. The physiological concentration of 20E during the LLI stage (0.4 pg/μl) did not alter the normal expression levels of H-fibroin, when compared with control. However, when the glands from LLI were treated with the hemolymph levels of 20E found during the EPP stage (5.7 pg/μl), there was a significant decrease in H-fibroin expression (Fig. 2B). Similarly, the physiological concentration of 20E during the ELI stage (0.008 pg/μl) did not change the expression pattern of H-fibroin in ELI glands as compared to control. However, when the ELI glands were treated for 12 h with the physiological levels of 20E found during LLI stage (0.4 pg/μl), the expression of H-fibroin declined significantly (Fig. 3B). The semi-quantitative PCR data (Figs. 2A and 3A) matches with that obtained in real-time PCR (Figs. 2B and 3B).

In *B. mori*, the function and development of silk glands were shown to be under hormonal control [1]. Maximum silk fibroin

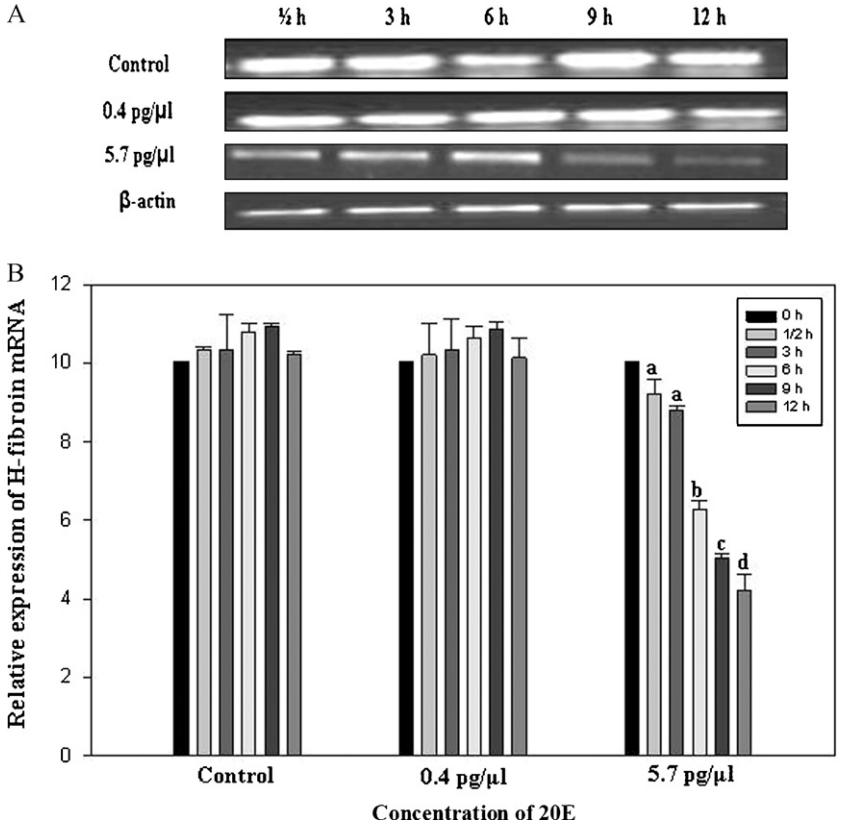

Fig. 2. Effect of 20E on H-fibroin expression of LLI larval silk glands. (A) Semi-quantitative PCR showing the expression at various time points (0.5, 3, 6, 9 and 12 h) in control (0.05% ethanol) and 20E (0.4 pg/μl and 5.7 pg/μl) treated glands. β-Actin was used as internal control. (B) Real-time PCR ($n = 3$) showing the expression at various time points in control (0.05% ethanol) and 20E (0.4 pg/μl and 5.7 pg/μl) treated glands. H-fibroin expression is reported as fold change relative to 0 h control. The values labelled with common letter do not differ significantly ($P < 0.05$), while the values with different letters differ significantly and are compared group-wise ($P < 0.05$).

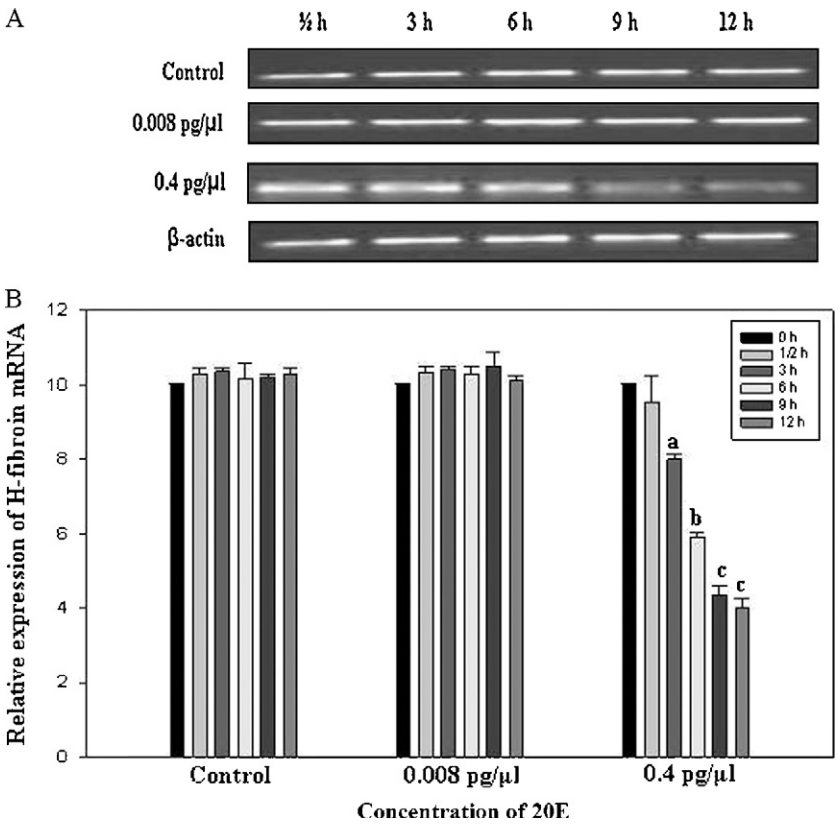

Fig. 3. Effect of 20E on H-fibroin expression of ELI larval silk glands. (A) Semi-quantitative PCR showing the expression at various time points (0.5, 3, 6, 9 and 12 h) in control (0.05% ethanol) and 20E (0.008 pg/μl and 0.4 pg/μl) treated glands. β-Actin was used as internal control. (B) Real-time PCR ($n = 3$) showing the expression at various time points in control (0.05% ethanol) and 20E (0.008 pg/μl and 0.4 pg/μl) treated glands. H-fibroin expression is reported as fold change relative to 0 h control. The values labelled with common letter do not differ significantly ($P < 0.05$), while the values with different letters differ significantly and are compared group-wise ($P < 0.05$).

synthesis and greater cellular changes were observed in the last larval instar [29]. In *G. mellonella* too, the silk glands were shown to readily respond to hormones during this stage [30]. Based on these reports, the last instar larvae were chosen to study the effect of 20E. The hemolymph titre of 20E varies among insects and at each stage [31,32]. Therefore, we estimated the concentration of 20E during ELI, LLI and EPP stages in *C. cephalonica*.

In *C. cephalonica*, *in vitro* treatment with physiological concentration of 20E found during LLI larval stage (0.4 pg/μl), did not alter the normal expression pattern of H-fibroin gene when compared with that of solvent. However, Sehnal and Akai [1] reported a high expression of silk fibroin during the last instar larval development of *B. mori* [1]. Hence, it was not clear whether the physiological dose of 20E has any effect on the H-fibroin expression. Therefore, we monitored the effect of physiological levels of 20E during the earlier stage, i.e. ELI stage, on H-fibroin expression. Once again the hemolymph levels of 20E during ELI stage (0.008 pg/μl) did not alter H-fibroin gene expression, when compared with the control. This study shows that the physiological dose of 20E facilitates the normal expression of H-fibroin gene during last instar. Earlier study on *B. mori* silk glands also suggested that low titers of ecdysteroid were necessary for proper silk gland function [33]. Further, in *G. mellonella*, *in vitro* application of 20E (0.5 μg/ml concentration) caused an increase in the level of PSG mRNAs. However, the stimulation was restricted to day 1 of last instar larvae after which no effect was found on the mRNA levels [34]. In cultured PSGs of *Galleria* larvae, it was observed that the effect of 20E on RNA synthesis was dose-dependent. Furthermore, the lower concentrations of 20E did not alter the RNA levels during the LLI stage [35]. Taken together and the finding that the physiological levels of 20E estimated during the ELI as well as the LLI stage of *C. cephalonica* were low, we suggest that probably low or physiological doses of 20E is required for the normal expression of H-fibroin gene.

On the other hand, higher concentrations of 20E exerted an inhibitory effect on the RNA synthesis in *G. mellonella* [35,36]. Further, in *B. mori*, treatment with high concentrations of ecdysteroids caused silk gland degeneration at the end of last instar larval development [1]. Our results in *C. cephalonica* further strengthen this point. Upon treatment of LLI larval silk glands with higher dosage of 20E (5.7 pg/μl seen during EPP stage), the expression of H-fibroin decreased significantly. Such a prominent effect of 20E was most likely mediated by ecdysone receptors present in the salivary glands cells [37]. Further, the pupal molt in various insect species is known to be associated with high titers of ecdysteroids [31,32]. The declined expression of L-fibroin, P25 [28] and H-fibroin (unpublished) during the pupal stage of insect development as well as the significant decrease in the expression observed upon treatment of LLI larval silk glands with dosage of 20E similar to that found during EPP stage supports the fact that higher concentrations of 20E cause a decline in the H-fibroin expression. Treatment of ELI larval silk gland with 0.4 pg/μl of 20E (the physiological concentration of 20E found during LLI larval stage) once again reconfirmed the declined expression of H-fibroin at higher concentration of ecdysteroid. Present study shows that H-fibroin is under 20E control and it regulates H-fibroin expression in a dose-dependant manner during the last instar larval development.

3.3. Identification of a novel functional ecdysone response element (EcRE) in the 5′ flanking region of H-fibroin gene

In the present study, a PCR product of 590 bp was amplified from the EcoRV library and the 5′ flanking region of H-fibroin

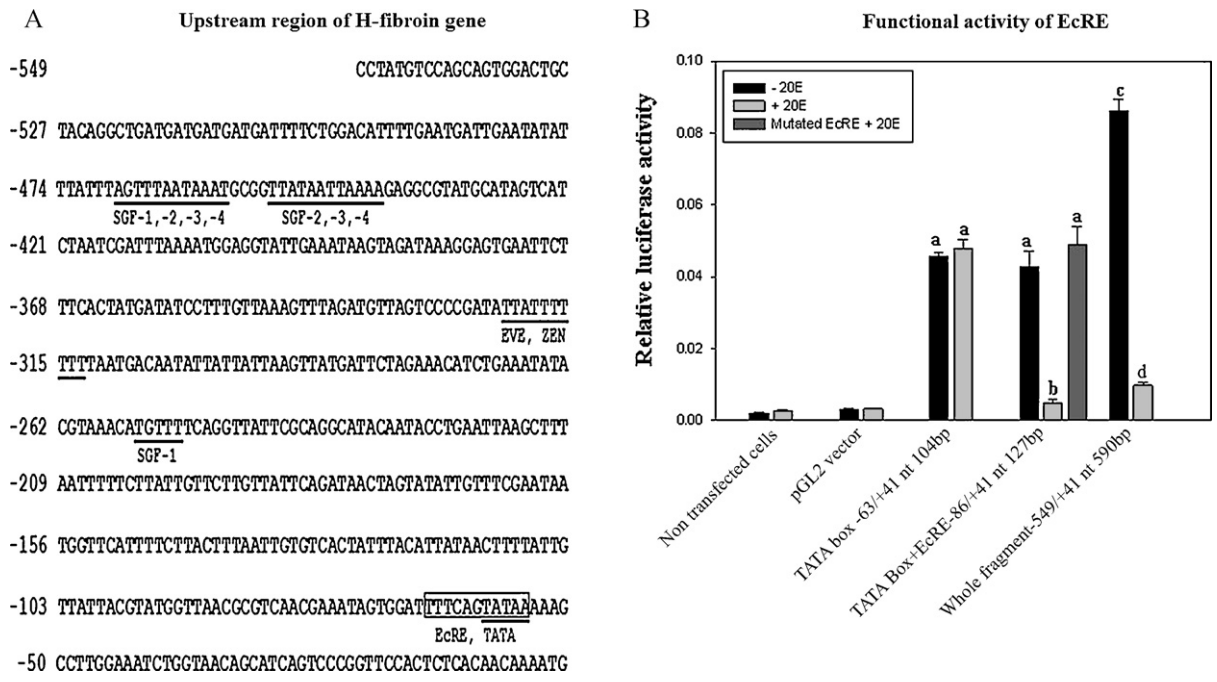

Fig. 4. Functional activity of ecdysone response element (EcRE) present in the 5′ flanking region of H-fibroin gene of *C. cephalonica*. (A) Sequence of 5′ upstream region of the *C. cephalonica* H-fibroin gene (GenBank accession no. GQ901977). The potential binding sites for specific transcription factors are underlined. EcRE sequence is represented in a rectangle. (B) Luciferase activity of different H-fibroin promoter constructs co-transfected with pRL-TK into EcR293 cells in the presence or absence of 20E. The enzyme activity is presented as relative to the activities measured for *Renilla* luciferase. The values labelled with common letter do not differ significantly ($P < 0.05$), while the values with different letters differ significantly and are compared group-wise ($P < 0.05$).

was cloned. The upstream analysis revealed a TATA sequence at −59 relative to the ATG sequence. An ecdysone response element (EcRE) was identified at −65 position that overlaps with the TATA box. A silk gland specific factor binding site (SGF-1) is located at −254 position. A ten base A+T region that consists of TAAT motif in the core is found to be the binding site for posterior silk gland specific (SGF2) and ubiquitous (SGF-3 and SGF-4) factors. At −322 position, *Drosophila* homeodomain proteins, EVE (*even-skipped*) and ZEN (*zerknullt*) binding site is harbored (Fig. 4A).

Structural analyses of the 5′ flanking region of the H-fibroin gene from several strains of *B. mori* [38,39] and a closely related species, *B. mandarina* [40] showed that the immediate 5′ flanking sequence, which is highly conserved, was probably important for its expression. The binding sites for SGF-1, -2, -3 and -4 factors, homeodomain proteins which are found in 5′ flanking sequence of *B. mori* fibroin [38,39], were also seen in the H-fibroin upstream region of *C. cephalonica*. It was also predicted that SGF-1 might play an important role in the expression of silk protein genes, because it also binds to a similar site in sericin-1 gene. The regions for SGF-2, -3, and -4 contain ten-base pairs of A+T repeats. The TAAT motif in the core region was shown to be important for the binding of these three proteins and also *Drosophila* homeodomain proteins, EVE (*even-skipped*) and ZEN (*zerknullt*) [41]. In this context, it is noteworthy that an EcRE sequence overlapping the TATA box is also present in the H-fibroin upstream region of *B. mori* [42]. However, it was not analyzed for its functional activity. The consensus sequence of EcRE obtained from the comparison of various hormone response elements was shown to be (G/T)NTCANTNN(A/C)(A/C) [13–16]. The identified sequence of EcRE in the H-fibroin of *C. cephalonica* is TTTCAGTATAA, which is well in agreement with the above mentioned consensus sequence. Based on the upstream sequence, PCR based progressive deletion constructs were generated and gene reporter assays were performed in engineered HEK-293 cells.

Luciferase activity assay was performed primarily to find out the functional activity of the *C. cephalonica* EcRE in the presence or absence of 20E. The H-fibroin gene expression declined significantly at LLI larval stage of *C. cephalonica* in the presence of 5.7 pg/μl of 20E (Fig. 2A). Therefore, this concentration of 20E was used in luciferase as well as gel shift assays. The −63/+41 construct which contained TATA box showed similar activity both in the presence or absence of 20E suggesting that this region is not affected by 20E. A significantly lower activity of the −86/+41 construct in the presence of 20E, that harbors both TATA box and EcRE suggests that EcRE might act as a negative regulatory element. The mutated EcRE construct as well as the wild type construct in the absence of 20E did not show significant changes in promoter activity when compared with the −63/+41 construct which consisted TATA box (Fig. 4B). Furthermore, the whole upstream fragment 590 bp also showed diminished activity in the presence of 20E (Fig. 4B).

These results strengthen the view that EcRE present in the upstream is functional and regulates H-fibroin expression in *C. cephalonica*. The presence of functional EcRE indicates that 20E effect on H-fibroin expression is probably mediated by EcR as described in the case of other 20E regulated genes [13,17].

3.4. EcR binds to EcRE upon 20E induction

The presence of EcR in the nuclear extract was confirmed by immunoblot (Fig. 5A). 20E induced silk gland nuclear extract demonstrated a complex formation with [γ^{32}P] dATP labelled oligomeric sequence containing EcRE motif, while no complex was observed in the absence of 20E induction. The shift in the band observed when probed with EcR specific antibody further confirms the binding. The mutated oligonucleotide did not show any binding in the presence of 20E (Fig. 5B).

Upon 20E exposure, puffing responses of the polytene chromosomes in *D. melanogaster* salivary glands were observed. In this model, 20E binds with an ecdysone receptor protein and directly activates the transcription of a small set of early genes, which then activate many late genes [43]. The presence of EcR and its induction by 20E was also shown in *B. mori* silk glands [20]. These studies

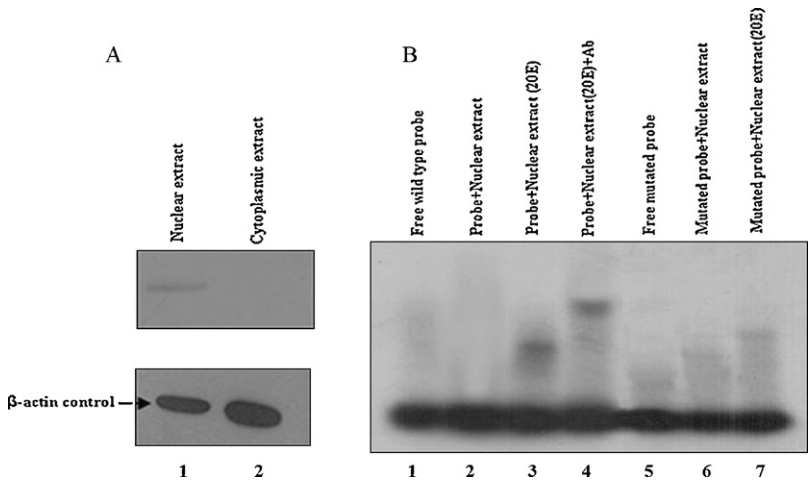

Fig. 5. EcR mediated 20E regulation of H-fibroin. (A) Nuclear and cytoplasmic extracts (40 μg each) from the larval silk glands were immunoblotted against anti-ecdysone receptor antibody. Please note the presence of cross reactivity in the nuclear extract (lane 1). β-Actin was used as loading control. (B) Gel shift assay showing the binding of EcR protein to EcRE oligomer in the presence of 20E. Lane 1: [γ^{32}P] labelled wild type oligo, lane 2: nuclear extract and the radiolabelled wild type oligo, lane 3: 20E induced nuclear extract and radiolabelled wild type oligo, lane 4: 20E induced nuclear extract and radiolabelled wild type oligo probed with anti-ecdysone receptor antibody, lane 5: [γ^{32}P] labelled mutated oligo, lane 6: nuclear extract and the radiolabelled mutated oligo and lane 7: 20E induced nuclear extract and radiolabelled mutated oligo.

clearly suggest that 20E regulates various processes in the salivary gland via EcR, which control gene transcription by binding to specific sequences. An EcRE in the upstream region of *Hsp*27 promoter was previously shown in *Drosophila* [13]. Treatment with ecdysone, *in vitro*, enhanced the DNA binding capability of the receptor. The most extensively studied promoters are the four small heat shock protein genes of *Drosophila*, were shown to be activated by ecdysone as well as heat shock [17]. In the present study, in the absence of 20E there was no complex formation and hence no signal is observed. The mutated oligonucleotide did not bind to EcR even after induction by 20E further confirms that EcR binds specifically to EcRE sequence in the upstream region of H-fibroin gene, only when induced by 20E. Thus, it would be reasonable to hypothesize that 20E induces EcR pairing with RXR in the absence of USP and predictably binds to the EcRE region [12,14] that might regulate the H-fibroin gene expression.

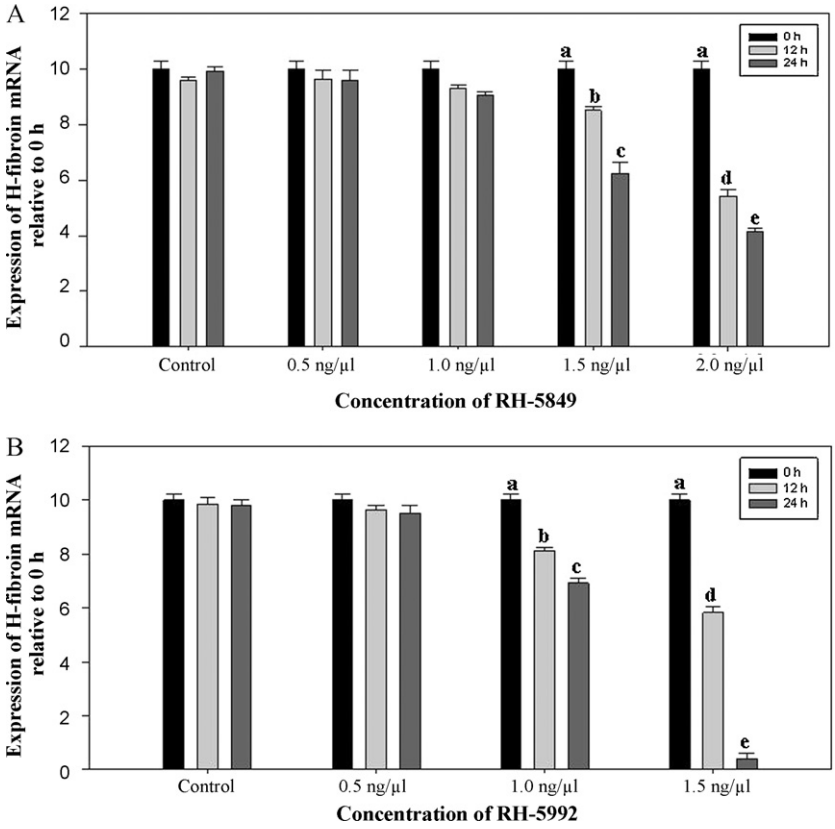

Fig. 6. Effect of RH-5849 and RH-5992 on H-fibroin expression. (A) Relative expression of H-fibroin gene upon treatment with increased concentrations of RH-5849, 0.5 ng/μl, 1 ng/μl, 1.5 ng/μl, 2 ng/μl and 0.05% ethanol (control). (B) Relative expression of H-fibroin gene upon treatment with increased concentrations of RH-5992, 0.5 ng/μl, 1 ng/μl and 1.5 ng/μl and 0.05% ethanol (control). The values labelled with common letter do not differ significantly ($P < 0.05$), while the values with different letters differ significantly and are compared group-wise ($P < 0.05$).

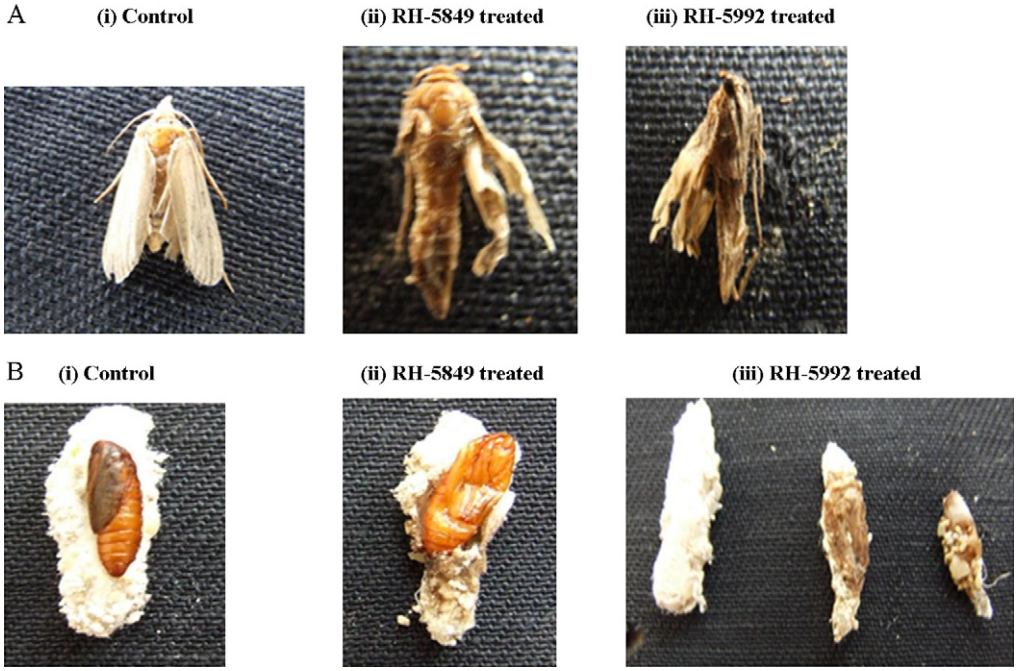

Fig. 7. Effect of RH-5849 and RH-5992 treatment on insect metamorphosis. For this experiment the LLI larvae were treated with RH-5849 (2 ng/μl) and RH-5992 (1.5 ng/μl) and were allowed to undergo development and adult emergence. (A) (i) control—normal adult, (ii) RH-5849 treated defective adult and (iii) RH-5992 treated defective adult (B) Cocoons obtained from (i) control—normal pupa, (ii) RH-5849 and (iii) RH-5992 treated insects.

3.5. Effect of RH-5849 and RH-5992 on H-fibroin expression, larval–pupal–adult transition and the ultrastructure of silk fibers

Increasing doses of RH-5849 (0.5, 1, 1.5 and 2 ng/μl) (Fig. 6A) and RH-5992 (0.5, 1 and 1.5 ng/μl) (Fig. 6B) were added to the glands and the expression of H-fibroin gene was monitored. The H-fibroin expression decreased significantly upon treatment with 2 ng/μl of RH-5849 *in vitro* (Fig. 6A). This decline was more prominent in the cultured glands that received RH-5992 at a concentration of 1.5 ng/μl (Fig. 6B).

RH-5849 and RH-5992 are bisacylhydrazine insecticides that exhibit their activity via interaction with the ecdysteroid receptor proteins. Upon treatment with these compounds, a state of ecdysteroid excess, called hyperecdysonism is induced and the insecticides bind to the ecdysteroid receptor complex with high affinity [24]. Changes in EcR expression were also observed in RH-

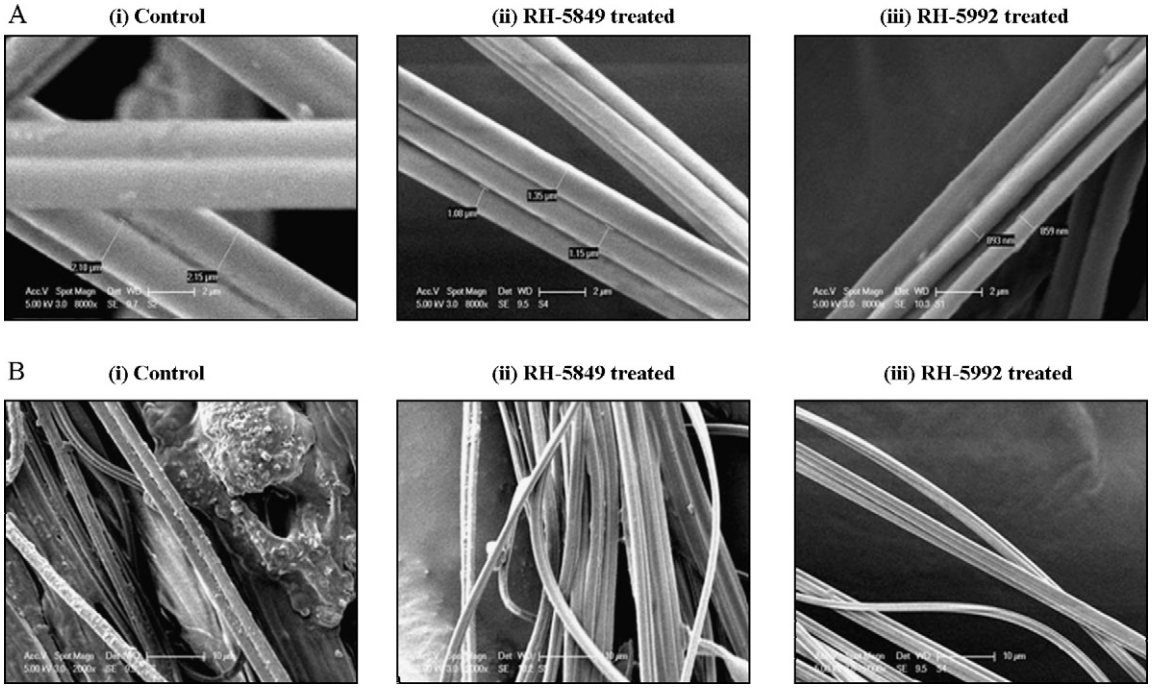

Fig. 8. Effect of RH-5849 and RH-5992 on ultrastructure of silk fibers. (A) Scanning electron micrograph showing the fibers from (i) control insects, (ii) RH-5849 fed insects and (iii) RH-5992 fed insects. All the images were captured at same magnification. (B) Sticky coating found in (i) control silk fibers, was absent in (ii) RH-5849 and (iii) RH-5992 fed insects. All the images were captured at same magnification.

5992 treated larvae of *Cydia pomonella* [44]. Results obtained in the present study indicate that RH-5849 and RH-5992 mimic the 20E excessive state that might cause the down regulation of H-fibroin gene via EcR complex.

In the current study, when the insects were fed with similar concentrations of these insecticides, they metamorphosed into abnormal adults (Fig. 7A). Precocious slippage of head capsule was observed (Fig. 7A; ii and iii). This was also associated with high mortality of insects during pupal stage due to improper metamorphosis and defective cocoon formation (Fig. 7B; ii and iii). Those which survived metamorphosed into defective adults (Fig. 7A; ii and iii). Previous studies also reported that insecticide intoxicated larvae prematurely slipped their old head capsules in an attempt to ecdyse [24]. In *Manduca sexta*, RH-5849 halted feeding and resulted in premature lethal molt [45]. Based on these reports and our observations, it is clear that the RH-5849 and RH-5992 treatment caused a disruption in the normal larval–pupal–adult transition, in lepidopteran insects.

Differences were also observed in the ultrastructure of the silk fibers of control and insecticide fed *C. cephalonica* larvae (Fig. 8). The mean diameter of a single fiber of RH-5849 fed insects was 1.19 μm (Fig. 8A; ii) and that of RH-5992 fed insects was 876 nm (Fig. 8A; iii). A remarkable decrease in the diameter of the fiber was evident, when the aforementioned values were compared with the control, which shows a mean fiber diameter of 2.16 μm (Fig. 8A; i). The fibers from insects treated with either RH-5849 or RH-5992 were found to be devoid of sticky coating (bleb like structures on the fiber) (Fig. 8B; ii and iii) which were seen in the control (Fig. 8B; i). It is known that the sticky coating of the silk fiber is provided by sericin proteins, which are secreted in the MSG [46,47]. However, it remains to be investigated whether sericin expression is down regulated upon treatment with these hormone analogs and thus leads to decrease in the overall diameter of the silk fiber.

4. Conclusion

In conclusion, our results describe 20E regulation of H-fibroin at transcriptional level during the last instar development of *C. cephalonica*. 20E modulates H-fibroin expression in a dose dependent manner. The action of 20E is mediated through EcR that binds to the functional EcRE shown in the upstream region of H-fibroin gene. To our knowledge, this study provides the first report of an EcRE in H-fibroin promoter. The various effects observed upon non-steroidal ecdysteroids agonist treatment further signify the role of 20E mediated regulation of H-fibroin in insect development. More studies on such silk producing insect pests in this direction may provide an insight for their efficient control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2010.09.009.

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Original Article

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Dimorphic Expression of Various Transcription Factor and Steroidogenic Enzyme Genes during Gonadal Ontogeny in the Air-Breathing Catfish, *Clarias gariepinus*

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Key Words
Catfish · Dimorphic expression · Gonadal development · Ovary · Sex differentiation · Teleosts · Testis

Abstract
In the present study the expression of 13 genes known to be involved in sex differentiation and steroidogenesis in catfish was analyzed during gonadal ontogeny by quantitative real-time RT-PCR. *Dmrt1* and *sox9a* showed exclusive expression in male gonads while ovarian aromatase *(cyp19a1)* and *foxl2* were abundant in differentiating female gonads. Most of the genes related to steroidogenesis were expressed only after gonadal differentiation. However, genes coding for 3β-hydroxysteroid dehydrogenase *(3β-hsd)*, 17α-hydroxylase/C17–20 lyase type 1 *(cyp17)* and steroidogenic acute regulatory protein *(star)* were barely detectable during gonadal differentiation. Ovarian aromatase, *cyp19a1,* which is responsible for estradiol-17β biosynthesis in females, was expressed very early in the undifferentiated gonads of catfish, around 30–40 days post hatch (dph). The steroidogenic enzyme, 11β-hydroxylase *(cyp11b1)* required for the production of 11-ketotestosterone (11-KT) was expressed only after differentiation of testis. These results suggest that estradiol-17β has a critical role in ovarian differentiation, while the role of 11-KT in testicular differentiation is doubtful. In conclusion, dimorphic expression of *dmrt1* and *sox9a* in gonads during early development is required for testicular differentiation, and sex-specific expression of *cyp19a1* and *foxl2* in females plays a critical role in ovarian development. Our study reveals that the critical period of gonadal differentiation in catfish starts around 30–40 dph when sex-specific genes showed differential expression.

Sexual reproduction is an important event which enables an organism to propagate and transfer genetic information from one generation to another. Successful reproduction usually depends on events that begin early in the reproductive life cycle. In vertebrates, sex determination and differentiation are the two important events in the development of gonads [Hughes, 2001]. Sex determination is the genetic (sex chromosomes) or environmental process by which the sex of an individual is established [Hughes, 2001; Kondo et al., 2009]. Sex determination is extremely diverse and lacks conservation in vertebrates. Sex differentiation refers to gonadal development after sex determination, when an indifferent/bipotential gonad develops into testis or ovary, and is controlled by var-

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ious factors/genes and hormones [Hughes, 2001]. Sex differentiation appears to be relatively conserved in vertebrates across the phyla. There are several factors like DMY/DMRT1, SOX9, AD4BP/SF-1 (NR5A1) and AMH which are implicated in testicular differentiation, while CYP19A1 and FOXL2 promote ovarian differentiation in most vertebrates [Hughes, 2001; Matsuda et al., 2002; Nanda et al., 2002; Nagahama, 2005; Guerrero-Estévez and Moreno-Mendoza, 2010]. Teleosts are excellent models for studying the events of sex determination and differentiation from an 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]. Further, their sex can be manipulated or completely reversed by exogenous sex steroid treatment around the critical period of sex determination/differentiation [Nagahama, 2005; Kobayashi et al., 2008; Raghuveer and Senthilkumaran, 2009].

Considering the knowledge gained in mammalian sex determination and differentiation studies, in-depth research work in teleosts related to this area is mostly restricted to daily or fortnight breeders like zebrafish, medaka and tilapia. In this regard, teleosts which show a seasonal (annual breeders) pattern of gonadal attenuation and recrudescence (reproductive cycle) are good models for comparative analysis and may provide interesting insights in understanding the expression pattern of sex-specific genes, not only during gonadal development, but also during gonadal recrudescence. Our laboratory is working on a teleost fish model, *Clarias gariepinus* (commonly known as North African/air-breathing catfish), in order to unravel the role and expression pattern of various transcription factors and steroidogenic enzyme genes during sex differentiation and gonadal development. The catfish is a gonochoristic annual breeder having a seasonal reproductive cycle. The present study combines the histological analysis of gonads and the expression pattern of genes/factors during the process of gonadal ontogeny. We examined the sex specificity and precise timing of the expression of genes encoding transcription factors *(wt1*, *dmrt1*, *sox9a*, *sox9b*, *sox3*, *ad4bp/sf-1, foxl2)*, steroidogenic enzyme genes *(3β-hsd*, *cyp17*, *17β-hsd1*, *cyp19a1, cyp11b1)* and the sterol transfer protein/steroidogenic acute regulatory protein coding gene *(star)* during gonadal differentiation and later stages of gonadal development after differentiation until maturity in catfish. For this purpose, the transcript levels of these 13 genes were analyzed by quantitative real-time PCR. Further, we also performed immunolocalization studies for few sex-specifically expressed proteins (Dmrt1, Sox9, Cyp19a1, and Foxl2) during gonadal development in catfish.

Materials and Methods

Animals and Sampling

Catfish *(C. gariepinus)* at different age groups were reared in fresh water tanks under ambient photothermal conditions. To obtain different age groups of catfish hatchlings, in vitro fertilization was performed during the breeding season using mature male and gravid female catfish injected (intraperitoneally) with human chorionic gonadotropin (500 IU/100 g body weight). The fertilized catfish embryos were transferred to small-size glass tanks containing filtered water with aeration. Generally catfish embryos take 24 h to hatch and the hatchlings can survive for 2–3 days without any external feed by utilizing the yolk sac for nutrition. Later on, the hatchlings were fed with live tubeworms until 3 months. Juvenile 3- to 8-month-old catfish were fed with commercially available fish feed pellets along with tubeworms. Catfish take about a year to mature which marks the beginning of the reproductive cycle. Catfish at different age groups (0, 10, 20, 30, 40, 50, 75, 100, 150, 200 and 300 days post hatch (dph)) were collected and gonads were isolated for total RNA extraction as described earlier [Raghuveer and Senthilkumaran, 2010a]. In brief, the mesonephric gonadal complex (MGC) was isolated using fine sterile forceps under a stereoscopic dissection microscope from catfish hatchlings at 10, 20, 30 and 40 dph. The whole body was used for 0 dph (less than 24 h after hatching). The MGCs of fishes of the same age group were pooled (5 hatchlings per sample; n = 3) to get a sufficient amount of total RNA. In catfish, morphological distinction of male and female gonads is possible from 50 dph onwards [Raghuveer and Senthilkumaran, 2009]. Therefore, male and female gonads of catfish (3 fishes per sample; n = 3) at 50, 75, 100, 150, 200 and 300 dph were isolated using fine sterile forceps for total RNA extraction.

Histological Studies

The male and female gonads of catfish hatchlings at different age groups (28, 40, 45, 52, 70, 90 and 160 dph) were fixed in Bouin's solution, dehydrated and embedded in paraplast (Sigma). For light microscopic histological examination, 5-μm sections were cut using a microtome (Leica, Wetzler, Germany) and stained with hematoxylin-eosin. All photomicrographs were taken with an Olympus CX41 bright field light microscope (Olympus, Tokyo, Japan) fitted with a digital camera.

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

All target genes analyzed and the endogenous control *(β-actin)* used in the present study were previously cloned from catfish. The GenBank accession numbers of the genes are as follows: Wilms' tumor gene 1, *wt1* (JF510005); doublesex and mab-3 related transcription factor 1, *dmrt1* (FJ596554); SRY-related homeobox gene 9, *sox9a* (HM149258) and *sox9b* (HM149259); SRY-related homeobox gene 3, *sox3* (HQ680982); *ad4bp/sf-1* (HQ680985); forkhead box L2, *foxl2* (HQ680981); cytochrome P450 aromatase type A1 or ovarian aromatase, *cyp19a1* (GU220075); 3β-hydroxysteroid dehydrogenase/Δ4–Δ5 isomer-

Table 1. List of primers used for quantitative real-time PCR analysis

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
wt1	ACGCGCACAGGGTGTTCGA	GGTACGGTTTCTCTCCTTGTG
dmrt1	GCAGAGCTCAGCAAAACCCGG	GCGGCTCCCAGAGGCAGCAGGAGA
sox9a	TCTGGCGGCTGCTGAATGAAGG	CTCGGTATCCTCGGTTTCACC
sox9b	GAGACCCAGTCAGGCCACAG	AGGGTCTCGATGTGGGCCA
sox3	CACGGTATGAGTAGCCCACCA	GCGATGGCAGGTGGTGGTGAG
ad4bp/sf-1	TCACTATG CACCTGCCT	CGCTTGTACATGGGGCCGAAC
foxl2	CATGGCTATACGCGACAGCTC	CCAGTAGTTCCCCTTCCTCTC
3β-hsd	GAGGTAAATGTGAAAGGTACCAA	TAGTACACAGTGTCCTCATGG
cyp17	CCATGGCTCCAGCTCTTTCC	CAGTAAGACCAACATCCTGAGTGC
star	TCGTCCGAGCCGAGAACGG	TGCCTCCTCCACTCCACTG
cyp19a1	AGGTCCCTGGTTTTGTCTG	TGCAGATGGCCTGCTGAGG
17β-hsd1	GACATCCTGGTGTGTAATGCAGG	CTGCCTGTGACCAGGATCCGT
cyp11b1	GGCAGTGGAGCGAATGCTGAA	GCACCCCGGGGAACCAGC
β-actin	ACCGGAGTCCATCACAATACCAGT	GAGCTGCGTGTTGCCCCTGAG

ase, *3β-hsd* (HQ680983); 17α-hydroxylase/C17–20 lyase type 1, *cyp17* (FJ790422); steroidogenic acute regulatory protein, *star* (FJ793811); 17β-hydroxysteroid dehydrogenase type1, *17β-hsd1* (HQ680984); and cytochrome P450 hydroxylase type B or 11β-hydroxylase, *cyp11b1* (HQ680986). Total RNA was extracted from gonadal samples of different stages using the Sigma TRI-reagent method and quantified using a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription (RT) was carried out with 1 μg of total RNA and random hexamer primers using superscript-III (Invitrogen) according to the manufacturer's protocol. Successful RT was confirmed for all samples by performing PCR amplification of *β-actin* as an endogenous control. The primer sets used for qRT-PCR (table 1) were designed using Primer Express software (Applied Biosystems, Foster City, Calif., USA) such that at least one primer in each set flanked the intron-exon boundary to prevent amplification from genomic DNA. All qRT-PCRs were carried out in 20-μl reactions in triplicates using power SYBR Green PCR master mix (Applied Biosystems) in an ABI-7500 fast real time PCR machine (Applied Biosystems) at an initial hold of 95°C (10 min) then followed by a 2-step PCR reaction of 95°C (15 s) and 60°C (1 min) for 40 cycles according to the manufacturer's protocol. Dissociation or melting curve analysis was performed for each gene to check for single amplification. During PCR, fluorescence accumulation resulting from DNA amplification was recorded using the ABI 7500 sequence detection system (SDS) software (Applied Biosystems). Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification by SDS software. We used *β-actin* as an endogenous control as it did not show any significant change in expression at different stages. The target gene expression was normalized against *β-actin* expression generating a Ct value (Ct = Ct of target gene – Ct of *β-actin*). Relative expression of the target gene was then calculated according to the equation $2^{-\Delta Ct}$.

Immunohistochemistry

Immunohistochemistry (IHC) was performed to localize Dmrt1, Sox9, Cyp19a1 and Foxl2 proteins in differentiated gonads of catfish. For this, juvenile male and female catfish gonads at 52 dph were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), processed and embedded in paraplast (Sigma). Sections of 5 μm thickness were spread on sterile glass slides coated with polylysine, deparafinized in xylene and rehydrated in successively lower-graded concentrations of ethanol. The sections were then treated with 0.1% H_2O_2 for 15 min to prevent endogenous peroxidase reaction. The sections were washed twice in 0.1 M PBS, 1% Tween 20 and then blocked using 10% normal goat serum (Bangalore Genei, Bengaluru, India) in 0.1 M PBS for 10 min at room temperature (25°C). Sections were then incubated overnight at 4°C either with Dmrt1, Sox9, Cyp19a1, or Foxl2 antibodies. The specificity of Dmrt1 [Raghuveer and Senthilkumaran, 2009], Sox9 [Raghuveer and Senthilkumaran, 2010b] and Foxl2 [Sridevi and Senthilkumaran, unpublished data] antibodies used for IHC were evaluated previously in our laboratory. In brief, we used catfish-specific antibodies that were raised in our laboratory for the localization of Dmrt1 and Foxl2 proteins. For the detection of Cyp19a1, we used a heterologous antibody specific to tilapia Cyp19a1 protein. Human SOX9-specific antibody (heterologous) was used for detection of Sox9a in catfish [Raghuveer and Senthilkumaran, 2010b]. Following incubation with primary antibody, sections were washed in PBS, 0.1% Tween 20 (PBST) for 10 min and then incubated with 1:500 dilution of biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, Calif., USA) for the horseradish peroxidase detection. Sections were 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 (DAB) as chromogen and H_2O_2 as substrate for horseradish peroxidase (Vector Laboratories). The sections were washed, dehydrated in graded ethanol series, cleared in xylene and mounted using DPX mountant. Immunofluorescence using FITC-conjugated anti-rabbit secondary antibody (Invitrogen) was employed for localizing Dmrt1 protein. After the secondary incubation the sections were washed with PBS and mounted in 90% glycerol. Photomicrographs were taken using Olympus CX41 fluorescence cum light microscope.

Statistical Analysis

The data from real-time PCR were expressed as mean ± SEM of at least 3 independent samples. Significant differences in the data between male and female groups after differentiation (50 dph onwards) were compared using Student's t test. A probability of $p < 0.05$ was considered statistically significant.

Results

Histological Observation of Gonadal Development in Catfish

Conventional histological methods were used to study the onset of gonadal sex differentiation in catfish. A primitive gonad with primordial germ cells (PGCs) surrounded by supporting cells was observed in the abdomen region near the coelomic cavity at 28 dph (fig. 1A). At 40 dph, the formation of 2 bipotential gonads protruding from the mesonephros was observed (fig. 1B). Higher magnification of the bipotenial gonad showed development of germ cells surrounded by somatic/supporting cells (fig. 1C). The first sign of morphological differentiation of gonads was evident by formation of the ovarian cavity in the developing female gonad around 45 dph (fig. 1D). The differentiated ovary at 52 dph showed the presence of few meiotic oogonia and perinucleolar/primary growth oocytes (fig. 1E). The female gonads were completely filled with pre-vitellogenic oocytes and few perinucleolar oocytes at 70 and 90 dph (fig. 1F, G) and showed different stages of oocytes (perinucleolar and pre-vitellogenic) at 160 dph (fig. 1H).

Testicular differentiation in catfish begins around 52 dph when developing germ cells (spermatogonia) surrounded by supporting/Sertoli cells were noticed (fig. 1I). Male and female gonads at 70 and 90 dph showed the proliferation and differentiation of spermatogonia and oocytes, respectively (fig. 1J, K). The completely developed testis at 160 dph showed different stages of spermatogonial cells, spermatocytes and few spermatids/sperm in the lumens indicating the progression of spermatogenesis (fig. 1L). These results suggest that testicular differentiation is delayed and takes more time than ovarian differentiation.

Expression Profile of Transcription Factors: wt1, dmrt1, sox9a, sox9b, foxl2, ad4bp/sf-1 and sox3

In catfish, *wt1* expression was seen in the MGC before gonad differentiation, around 20–30 dph (fig. 2A). Its expression was found to be high in undifferentiated gonads at 40 dph. Later on, after differentiation of gonads (50 dph onwards), *wt1* was expressed at significantly higher levels in male gonads than in female gonads (* $p < 0.05$) (fig. 2A). However, its expression disappeared in both male and female gonads at maturity. The expression of *dmrt1* was observed at low levels in 40 dph undifferentiated gonads (fig. 2B). Thereafter, its expression increased significantly in differentiated male gonads at 50 dph and then gradually increased until 150 dph (fig. 2B). On the other hand, *dmrt1* expression was negligible or undetectable in female gonads during gonadal ontogeny. Previously, we isolated 2 duplicate isoforms of *sox9* which were named *sox9a* and *sox9b* [Raghuveer and Senthilkumaran, 2010b]. In this study, *sox9a* and *sox9b* transcripts were first noticed at low levels in indifferent gonads at 30 dph and at moderately high levels in bipotential gonads around 40 dph (fig. 2C, D). Later on, *sox9a* expression was observed only in male gonads at 50 dph and gradually decreased until 100 dph (fig. 2C). Then its expression remained stable in male gonads till maturity. Conversely, *sox9b* mRNA was detected at significantly high levels in female gonads at 50 and 75 dph. Thereafter, its expression decreased gradually marking the progression of ovarian development (fig. 2D). In developing male gonads, *sox9b* expression was barely detectable or negligible (fig. 2D). Expression of *sox3* was noticed from 0 dph till 300 dph (fig. 3A). It was almost similar in both male and female gonads from 50 dph to 150 dph. However, its expression was significantly higher in males compared to females at 200–300 dph (* $p < 0.05$) (fig. 3A). Real-time PCR analysis revealed low levels of *foxl2* transcripts in the MGC of hatchlings at 20 and 30 dph (fig. 3B). The transcript was present in undifferentiated gonads at 40 dph, increased considerably in female gonads at 50 dph and thereafter decreased gradually till 100 dph (fig. 3B). In male gonads, *foxl2* was expressed at barely detectable or negligible levels throughout gonadal development (** $p < 0.001$) (fig. 3B). In catfish, prominent expression of *ad4bp/sf-1* was seen in indifferent gonads at 30 and 40 dph (fig. 3C). Thereafter, it is expressed in both differentiated male and female gonads until maturity, with significantly higher levels in females (* $p < 0.05$) (fig. 3C).

Expression Profile of Genes Involved in Steroidogenesis: 3β-hsd, cyp17, star, cyp19a1, 17β-hsd1 and cyp11b1

3β-hsd, *cyp17* and *star* transcripts were detectable at very low levels in undifferentiated gonads at 40 dph (fig. 4A–C). These genes showed significantly higher expression in male gonads compared to female gonads during ontogeny (* $p < 0.05$; ** $p < 0.001$) (fig. 4A–C). Further, their expression gradually increased in both gonads

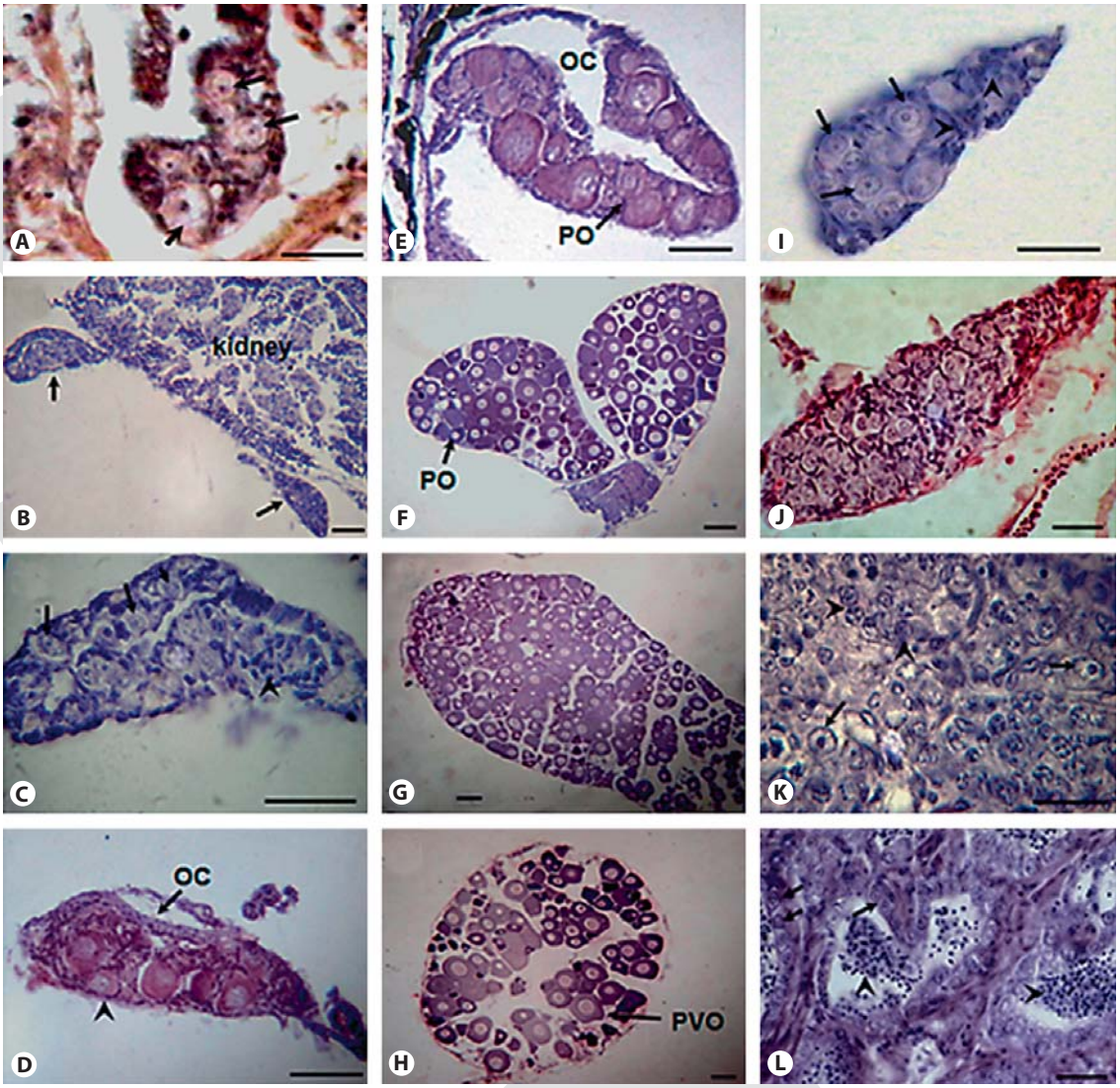

Fig. 1. Histology of gonads at different stages of development in catfish. **A** Primitive gonad at 28 dph showing PGCs (arrows) surrounded by somatic/supporting cells. **B** Gonadal section at 40 dph showing bipotential/indifferent gonads (arrows) attached to the mesonephros. **C** Section of bipotential gonad at high magnification showing germ cells (arrows) surrounded by supporting cells (arrowheads). **D** Differentiating female gonad at 45 dph with perinucleolar oocytes (arrowhead). Arrow indicates the ovarian cavity. **E** Differentiated ovary at 52 dph showing primary growth oocytes (arrow). **F** Developing ovary at 70 dph filled with primary growth/perinucleolar oocytes (arrow). **G**, **H** 90 and 160 dph ovary, respectively, showing primary growth and pre-vitellogenic oocytes (arrow). **I** 52 dph differentiating testis having germ cells (arrows) surrounded by supporting cells (arrowheads). **J** 70 dph developing testis showing many proliferating primary spermatogonia (arrows). **K** Testis section at 90 dph showing primary spermatogonia (arrows) and differentiating spermatogonia (arrowheads). **L** 160 dph testis showing lumens filled with spermatids/spermatozoa (arrowheads) indicating progression of spermatogenesis. Arrows indicate primary spermatogonia. OC = Ovarian cavity; PO = primary growth/perinucleolar oocytes; PVO = pre-vitellogenic oocytes. All scale bars indicate 50 μm.

as the development proceeds till maturity (fig. 4A–C). *Cyp19a1* transcript was detectable in undifferentiated gonads at 30 and 40 dph (fig. 4D). Thereafter, its expression was significantly higher in females than in males throughout development (** $p < 0.001$) (fig. 4D). *17β-hsd1* expression was noticed in both gonads after sex differentiation from 50 dph until 300 dph with abundant levels in females and very low levels in males (** $p < 0.001$) (fig. 4E). *Cyp11b1* expression was not detectable in undifferentiated gonads until 40 dph (fig. 4F). Its expression became

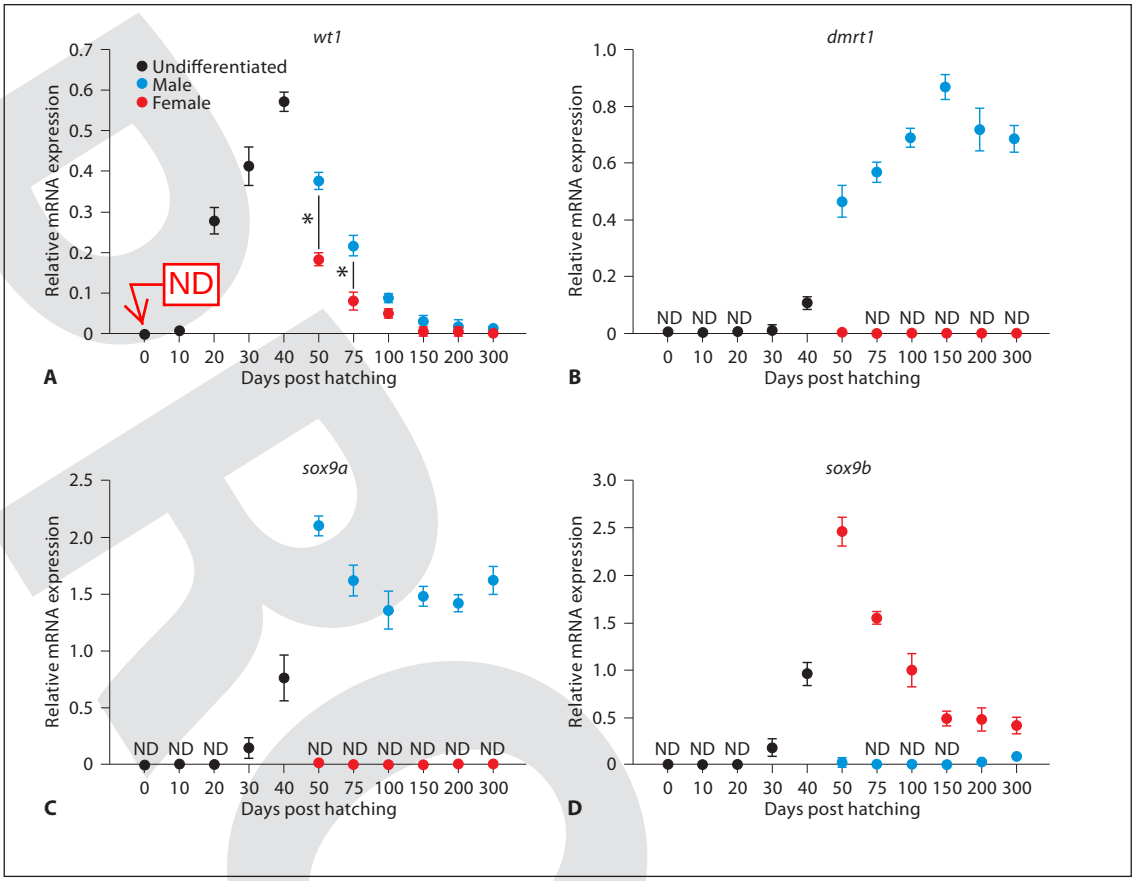

Fig. 2. Relative mRNA expression in catfish gonads from 0 to 300 dph. **A** *wt1* (Wilms' tumor gene type 1), **B** *dmrt1* (doublesex and mab-3 related transcription factor 1), **C** *sox9a* (SRY-related homeobox gene 9 isoform a) and **D** *sox9b* (SRY-related homeobox gene 9 isoform b). ND = Non-detectable level. Statistical differences between male and female groups were determined by Student's t test (* $p < 0.05$).

apparent only in differentiating male gonads from 50 dph to 100 dph, further increasing during the initiation of spermatogenesis in the developing testis at 150–200 dph (fig. 4F). However, barely detectable levels of *cyp11b1* were noticed in female gonads around 200–300 dph when compared to male gonads ($p < 0.001$) (fig. 4F).

IHC Localization of Dmrt1, Sox9, Cyp19a1 and Foxl2 in Differentiated Gonads

We studied the localization of Dmrt1 and Sox9 in differentiated male gonads at 52 dph using IHC. Similarly, Foxl2 and Cyp19a1 expression was analyzed in developing female gonads. IHC studies revealed Dmrt1 in both germ cells and supporting cells of male gonads at 52 dph (fig. 5A), while Sox9 was detectable only in the supporting cells (fig. 5B). Cyp19a1 was localized in the follicular layer extending to the cytoplasm of the primary growth oocytes in the differentiated ovary at 52 dph (fig. 5C). On the other hand, Foxl2 was detectable in the follicular layer of the oocytes in the differentiated ovary at 60 dph (fig. 5D).

Discussion

In the present study, we analyzed the expression profiles of genes encoding transcription factors and steroidogenic enzymes during the process of gonadal development in catfish. Parallel to this, we also tracked the structural changes in gonads during differentiation/development using histology. Based on our histological observations in catfish, the first morphological signs of gonadal differentiation are seen around 45–50 dph, as it is evident by the formation of the ovarian cavity in the

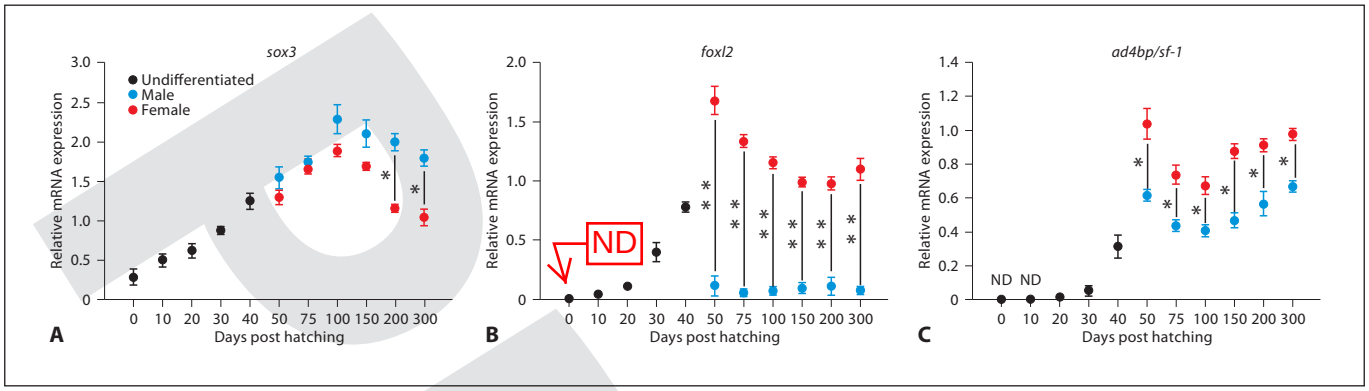

Fig. 3. Relative mRNA expression in catfish gonads from 0 to 300 dph. **A** *sox3* (SRY-related homeobox gene 3), **B** *foxl2* (forkhead box L2) and **C** *ad4bp/sf-1* (steroidogenic factor-1). ND = Non-detectable level. Statistical differences between male and female groups were determined by Student's t test (* $p < 0.05$; ** $p < 0.001$).

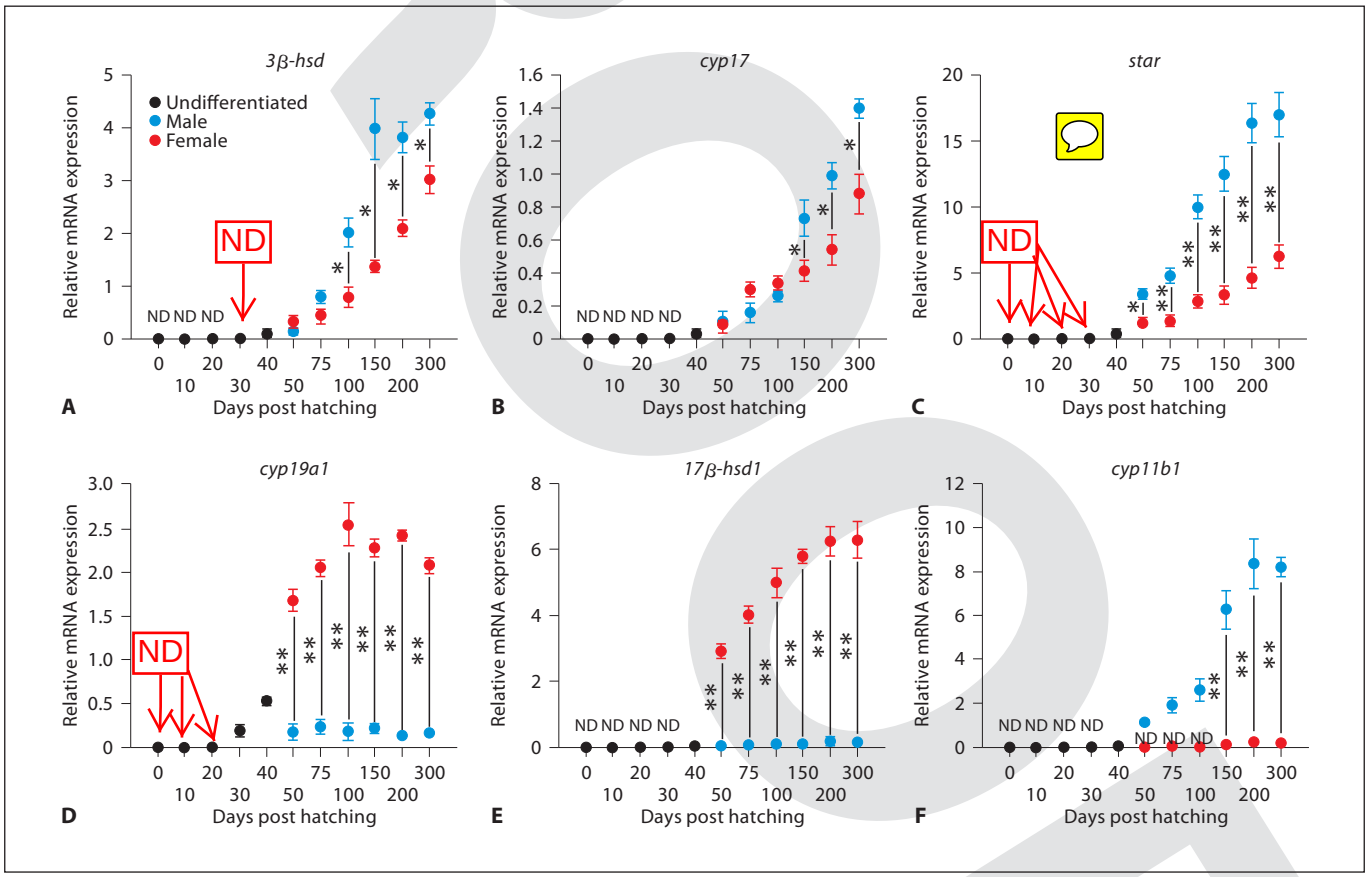

Fig. 4. Relative mRNA expression in catfish gonads from 0 to 300 dph. **A** *3β-hsd* (3β-hydroxysteroid dehydrogenase/Δ4–Δ5 isomerase), **B** *cyp17* (17α-hydroxylase/C17–20 lyase type 1), **C** *star* (steroidogenic acute regulatory protein), **D** *cyp19a1* (ovarian aromatase), **E** *17β-hsd1* (17β-hydroxysteroid dehydrogenase type 1) and **F** *cyp11b1* (cytochrome P450 hydroxylase type B or 11β-hydroxylase). ND = Non detectable level. Statistical differences between male and female groups were determined by Student's t test (* $p < 0.05$; ** $p < 0.001$).

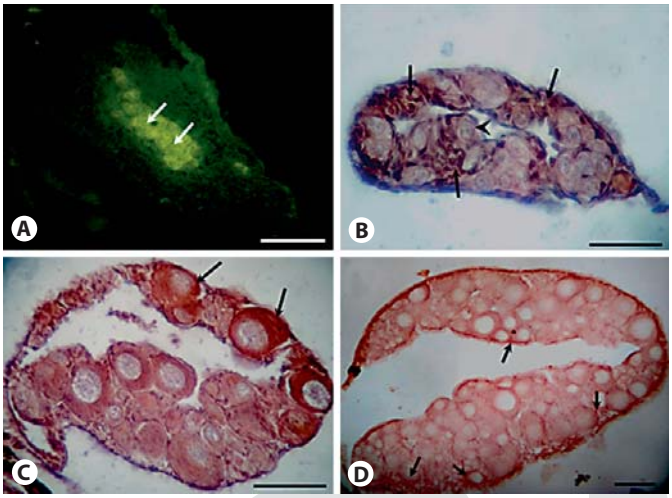

Fig. 5. Immunohistochemical localization of Dmrt1, Sox9, Cyp19a1, and Foxl2 proteins in developing gonads of catfish after FITC (**A**) and DAB (**B**–**D**) detection. **A** Dmrt1 expression (arrows) in germ cells of the male gonad at 52 dph. **B** Sox9 localization in the supporting (Sertoli) cells of the male gonad at 52 dph. Arrows indicate supporting cells; arrowhead indicates germ cell. **C** Cyp19a1 localization (arrows) was observed in the follicular layer extending inside the cytoplasm of peri-nucleolar oocytes in the female gonad at 52 dph. **D** Foxl2 localization in the follicular layer (arrows) of the oocytes in the female gonad at 60 dph. All scale bars indicate 50 μm.

differentiating female gonad. In catfish, gonads at 50 dph are fully differentiated into either testis or ovary which was apparent by histology. In this study, we observed the beginning of ovarian differentiation much earlier than testicular differentiation as seen in the majority of gonochoristic teleosts. Interestingly, testis takes more time to differentiate fully when compared to ovary. However, a few teleosts like Mozambique tilapia and Nile tilapia show simultaneous differentiation of both sexes [Strüssmann and Nakamura, 2002].

We observed differential expression patterns of various genes/transcription factors during gonadal sex differentiation in catfish and such differences in expression become more important as the gonadal development progresses. The transcription factor WT1 is critical for the development of the urogenital system and its expression pattern is conserved in vertebrates, including teleosts [Pritchard-Jones et al., 1990; Perner et al., 2007; Klüver et al., 2009]. In catfish, *wt1* expression was found very early in the indifferent gonad/MGC. Later, after differentiation of gonads, its expression was mainly seen in males and gradually decreased as the testis developed and attained maturity. The *DMRT1* gene encodes a putative transcription factor that shares a highly conserved zinc finger DNA-binding domain known as DM-domain across different vertebrates [Raymond et al., 2000]. In this study, we observed dimorphic expression of *dmrt1* restricted to male gonads during development which is consistent with our previous results using semi-quantitative PCR where few stages of gonadal development after differentiation of gonads were analyzed [Raghuveer and Senthilkumaran, 2009]. Its expression was first evident in undifferentiated gonads and persisted throughout the development of testis till adult, suggesting an important role in testicular development in catfish. This kind of male-specific expression of *dmrt1* during sex differentiation was also observed in human, tilapia, orange-spotted grouper and rainbow trout [Moniot et al., 2000; Fernandino et al., 2006; Xia et al., 2007; Kobayashi et al., 2008]. However, there are few reports where *dmrt1* expression was also shown in female gonads [Guo et al., 2005; Huang et al., 2005]. The IHC results revealed that Dmrt1 protein was expressed in germ cells of developing testis as reported previously from our group [Raghuveer and Senthilkumaran, 2009] and also from others using teleosts like zebrafish, orange-spotted grouper and hermaphrodite grouper [Guo et al., 2005; Xia et al., 2007; Zhou and Gui, 2010]. However, in mice DMRT1 expression was seen in both germ and somatic cells [Lei et al., 2007; Matson et al., 2010]. Another testicular related gene, *SOX9,* that belongs to the high-mobility-group (HMG) family of transcription factors is up-regulated in male gonads during differentiation [Kent et al., 1996]. As in other teleosts, we also identified 2 isoforms of *sox9* in catfish, *sox9a* and s*ox9b,* which showed dimorphic expression patterns in developing catfish gonads: *sox9a* expression was detectable only in developing testis, while *sox9b* expression was mostly restricted to ovary. This kind of differential expression pattern was also reported in zebrafish and the Nile tilapia [Chiang et al., 2001; Kobayashi et al., 2008] which suggests that *sox9a* retained its function as testis-specific gene, while *sox9b* may have a new role to play in ovarian development. Sox9 protein was localized in somatic/supporting cells in the differentiating catfish male gonad using a heterologous antibody (specific to human SOX9). In catfish female gonads, it was localized in the cytoplasm of the oocytes as reported earlier by Raghuveer and Senthikumaran [2010b]. In other teleosts, Sox9 protein was also detected in gonads of both males and females [Chiang et al., 2001; Yokoi et al., 2002; El Jamil et al., 2008]. Although its role is well defined in testis, it is not yet clear what exactly it does in ovary. The *SOX3* gene is a member

of the HMG family of transcription factors which is expressed in gonads and brain during early vertebrate development [Weiss et al., 2003]. Moreover, *Sox3* is important for normal oocyte development, testis differentiation and gametogenesis in mouse [Weiss et al., 2003]. In this study, *sox3* was expressed in primordial gonads at very early stages of development as it is known to be detectable in PGCs of other teleosts [Yao et al., 2007; Zhou and Gui, 2010]. Its expression was noticed in both male and female developing gonads of catfish. *Foxl2* belongs to the forkhead family of transcription factors that regulate *cyp19a1* expression during ovarian differentiation in teleosts [Wang et al., 2007]. Recently, in human it has been reported that conditional knock out of *FOXL2* results in the upregulation of *SOX9* in the transdifferentiated ovary [Uhlenhaut et al., 2009]. In this study, *foxl2* was expressed at significantly higher levels in female gonads than in male gonads during development, suggesting its role in ovarian differentiation. IHC showed Foxl2 immunoreactivity in the follicular layer of oocytes of developing female catfish. In accordance to this, in medaka and also in the Nile tilapia, Foxl2 protein was detected in the follicular layer of oocytes by IHC [Nakamoto et al., 2007; Wang et al., 2007]. *Ad4bp/sf-1* was initially identified as a key regulator for steroid hormone biosynthesis [Bakke et al., 2001]. It is involved in the differentiation/development of testis and ovary by regulating the transcription of *amh* in males and *cyp19a1* in females [Yoshiura et al., 2003; Takada et al., 2006; Wang et al., 2007]. In catfish its expression was also seen in indifferent gonads during early development. Later on, after differentiation, it was observed in both male and female gonads suggesting a role in gonadal development in both sexes.

In teleosts, sex steroids affect the development of germ cells and other cell types during the process of gonadal sex differentiation [Yamamoto, 1969; Devlin and Nagahama, 2002]. Estradiol-17β is considered to be responsible for inducing and maintaining ovarian development, and its levels are considerably higher in females than in males. In teleosts, testicular development is mainly regulated by the potent androgen 11-ketotestosterone (11-KT) produced by males [Nakamura et al., 1998]. In this study, we showed that *cyp19a1* which is required for the production of estradiol-17β was expressed very early in primordial/indifferent gonads, around 30–40 dph, prior to morphological differentiation of the gonads which was followed by a sharp increase at 50 dph in female gonads. Later on, the levels were found to be high in ovary throughout development, which substantiates part of our earlier findings [Rasheeda et al., 2010a]. On the contrary, its expression was very low in male gonads throughout development. Our IHC studies revealed that Cyp19a1 protein was detectable in the ooplasm of primary growth oocytes in addition to the follicular layer during ovarian development in catfish. Similar observations were also reported in killifish using in situ hybridization [Dong and Willett, 2008]. However, there are reports showing that Cyp19a1 protein was localized only in the follicular layer of vitellogenic oocytes [Wang et al., 2007]. Other steroidogenic enzyme genes *(3β-hsd*, *cyp17* and *17β-hsd1)* and the sterol transfer coding gene *star*, necessary for the synthesis of estrogens, were expressed at barely detectable levels in undifferentiated gonads at 40 dph and thereafter increased gradually in both differentiated male and female gonads as development progressed. Similarly in the Nile tilapia most of these steroidogenic enzyme genes were also expressed in undifferentiated XX gonads (around 5–10 dph) and later gradually increased in both sexes [Ijiri et al., 2008]. Further, earlier reports on the manipulation/skewing of sex in catfish hatchlings during early development (before sex differentiation) and complete sex reversal of XY tilapia fry by following ethynylestradiol treatment prove that estrogen is produced in female gonads during the critical period of ovarian differentiation and plays a crucial role in female development [Nagahama, 2005; Raghuveer et al., 2005]. 11-KT is the most potent androgen in teleosts which is synthesized from testosterone [Nakamura et al., 1998]. *Cyp11b1* encodes a key enzyme in the production of 11β-hydoxytestosterone from testosterone. An important observation in this study showed that *cyp11b1* was expressed in males after gonadal differentiation (from 50 dph onwards). On the other hand, its expression was negligible in female gonads of catfish, similar to female Nile tilapia where *cyp11b1* was undetectable during ontogeny [Ijiri et al., 2008]. Previous studies on catfish *11β-hsd*, an important steroidogenic enzyme required for the production of 11-KT in males, also showed a similar expression pattern as *cyp11b1* [Rasheeda et al., 2010b]. These results together indicate that 11-KT is not produced in gonads during differentiation, but is detectable only in the developing testis or at least late stages of testicular differentiation in catfish. In this study, the expression of *cyp11b1* was abundant in testis around 150–200 dph, when spermatogenesis is initiated in catfish. In most teleosts, including catfish, the endogenous androgens do not appear to have an important role in testicular differentiation unlike that of estrogens during ovarian differentiation [Nakamura et al., 1998; Ijiri et al., 2008; present study]. Taken together, these results suggest that steroidogenic enzyme genes play a significant role in the maintenance of post-differentiation

gonadogenesis. However, their role in ovarian differentiation, consistent with earlier reports, cannot be ruled out.

In conclusion, male-specific expression of *dmrt1, sox9a* and *wt1* in developing gonads indicates that these genes are essential for testicular differentiation. In contrast, abundant expression of *sox9b, foxl2* and *cyp19a1* in female gonads suggests an important role for these correlates in ovarian differentiation. Results from IHC for various gene products support this contention to some extent. Thus, most of these transcription factors/genes were expressed in gonads during 30–40 dph, a period critical for the differentiation of indifferent gonads into either testis or ovary in catfish.

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