# Cloning, expression and activity analysis of terminal enzymes producing estradiol-17β and 11-ketotestosterone during gonadal differentiation and reproductive cycle of *Clarias gariepinus*

**THESIS SUBMITTED FOR THE DEGREE OF**

*Doctor of Philosophy*

*In*

*Animal Sciences*


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**University of Hyderabad**

(Central University established in 1974 by act of parliament)

**HYDERABAD – 500 046, INDIA**

# CERTIFICATE

This is to certify that **Mrs. Rasheeda M.K.** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend her thesis entitled "**Cloning, expression and activity analysis of terminal enzymes producing estradiol-17β and 11-ketotestosterone during gonadal differentiation and reproductive cycle of *Clarias gariepinus***" for submission for the degree of Doctor of Philosophy in Animal Sciences of this University.

**Prof. B. Senthilkumaran** _____

Supervisor

**Head** _____

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**Dean** _____

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**HYDERABAD – 500 046, INDIA**

I hereby declare that the work embodied in this thesis entitled **"Cloning, expression and activity analysis of terminal enzymes producing estradiol-17β and 11-ketotestosterone during gonadal differentiation and reproductive cycle of *Clarias gariepinus*"** has been carried out by me under the supervision of Prof. B. Senthilkumaran and this has not been submitted for any degree or diploma of any other university earlier.

**Prof. B. Senthilkumaran**
**(Research Supervisor)**

**Rasheeda M.K.**
**(Research Scholar)**

*To my*
*Parents and husband*

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✍ *Rasheeda M.K*

# Contents

# *General Introduction*

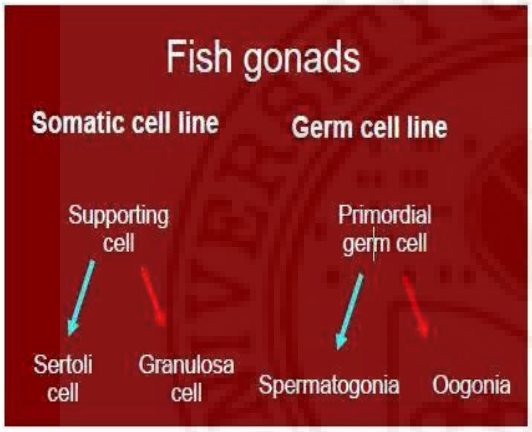
## Introduction:

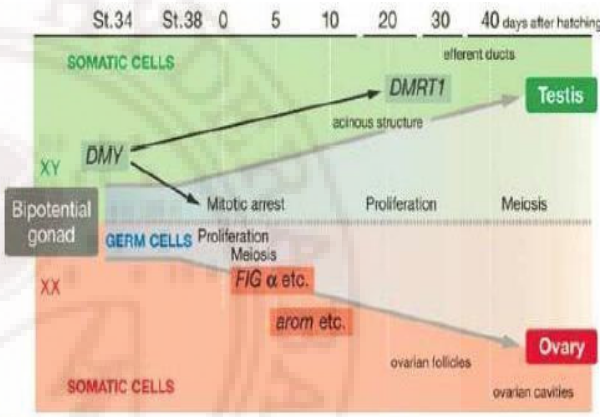
Sexual reproduction in animal kingdom which empowers the organism to propagate and survive in the perpetually changing environment by enriching its genome through recombination includes two processes, one is the determination of the sex of organism and other is the production of gametes. Inception of studying the sex determining mechanism took a lead from 1930 with the discovery of variety of sex determination mechanisms for example in *Drosophila* (genic balance X:A ratio), in *Dinophilis* (size of larvae), in *Bonelia viridis* (parasitic association), in Hymenoptera insects (haplo-diploidy), in Lepidopteran (ZW/ZZ), in *Matsococcus gallicola* (male $X_1X_2X_3X_4X_5X_6O$), in fishes and amphibians variable patterns of sex determination system prevails, in reptiles (temperature dependent), in birds (ZW/ZZ system with female heterogamety), in mammals 2-factor (XX/XY with male heterogamety) system (Bull, 1983; Manalakou *et al*., 2006). Sexual plasticity in fish is due to its diverse biology and ecology, which influences these species to engage in various sex-determination mechanisms, thus empowering it to survive and maintain a stable sex ratio. Because they are amenable to artificial culture and experimental analysis in many cases, fish also provide unique opportunities to investigate and test theoretical concepts of sex determination, ranging from evolutionary mechanisms to biochemical processes. Similarly, in aquaculture systems, understanding and controlling reproduction is central to the efficient propagation of organisms due to differential growth rates of sexes and a need for synchronous and reliable maturation.

**Gonadal development and differentiation:**

A brief introduction related to gonadal development and differentiation of fish in comparison to higher vertebrates including mammals will help us appreciate the need to probe sex development in varied fish species. Gonadal (ovary/testis) development in vertebrates begins with the formation of a genital ridge (Balinsky, 1975), which appears as a longitudinal thickening of mesoderm protruding into the coelomic cavity ventral to the developing kidney and lateral to the dorsal mesentery. They are made up of two distinct type of cells 1) gamete forming cells and 2) supporting somatic cells, having different developmental origin. Gamete forming cells are derived from primordial germ cells (PGCs) that stem from the vegetal portion of the unfertilized egg rich in granular cytoplasmic (Wei and Mahowald, 1994) substances. PGCs subsequently get surrounded by somatomesodermal cell and are transferred to the genital ridge through morphogenetic movements of mesoderm and cell migratory activities (Hamaguchi, 1992). They do not differ in both sexes and remain undetermined until subjected to hormonal and other endogenous or exogenous factors (environment) driving them to transform into spermatogonia or oogonia. Somatic cells on the other side are derived from cortex epithelial layer, and they are also similar in presumptive males and females. In mammals, the Y linked sex-determination gene, *Sry*, recruits mesonephric cells into the gonad in males (Sinclair *et al*., 1990; Koopman, 1999). In medaka Y linked *DMY* is implicated with the putative role to induce development of somatic cell into Sertoli cells in male (Matsuda *et al*., 2002). Subsequently, the somatic cells surrounding PGCs differentiate into seminiferous tubules and supporting connective tissue, and into cells similar to Leydig and Sertoli cells found in mammals (Van Vuren and Soley, 1990). During ovarian

development, the somatic cells and PGCs differentiate to form follicles, comprised of oocytes surrounded by an inner granulosa and outer thecal layers. Histologically, ovarian development in females is first detectable with the proliferation of somatic and oogonial cells followed by early oocyte differentiation and the formation of the ovarian cavity. Testicular development is found later than ovarian differentiation, usually weeks or months after the onset of ovary development (Guraya, 1994; Nakamura *et al*., 1998).



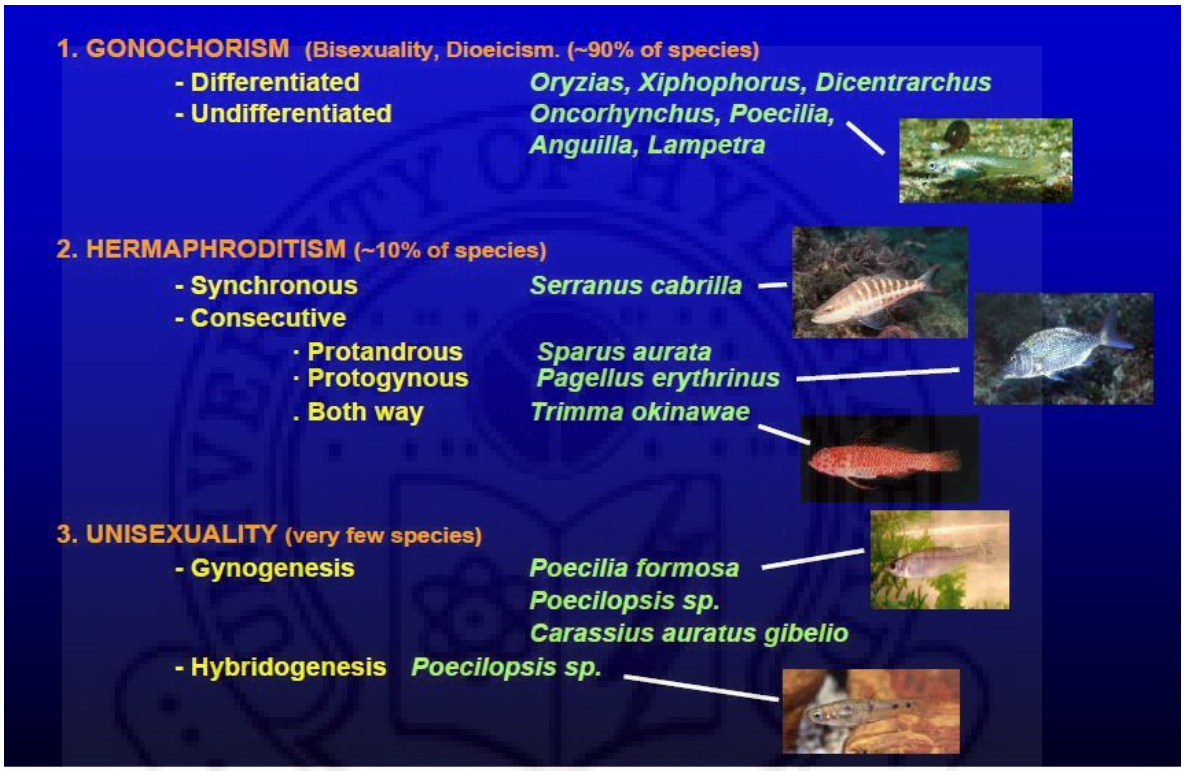




**Adopted from Dr. Piferrer's presentation on Determinacion Y Differenciacion Sexual En Los Pesces (www.reprofish.eu/.../Sex_determination_differentiation_Piferrer2007.)**

**Adopted from Matsuda *et al.*, Develop.Growth Differ. (2003), 45:397-403.**

A plethora of gonadal differentiation mechanism are exhibited by ~28,000 (see Penman and Piferrer, 2008) species of teleosts, ranging from species that directly develop and finally possess only testis or ovary at sexual maturation termed as gonochoristic to species with synchronous or sequential hermaphrodism (Devlin and Nagahama, 2002; Price, 1984). Among gonochoristic species, Yamamoto (1969) described two forms, differentiated (indifferent gonad→ testis/ovary) and undifferentiated (initially all develop ovary→ ~50% masculinization occurs). On the other hand, hermaphrodites are species that

have functional ovarian and testicular tissues during the life-cycle and are further classified into sequential hermaphrodites; protogynous (mature female→male) and protandrous (mature male→ female), synchronous hermaphrodite (intersex) and bidirectional hermaphrodites which can alter their sex in both directions (Devlin and Nagahama, 2002).

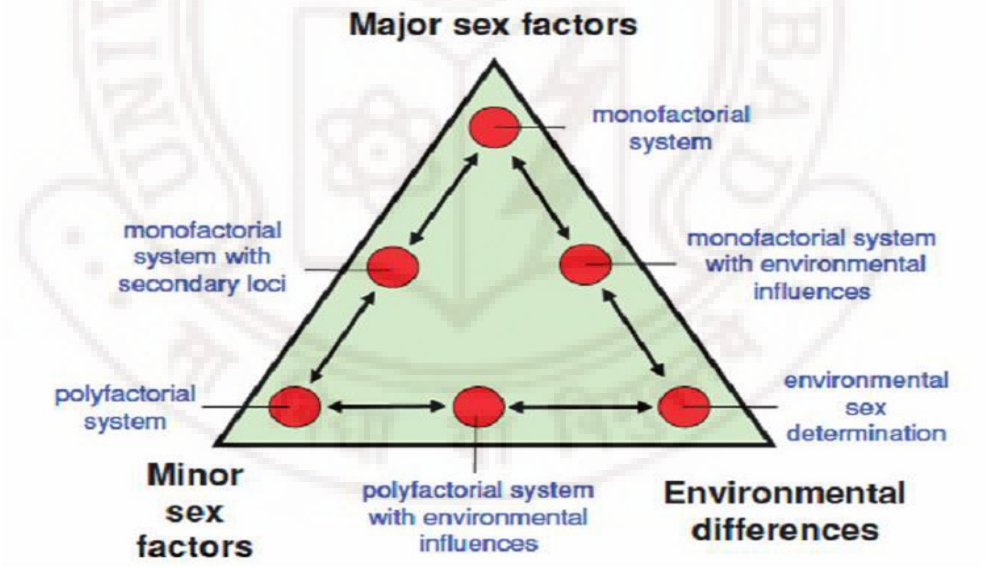



**Adopted from Dr. Piferrer's presentation on**
**Determinacion Y Differenciacion Sexual En Los Pesces**
**(www.reprofish.eu/.../Sex_determination_differentiation_Piferrer 2007. *pdf*)**

**Stability and inheritance of sex in gonochoristic species:**

Gonadal differentiation occurs through a single development pathway to produce completely differentiated gonad in mammals (Capel *et al*., 1998) where as in fish, gonadal development may be controlled by intrinsic factors, steroids and growth factors (gonochorist) social behaviour (natural hermaphrodites) or extrinsic environmental factors such as temperature, endocrine disrupters or pollutants. Irrespective of the mode or type of

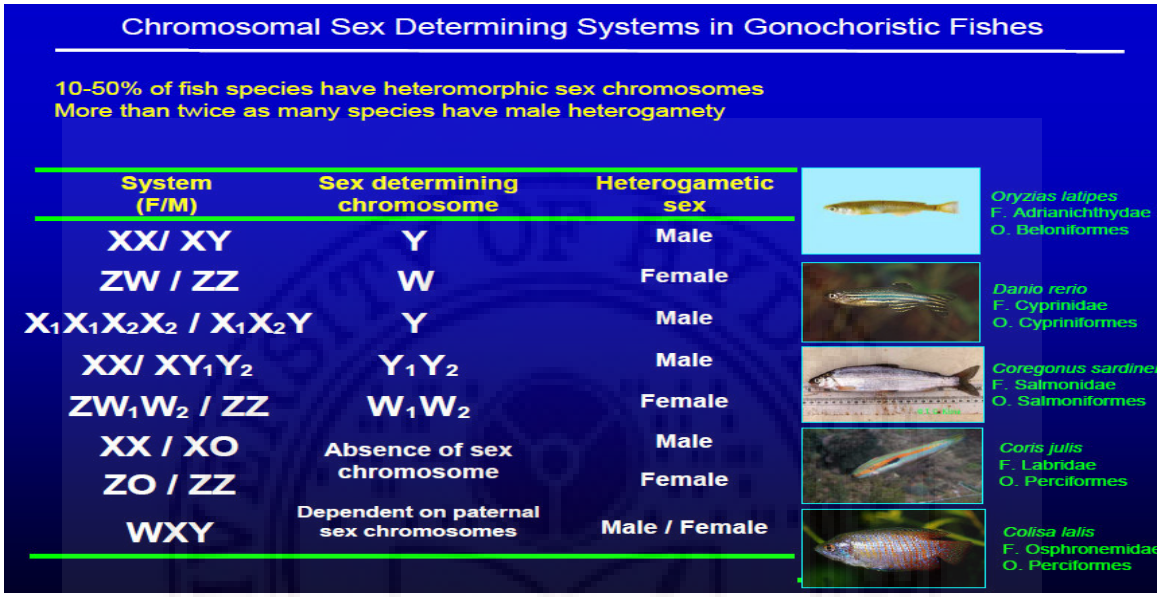
reproduction, maintenance of stable sex ratio ideally 1:1 drives the individual towards a strategy that help them to sustain a balance in their population against the varying selective pressures such as natural and anthropogenic. The prevalence of sex-related growth dimorphism is quite common (Parker, 1992) which in turn influence reproductive capacity and growth pattern of population before and after attaining sexual maturation. Hence, for a species or a small population in a species, sex is influenced either by a single sex factor or combination of several sex factors or environmental differences, and leading to evolutionary transitions involving changes in the relative abundance of these three factors (Bull, 1983).

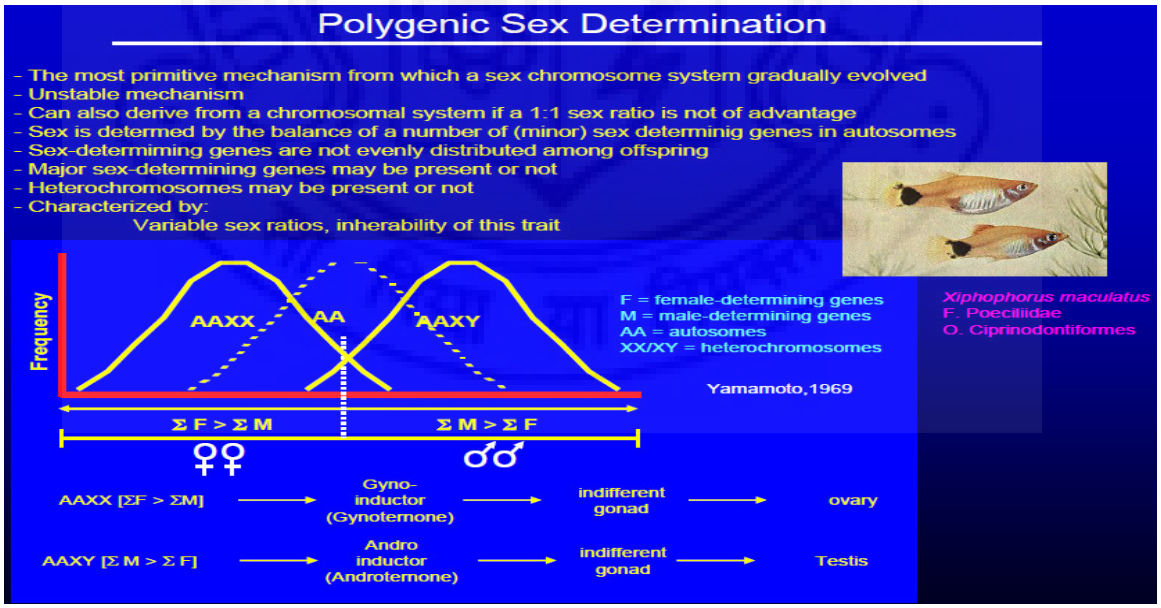


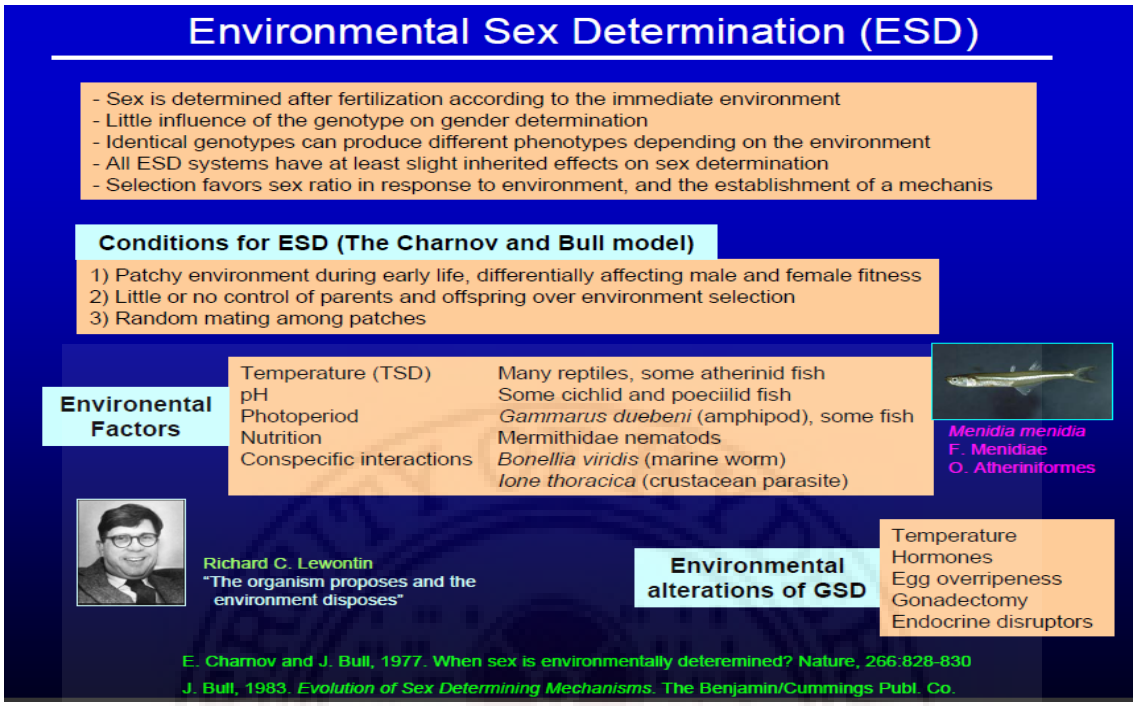

**Adopted from Penman and Piferrer, (2008), R. Fish. Sci., 16:16-34**

Gonochorism in teleosts may solely be due to genetic differences between sexes initially or absence of genetic difference but respond to environmental conditions prevailing at the

time of fertilization (Bull, 1983; Valenzuela, 2008). The slides presented below adopted from a presentation on sex determination and differentiation in fish by Piferrer (2007) illustrate theses mechanisms explicitly.



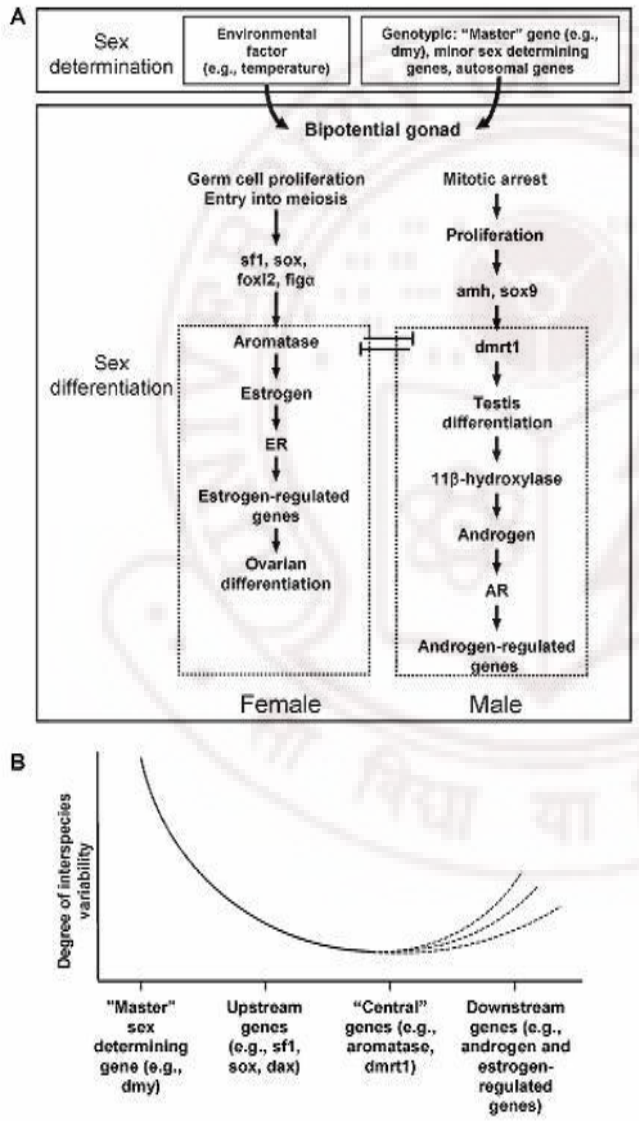






**Adopted from Dr. Piferrer's presentation on**
**Determinacion Y Differenciacion Sexual En Los Pesces**
**(www.reprofish.eu/.../Sex_determination_differentiation_Piferrer 2007. pdf)**

Sexual development in mammals cannot be altered by treatment with exogenous steroids or environmental parameters suggesting that it is under the control of very stable genetic sex determination-XX/XY system with *Sry* being the master switch on Y chromosome. In contrast to mammals, both gonochorist and hermaphrodites are labile for sex change throughout their life (Pandian and Koteswaran, 1998; Devlin and Nagahama, 2002) which is explained on the basis of unique characteristic features in teleosts: the property of retaining bipotentiality by holding PGCs at many stages of gonadogenesis and their potentiality to redifferentiate throughout the reproductive lifespan. Among fishes a variety of sex determination/differentiation-related genes exist and this phenomenon is explained by the mechanism of chromosome evolution (Schartl, 2004; Volff *et al*., 2007). Genes downstream of the master regulatory gene that ultimately establish sexual dimorphism are

quite conserved in fish for example the steroidogenic enzyme, cytochrome P450 aromatase and the transcription factor *dmrt1* which have pivotal role in ovarian and testicular differentiation, respectively (Guiguen *et al*., 2010; Wang *et al*., 2009). But there are many more candidate genes potentially implicated in sex differentiation which warrants a thorough study in different fish models.




**Adopted from Piferrer and Guiguen, Fish.Sci. (2008), 16:35-55**

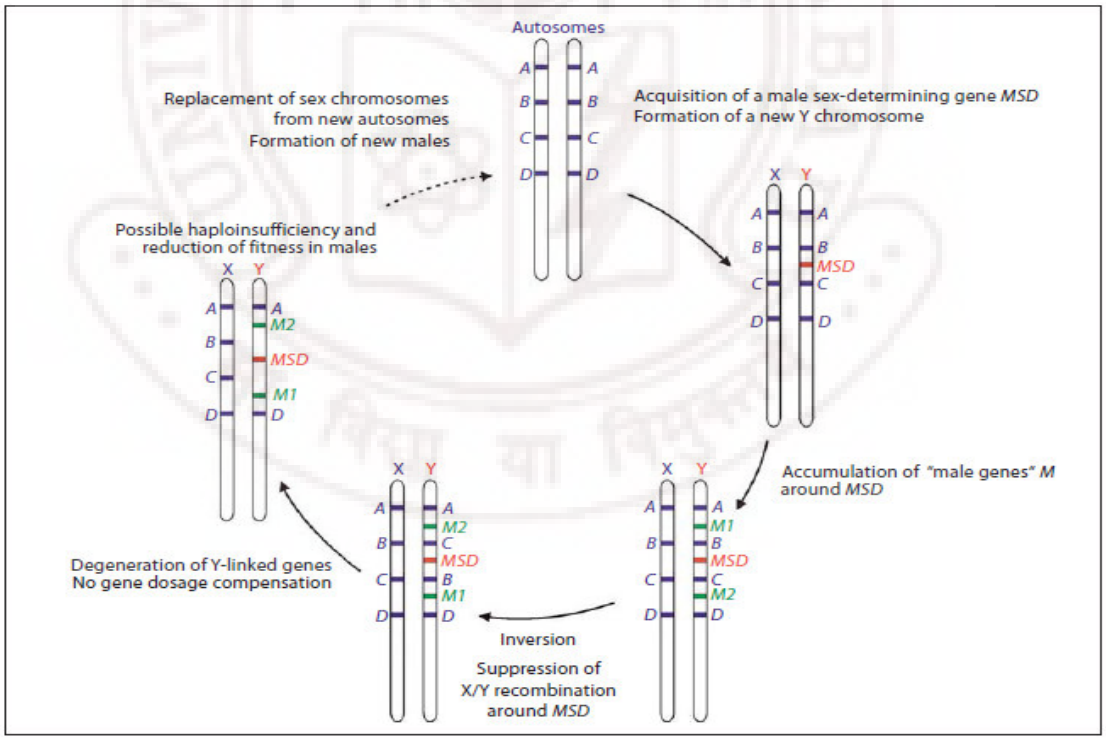
**Table 1.** Genes involved in mammalian sex differentiation

| Gene | Role |
|---|---|
| *SRY* | Primary testis determining factor |
| *SOX9* | Main target for SRY |
| *DAX1* | Antagonist to SRY |
| *SF1, WT1, GATA 4* | Required by SRY |
| *AMH* | Activated by SOX9 |
| *WNT4, WNT7* | Required by AMH |
| *DMRT1* | Required by Sertoli and germ cells |
| *ATRX* | Required for testis development |
| *LHX9, M33* | Required for gonadal development |
| *FGF9* | Required for peritubular myloid cell migration |
| *TDA1,2,3* | Promote ovarian development |
| *AR (DHTR)* | Required for development of male genitalia |
| *PAX2, LIM1, EMX2* | Required for Wolffian and Mullerian ducts |
| *INSL3* | Required for testis descent |
| *DHH* | Required for spermatogonial division |

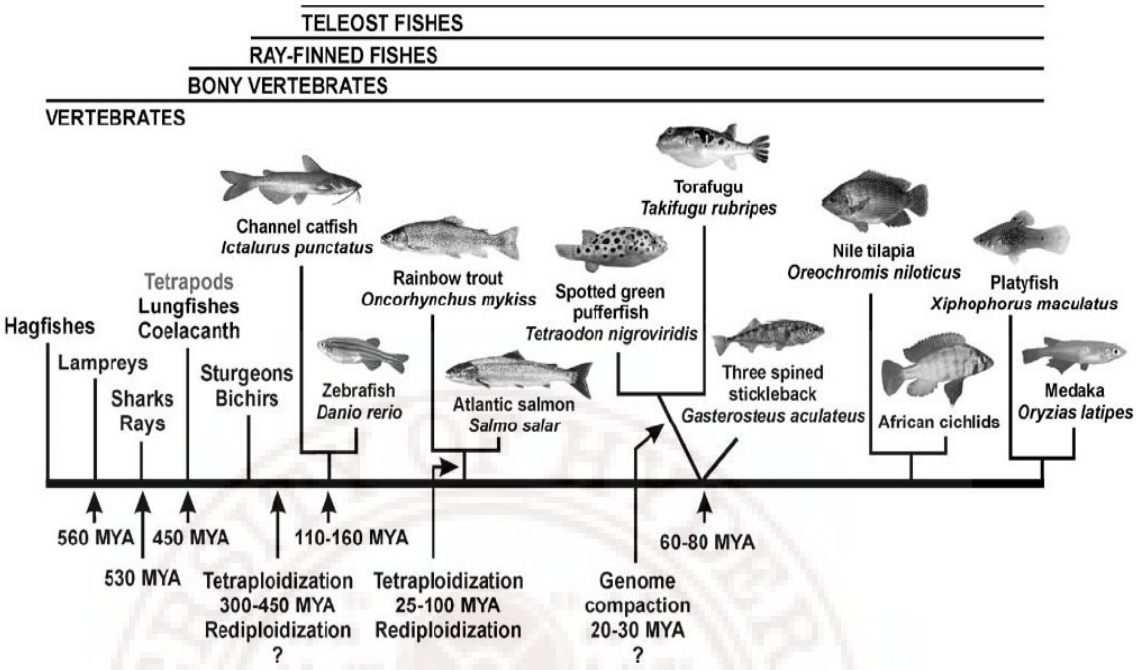
**Adopted from Ferguson-Smith, R. Sex Dev. (2007), 1:2-11**

**Endocrine regulation of sex differentiation:**

Cross-talk between the PGCs and the somatic cells of the gonadal ridge, resulting in sex determination/differentiation requires paracrine and endocrine factors distinct for each gender. The genes coding these factors are common to all vertebrates (Place and Lance, 2004) but variations are observed in temporal expression and sex-specificity especially in teleosts which is explained due to the tetraploidization/rediploidization during the early evolution of the ray-finned fish lineage. Differential loss or sub-function partitioning or neo-functioning of such gene duplicates might be involved in emergence of new sex chromosomes from autosome, also known as divergent evolution (Volff *et al*., 2005, 2007).




**Adopted fromVolff *et al*., Sex Dev., (2007), 1:85-99**




**Adopted from Volff, Heredity, (2005), 94:280-294**

For example, the male determining *DMY* gene in medaka, *Oryzias latipes*, evolved as a consequence of translocation and duplication of autosomal *dmrt1* gene on another autosome, generating a new Y chromosome. However, this gene, *DMY* is not an universal male sex determining gene in fish since it is found only in two species of medaka. Studies on platyfish, threespine stickleback, salmonids, tilapias and various other models have confirmed that the fish sex chromosomes are young and have evolved independently in different fish lineages. Hence, the current research focuses on comparing genes, i.e., *sox9, dmrt1, amh, nr5a1, nrob1, igf1, igf1ra, cyp19a, foxl2, fst* and *lhr* (Baron *et al*., 2005, Wang *et al*., 2007;Vizziano *et al*., 2007, 2008; Raghuveer and Senthilkumaran, 2009) that are involved in vertebrate sex determination. In addition, transcription factors, steroidogenic enzyme genes and the sex steroid receptors which ultimately regulate the sex steroids (androgens and estrogens) to exert their action on germ cells and other cell types and on

organs involved in sex differentiation. Steroid production is correlated significantly with very early stages of gonadal differentiation (Nakamura, 1998) and any perturbation in the steroid-synthesizing capability can impair the sex determination/differentiation mechanism. Extensive work is done on diverse area covering most aspect of sex steroids: its production at the time of embryogenesis, larval development, and temporal expression of the steroidogenic enzyme producing it, the transcription factors regulating the expression of the steroidogenic gene and finally the receptors-mediated function by binding to appropriate protein/DNA.

For females, estradiol-17β ($E_2$) is supposed to be the major sex steroid responsible for inducing and maintaining ovarian development (Yamamoto, 1969). Various biochemical and histochemical evidences from fish belonging to different reproductive strata elucidate the role of $E_2$ and aromatase. First and foremost aromatase expression studied during early development revealed its role in female differentiation in vast number of teleosts (Kwon *et al.,* 2001; Sudhakumari *et al.,* 2005; Ijiri *et al*., 2008). Inhibition of $E_2$ synthesis in early developmental stages using aromatase inhibitors can lead to masculinization (Guiguen *et al*., 1999; Kitano *et al*., 1999; Nagahama, 2005). Further, immunohistochemical studies in the gonad of tilapia detected aromatase before the onset of sex differentiation in female with delayed occurrence in males (Nakamura *et al*., 1998). Corroboratively aromatase activity is enhanced at feminizing temperature and suppressed at masculinizing temperatures (D'cotta *et al*., 2001). In hermaphrodites, aromatase expression is the driving force for gonadal reorganization during sex change i.e., increase in aromatase mRNA levels during protandrous sex change (Kroon *et al*., 2005) and decrease during protogynous sex change (Bhandari *et al*., 2003). All these evidences indicate that ovarian

aromatase (*cyp19a1*) can be used as a molecular marker to infer ovarian differentiation in species lacking sex-linked color pattern or non-availability of mono-sex population.

For males, 11-ketotestosterone (11-KT) is suggested a role in testis development (Jiang *et al*., 1996; Baroiller *et al*., 1998; Liu *et al*., 2000; Socorro *et al*., 2007). Exogenous treatment with 17α-methyltestosterone and 11-oxo-androgen treatment can cause induction of testes in mixed population, (Cardwell and Liley, 1991; Raghuveer *et al*., 2005; Raghuveer and Senthilkumaran, 2009) and regression of ovarian tissue leading to precocious masculinization in protogynous grouper *Epinephelus suillus* (Tan-Fermin, 1994). Further D'Cotta *et al*., (2001) illustrated up regulation of 11β-hydroxylase expression (the penultimate enzyme in 11-KT production) in genetic males of the Nile tilapia at masculinizing temperature (35ºC) compared to males reared at lower temperature (25ºC). Taken together, these findings suggest that in few species in which the androgen levels and expression of androgen synthesizing enzymes were measured during gonadal differentiation, those species exhibited sexual dimorphism with high level of androgen synthesizing transcripts detected in male larvae than that of females. Specific genes involved in the biosynthesis of steroids are differentially expressed in the somatic cells of testis and ovary (Nakamura *et al*., 1998) eventually maintaining the balance between androgens and estrogens during the critical window of gonadal development, mediated by the activity of aromatase (Simpson *et al*., 2002).

Molecular analysis of steroidogenic enzyme genes from fish has revealed the existence of two forms of aromatase in most teleosts studied. For example in goldfish and zebrafish, (terminal enzyme producing $E_2$) the brain and the ovarian isoform displays ~ 40% sequence homology with distinct spatiotemporal expression (Callard and Tchoudakova

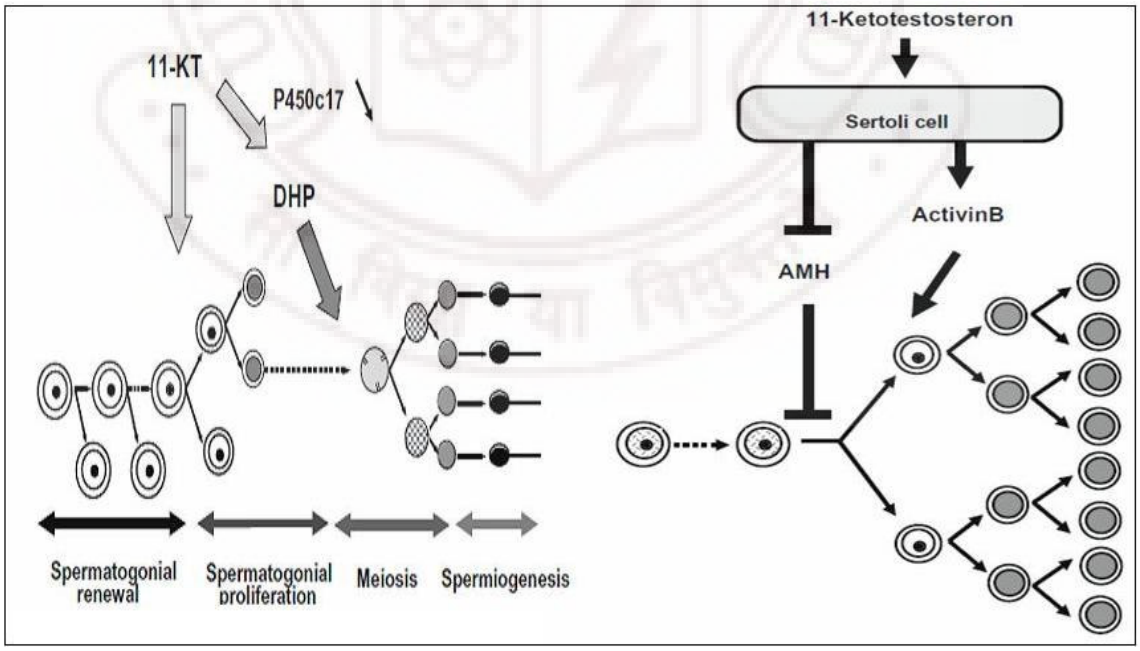
1997, 2001; Trant et *al*., 2001). Similarly the presence of a novel isoform of 11β-hydroxysteroid dehydrogenase (HSD) type 2 gene (terminal enzyme producing 11-KT) in medaka, fugu, zebrafish-*11β-HSD3* which is paralogous to mammalian *11β-HSD1* (Baker, 2004) and also the isolation of 11β-hydroxylase (*11β-H*, penultimate enzyme in 11-KT production) in the Nile tilapia (Zhang *et al*., 2010) revealed that though the biosynthetic pathway of sex steroid synthesis is conserved among fishes, the spatiotemporal expression of various isoforms vary in different fish models supporting the concept of independent evolution of sex genes in different fish lineages. Therefore, it would be highly relevant to explore the expression pattern of these steroidogenic enzyme genes during development in many more fish models to gain an insight into possible role of these steroids in endorsing the fish with the unique trait of plasticity in terms of sex determination/differentiation.

This prompted us to study the ontogeny of these three steroidogenic enzyme genes involved in the synthesis of sex steroids and their putative role during gonadal differentiation and development in catfish *Clarias gariepinus*. This teleostean fish model has been chosen since it exhibits distinct recrudescence and quiescent phases during the reproductive cycle. It can be bred and reared easily from day 1 after hatch to adult in laboratory/natural conditions (outdoor tanks) for ontogeny and reproductive cycle studies.

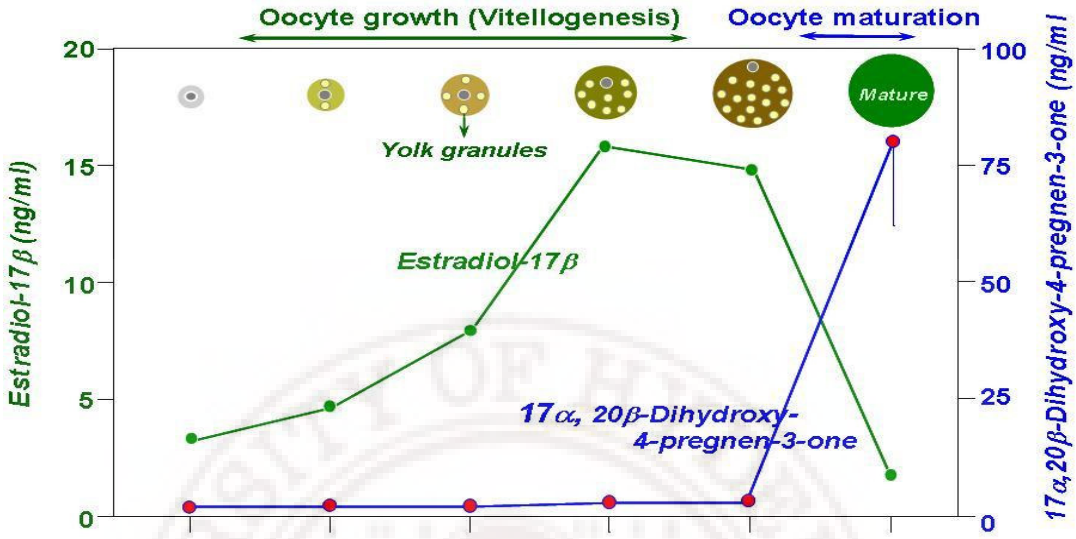
**Hormonal regulation of gametogenesis in fish:**

The functional unit of testis in teleosts is the spermatogenic cyst formed by group of Sertoli cells surrounding and nourishing synchronously developing germ cells whereas the functional unit within the ovary is ovarian follicle consisting of developing oocyte surrounded by follicular cell layers. The granulosa and thecal layers are separated by a basement membrane.

Spermatogenesis is a complex and highly coordinated process of producing haploid spermatozoa from diploid spermatogonia. In males, it consists of three phases: 1) mitotic phase with different generations of spermatogonia which are genetically determined 2) the meiotic phase that includes the primary and secondary spermatocytes and 3) the spermiogenic phase with the haploid spermatids emerging from meiosis and differentiating into spermatozoa (Schulz *et al*., 2010). Oogenesis on the other hand, is the process by which primordial germ cells become ova and it can be broadly divided into six major steps: (1) formation of primordial germ cells (germline segregation), (2) transformation of primordial germ cells in to oogonia (sex differentiation), (3) transformation of oogonia into oocytes (onset of meiosis), (4) growth of oocyte under meiotic arrest (vitellogenesis), (5) resumption of meiosis (maturation), and (6) ovulation (Patino and Sullivan, 2002).

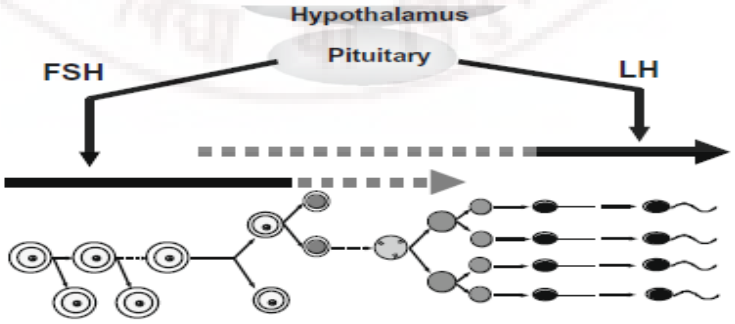



**Adopted from Schulz *et al*., Gen. Comp. Endocrinol., (2010), 165:390-411**



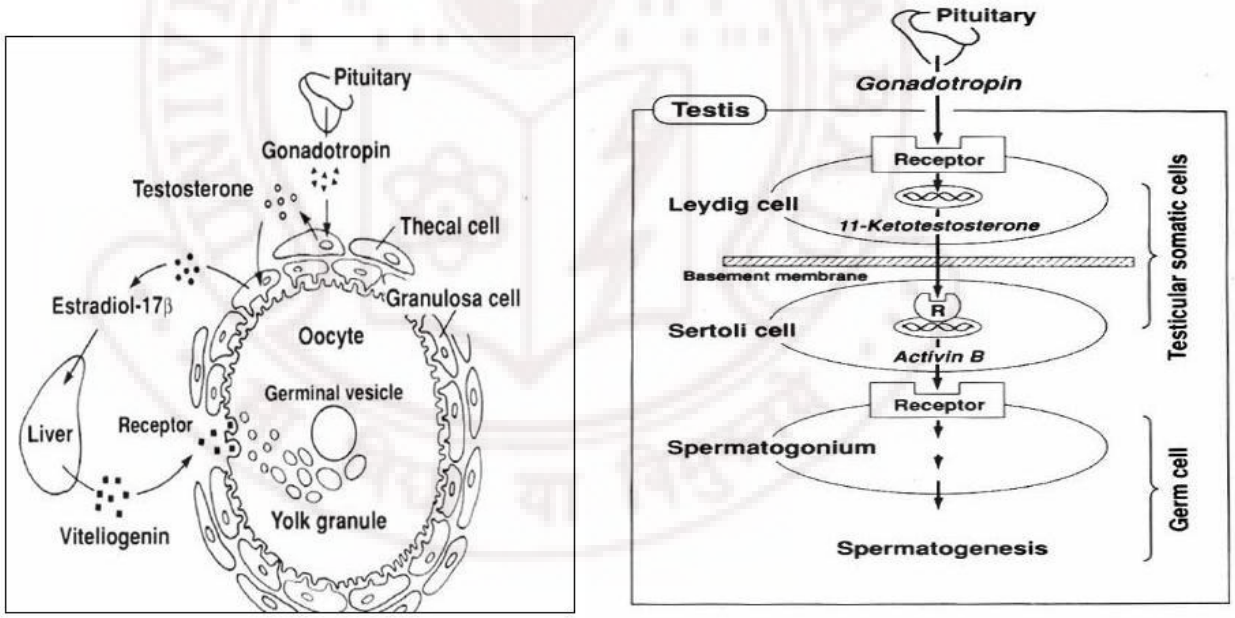

**Adopted from Nagahama, Steroids, (1997), 62:190-196;**
**Joy, Senthilkumaran and Sudhakumari, J. Endocrinol. (1998), 156:365-372**

A complex cross-talk between the gonad and pituitary occurs via endocrine and paracrine control after perceiving visual, social and chemical cues by brain. This leads to the secretion of gonadotropins (follicle stimulating hormone-FSH and Lutenizing hormone-LH) and growth factors which triggers the production of sex steroids in gonads facilitating the development of mature ova and sperm from spermatogonial and oogonial cells.




**Adopted from Schulz *et al*., Gen. Comp. Endocrinol., (2010), 165:390-411**

Pioneering reproductive biologist, Nagahama (1994) elucidated the production of $E_2$ in teleosts by a two-cell type model where the outer thecal cell layer under the influence of gonadotropin secretes the androgen substrate (testosterone) which diffuses into the granulosa cell layer where the aromatase is localized (Kagawa *et al*., 1982; 1985) to get converted into $E_2$. Likewise 11β-hydroxytestosterone is synthesized by the Leydig cells under the influence of gonadotropin (FSH) which activate the Sertoli cells to produce activin B that acts on the spermatogonial cells to undergo mitosis leading to proliferation, differentiation and production of spermatocytes (Nagahama, 1994; Miura and Miura, 2001).




**Adopted from Nagahama,**
**Int. J. Dev. Biol., (1994), 38:217-229**

Numerous studies have demonstrated the fluctuation in the 11-KT levels and the shift in steroidogenesis to 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) during transition

of testis from stage I to stage IV in cyclic reproducing teleosts (Sakai *et al*., 1987; Cavaco *et al*., 1997). Further discovery of microarray technique and transcriptome study of 9152 contigs from trout using both spermiating testis and isolated testicular cells have provided an insight on the temporal changes in the expression of varied genes (DNA repair gene, meiotic recombination etc.) during the transformation of spermatogonial stem cells to spermatids (Schulz *et al*., 2010). But none have reported simultaneous change in expression and protein activity of the enzyme producing 11-KT during testicular transition. Further reports exist from salmonids species on the stimulatory role of gonadotropins on 11-KT synthesis and up-regulation of the penultimate and terminal steroidogenic enzyme genes *11β-H* and *11β-HSD2* synthesizing it when administered in the immature fish (Jiang *et al.,* 1996; 2003) but no data exist on stage-dependent responsiveness of these genes to gonadotropin induction. Besides gonadotropins, thyroxine is also essential for the normal functioning of teleost testis and alteration in the thyroxine levels caused marked biochemical and histological changes in the testis of Nile tilapia and catfish (Matta *et al*., 2002; Swapna *et al*., 2006; Blanton and Specker, 2007). Therefore to elucidate the significance of thyroxine during testicular recrudescence, we studied the expression of *11β-H* and *11β-HSD2*, by treating the fish with goitrogenic agent, thiourea, during testicular recrudescence. In this study, we also analyzed several other steroidogenic enzyme genes, 3β-hydroxysteroid dehydrogenase (enzyme involved in the rate limiting step), cytochrome p450c17α enzyme and 20β-hydroxysteroid dehydrogenase (which synthesizes the maturation inducing hormone in teleosts) the former gene was chosen to study if triiodothyronine depletion blocked the initial steps of steroidogenesis and the latter two genes transcripts were measured to observe if there is a shift in steroidogenic

pathway leading to the synthesis of 17α, 20β-DP, (the steroid required for resumption of prophase-I arrested oocytes in teleosts) eventually causing precocious maturation. Taken together, an attempt was made to study the gene expression and activity of *11β-H* and *11β-HSD2* during different phases of catfish testicular cycle to delineate specific role of steroidogenic enzyme genes, gonadotropins and thyroid hormone in maintaining the seasonal reproductive cycle.

Similarly during oogenesis plasma FSH levels induced follicular $E_2$ production, which in turn, stimulated hepatic vitellogenin synthesis (Specker and Sullivan, 1994) followed by a rise in plasma LH levels, (well known as pre-ovulatory LH surge) coinciding the production of pre-ovulatory follicle (Khan and Thomas, 1999). Subsequently gonadotropin binds to its receptor on granulosa cells (Oba *et al*., 1999; Kumar *et al*., 2000) 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 (Kanamori and Nagahama, 1988; Nagahama, 1994; Senthilkumaran *et al*., 2004; Lubzens *et al*., 2010). Seasonal fluctuations in aromatase expression and activity levels are well documented in female sea bass and the Nile tilapia where they have attributed these fluctuations accountable for follicular growth and maturation (Chang *et al*., 1997; Dalla Valle *et al.,* 2002; Gonzalez and Piferrer, 2003; Chang *et al*., 2005). But this was not attempted simultaneously from the same fish which may not depict a correct picture of the change in the transcription and translation capacity of the ovary during that phase of follicular growth. Therefore, we attempted to simultaneously analyze the expression profile and rate of production of $E_2$ during ovarian cycle. We also monitored the responsiveness of catfish ovarian

aromatase gene and enzyme activity after induction with gonadotropin (hCG) to substantiate early findings from amago salmon where gonadotropins potentiated oocyte growth and maturation (Kagawa, 1982; Young, 1983). 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 (Koide *et al*., 1992; Bogerd, 2005; Kirubagaran *et al*., 2005). Apart from gonadotropin, several other hormones such as thyroid hormones, insulin and growth hormone have been implicated in the regulation of vitellogenesis (Patino and Sullivan, 2002). This prompted us to study the effect of thyroid hormone depletion by using thiourea during gonadal recrudescence.

**In the backdrop of this existing state of knowledge, the present thesis is an effort to understand the role of terminal enzymes responsible for the production of $E_2$ and 11-KT at molecular level, and further monitor the implications of thyroid hormone depletion and hCG administration on gonadal recrudescence. These aspects have been studied as four major chapters discussing the results and findings.**

**References**

Balinsky B.I., 1975. In: An introduction to embryology. Saunders, Philadelphia, pp.648.

Baker M.E., 2004. Evolutionary analysis of 11β-hydroxysteroid dehydrogenase-type 1, -type 2, -type 3 and 17β-hydroxysteroid dehydrogenase-type 2 in fish. FEBS. Letters, 574:167-170.

Baron D., Houlgatte R., Fostier A., Guiguen Y., 2005. Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol. Reprod., 73: 959-966.

Baroiller J.F., Guiguen Y., Iseki K., Fostier A., 1998. Physiological role of androgens on gonadal sex differentiation in two teleost fish, *Oncorhynchus mykiss* and *Oreochromis niloticus*. J. Exp. Zool., 281:506–507.

Bhandari R.K., Komuro H., Nakamura S., Higa M., Nakamura M., 2003. Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper, *Epinephelus merra*. Zool. Sci., 20:1399-1404.

Blanton M.L., Specker J.L., 2007. The hypothalamic-pituitary -thyroid (HPT) axis in fish and its role in fish development and reproduction. Cr. Rev. Toxic., 37:97-115.

Bull J.J., 1983. In: Evolution of sex determining mechanisms, Benjamin/Cumming, Menlo Park, CA, pp.316.

Bogerd J., 2005. Selective ligand-binding determinants in catfish and human gonadotropin receptors. 31:247-254.

Capel B., 1998. Sex in the 90s: SRY and the switch to the male pathway. Annu. Rev. Physiol., 60: 497-523.

Cardwell G.R., Liley N.R., 1991. Hormonal control of sex and color change in the stoplight parrotfish, *Sparisoma viride*. Gen. Comp. Endocrinol., 81:7–20.

Callard G.V., Tchoudakova A., 1997. Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish. J. Steroid Biochem., 61:387-392.

Callard G., Tchoudakova A., Kishida M., Wood E., 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J. Steroid Biochem. Mol. Biol., 79: 305-314.

Cavaco J.E.B., Lambert J.G.D., Schulz R.W., Goos H.J.Th., 1997. Pubertal development of male African catfish, *Clarias gariepinus*. *In vitro* steroidogenesis by testis and interrenal tissue and plasma levels of sexual steroids. Fish Physiol. Biochem., 2:129-138.

Chang X.T., Kobayashi T., Kajiura H., Nakamura M., Nagahama Y., 1997. Isolation and characterization of the cDNA encoding the tilapia (*Oreochromis niloticus*) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein and enzyme activity in ovarian follicles during oogenesis. J. Mol. Endocrinol., 18:57-66.

Chang X.T., Kobayashi T., Senthilkumaran B., Kobayashi-Kajura H., Sudhakumari C.C., Nagahama Y., 2005. Two types of aromatase with different encoding genes, tissue distribution and developmental expression in the Nile tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol., 141:101-115.

Dalla Valle L., Lunardi L., Colombo L., Belvedere P., 2002. European sea bass (*Dicentrarchus labrax* L.) cytochrome P450arom: cDNA cloning, expression and genomic organization. J. Steroid Biochem. Mol. Biol., 80:25-34.

D'cotta H., Fostier A., Guiguen Y., Govoroun M., Baroiller J.-F., 2001. Aromatase play a key role during normal and temperature-induced sex differentiation of tilapia *Oreochromis niloticus*. Mol. Reprod. Dev., 59: 265-276.

Devlin R.H., Nagahama Y., 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. Aquaculture, 208: 191-364.

Ferguson-Smith M., 2007. The evolution of sex chromosome and sex determination in vertebrates and the key role of DMRT1. Sex Dev., 1:2-11.

Gonzalez A., Piferrer F., 2003. Aromatase activity in the European sea bass *(Dicentrarchus labrax*) brain. Distribution and changes in relation to age, sex and the annual reproductive cycle. Gen. Comp. Endocrinol., 132: 223-230.

Guiguen Y., Fostier A., Piferrer F., Chang C.F. 2010. Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol., 165: 352-366.

Guiguen Y., Baroiller J.F., Ricordel M.J., Iseki K., Mc Meel O.M., Martin S.A., Fostier A., 1999. Involvement of estrogens in the process of sex differentiation in two fish species: The rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol. Reprod. Dev., 54:154-162.

Guraya S.S., 1994. Gonadal development and production of gametes in fish. Proc. Indian Natl. Sci. Acad., 60B:15-32.

Hamaguchi S., 1992. Sex differentiation of germ cells and their supporting cells in *Oryzias latipes*. J. Fish Biol., 4:11-17.

Ijiri S., Kaneko H., Kobayashi T., Wang D.S., Sakai F., Paul-Prasanth B., Nakamura M., Nagahama Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia (*Oreochromis niloticus)*. Biol. Reprod., 78:333–341.

Jiang J-Q., Kobayashi T., Gea W., Kobayashi H, Tanaka M., Okamotob M., Nonakab Y., Nagahama Y., 1996. Fish testicular 1lβ-hydroxylase: cDNA cloning and mRNA expression during spermatogenesis. FEBS Letters, 397:250-252.

Jiang J. Q., Wang D. S., Senthilkumaran B., Kobayashi T., Kobayashi H. K., Yamaguchi A., Ge W., Young G. and Nagahama Y., 2003. Isolation, characterization and expression of 11β-hydroxysteroid dehydrogenase type 2 cDNAs from the testes of

Japanese eel (*Anguilla japonica*) and Nile tilapia (*Oreochromis niloticus*). J. Mol. Endocrinol., 31:305–315.

Joy, K.P., Senthilkumaran, B., Sudhakumari, C.C., 1998. Perivoulatory changes in hypothalamic and pituitary monoamines following GnRH analogue treatment in the catfish *Heteropneustes fossilis*: A study correlating changes in plasma hormone profiles. J. Endocrinol., 156:365-372.

Kagawa H., Young G., Adachi S., Nagahama Y., 1982. Estradiol-17β production in amago salmon (*Oncorhynchus rhodurus*) ovarian follicles: Role of the thecal and granulosa cells. Gen. Comp. Endocrinol., 47:440-448.

Kagawa H., Young G., Adachi S., Nagahama Y., 1985. Estrogen synthesis in the teleost ovarian follicle: the two-cell type model in salmonids. In: Salmonid Reproduction (R.N. Iwamoto and S. Sower Eds.). Washington Sea Grant Program, University of Washington, Seattle, pp.20-25.

Kanamori A., Nagahama Y., 1988. Developmental changes in the properties of gonadotropin receptors in the ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) to chum salmon gonadotropin during oogenesis. Gen. Comp. Endocrinol., 72:25-38.

Khan I.A., Thomas P., 1999. Ovarian cycle, teleost fish. In: Encyclopedia of reproduction III, (E. Knobil and J.D. Neill, Eds.) Academic press, San Diego. pp. 552-564.

Kirubagaran R., Senthilkumaran B., Sudhakumari C.C., Joy K.P., 2005. Seasonal dynamics in gonadotropin secretion and $E_2$-binding in the catfish *Heteropneustes fossilis*. 31:183-188.

Kitano T., Takamune K., Kobayashi T., Nagahama Y., Abe S.I., 1999. Suppression of p450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (Paralicthys olivaceus). J. Mol. Endocrinol., 23: 167-176.

Koide Y., Noso T., Schouten G., Peute J., Zandbergen M.A., Bogerd J., Schulz R.W., Kawauchi H., Goos H.J.Th., 1992. Maturational gonadotropin from the African catfish, *Clarias gariepinus*: Purification, characterization, localization and biological activity. Gen. Comp. Endocrinol., 87:327-341.

Koopman P., 1999. *Sry* and *Sox9*: Mammalian testis-determining genes. Cell. Mol. Life Sci., 55:839-856.

Kroon F.J., Munday P.L., Westcott D.A., Hobbs J.P.A., Liley N.R., 2005. Aromatase pathway mediates sex change in each direction. Proc. Biol. Sci., 272:1399-1405.

Kumar R.S, Ijiri S., Trant J.M., 2000. Molecular biology of channel catfish gonadotropin receptors: Cloning of a functional lutenizing hormone receptor and preovulatory induction of gene expression. Biol. Reprod. 64:1010-1018.

Kwon J., Mc Andrew B., Penman D., 2001. Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia (*Oreochromis niloticus*). Mol. Reprod. Dev., 59: 359–370.

Liu S.J., Govoroun M., D'Cotta H., Ricordel M.J., Lareyre J.J., McMell O.M. Smith T., Nagahama Y., Guiguen Y., 2000. Expression of cytochrome P45011β (11β-hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. J. Steroid Biochem. Mol. Bio., l75:291–298.

Lubzens E., Young G., Bobe J., Cerda J., 2010. Oogenesis in teleost: How fish eggs are formed. Gen. Comp. Endocrinol., 165:367-389.

Manolakou P., Lavranos G., Angelopoulo R., 2006. Molecular patterns of sex determination in the animal kingdom: A comparative study of the biology of reproduction. Reprod. Biol. Endocrinol., 13: 4-59.

Matsuda M., Nagahama Y., Shinomiya A., Sato T., Matsuda C., Kobayashi T., Morrey C.E., Shibata N., Asakawa S., Shimizu N., Hori H., Hamaguchi S., Sakaizumi M., 2002.

DMY is a Y-specific, DM-domain gene, required for male development in the medaka (*Oryzias latipes)* fish. Nature, 417:559-563.

Matta S.L.P., Vilea D.A.R., Godinho H.P., Franca L.R., 2002. The goitrogen 6-*n*-propyl-2-thiouracil (ptu) given during testis development increases sertoli and germ cell numbers per cyst in fish: The tilapia (*Oreochromis niloticus*) model. Endocrinology, 143:970-978.

Miura T., Miura C., 2001. Japanese eel: A model for analysis of spermatogeneis. Zool. Sci., 18:1055-1063.

Nagahama Y., 1987. Gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. Zool. Sci., 4:209-222.

Nagahama Y., 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol., 38:217-229.

Nagahama Y., 1997. 17α-20β-dihydroxy-4 pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanism of synthesis and action. Steroids, 62:190-196.

Nagahama Y., 2005, Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol. Biochem., 31:105–109.

Nakamura M., Kobayashi T., Chang X.-T., Nagahama Y., 1998. Gonadal sex differentiation in teleost fish. J. Exp. Biol., 281:362-372.

Oba Y., Hirai T., Yoshiura Y., Yoshikuni M., Kawauchi H., Nagahama Y., 1999. Cloning, functional characterization and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). Biochem. Biophys. Res. Commun. 263:584-590.

Pandian T., Koteeswaran R., 1998. Ploidy induction and sex control in fish. Hydrobiologia, 384:167-243.

Parker G.A., 1992. The evolution of sexual size dimorphism in fish. J. Fish Biol., 41:1-20.

Patino R., Sullivan C.V., 2002. Ovarian follicle growth, matuation, and ovulation in teleost fish. Fish Physiol. Biochem., 26: 57-70.

Penman D.J., Piferrer F., 2008: Fish gonadogenesis. Part I: Genetic and environmental mechanisms of sex determination. R. Fish. Sci., 16:16-34.

Piferrer F., Guiguen Y., 2008. Fish Gonadogenesis. Part II: Molecular biology and genomics of sex differentiation. R. Fish. Sci., 16:35-55.

Place A.R., Lance V.A., 2004. The TSD drama: Same cast, different stars, In: Temperature-Dependent Sex Determination in Vertebrates, (N.Valenzuela and V. Lance, Eds.), Washington, DC: Smithsonian Books, pp. 99-110.

Price D.J., 1984. Genetics of sex determination in fishes: A brief review, In: Fish reproduction: Strategies and tactics, (G.W. Potts and R.J. Wooton, Eds.), London: Academic Press, pp. 77-89.

Raghuveer K., Rahul G., Wang D.S., Bogerd J., Kirubagaran R., Rasheeda M.K., Sreenivasulu G., Nupur B., Tharangini S., Nagahama Y., Senthilkumaran B., (2005). Effect of methyl testosterone and ethynyl estradiol-Induced sex differentiation on catfish, *Clarias gariepinus*: Expression profiles of *Dmrt1*, cytochrome P450 aromatases and 3β-hydroxysteroid dehydrogenase. Fish Physiol. Biochem., 31:143-147.

Raghuveer K., Senthilkumaran B., 2009. Identification of multiple *dmrt1s* in catfish: Localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment. J. Mol. Endocrinol., 42: 437-448.

Sakai N., Ueda H., Suzuki N., Nagahama Y.,1989. Steroid production by amago salmon (*Oncorhynchus rhodurus*) testes at different development stages. Gen. Comp. Endocrinol., 75: 231-240.

Schartl M.A., 2004. A comparitive view on sex determination in medaka. Mech. Dev., 121:639-645.

Schulz R.W., de Franca L.R., Lareyre J.-F., LeGac F., Chiarini-Garcia H., Nobrega R.H., Miura T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol., 165:390-411.

Senthilkumaran B., Yoshikuni M., Nagahama Y., 2004. A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. Mol. Cell. Endocrinol., 215:11-18.

Specker J.L., Sullivan C.V., 1994. Vitellogenesis in fishes: status and perspectives. In: Perspectives in Comparative Endocrinology, (K.G. Davey, R.E. Peter, and S.S., Tobe. Eds.), National research council, Ottawa., pp. 304-315.

Simpson E.R., Clyne C., Rubin G., Boon W.C., Robertson K., Britt K., Speed C., Jones M., 2002. Aromatase - A brief overview. Annu. Rev. Physiol., 64:93-127.

Sinclair A.H., Berta P., Palmer M.S., Hawkins J.R., Griffiths B.L., 1990. A gene from the human sex determining region encodes a protein with homology to a conserved DNA-binding motif. Nature, 346:240-244.

Socorro S., Martins R.S., Deloffre L., Mylonas C.C., Canario A.V.M., 2007. A cDNA for European sea bass (*Dicentrachus labrax*) 11β-hydroxylase:gene expression during the thermosensitive period and gonadogenesis. Gen. Comp. Endocrinol., 150:164–173.

Sudhakumari C.C., Kobayashi T., Kajiura-Kobayashi H., Wang D.S., Yoshikuni M., Nagahama Y., Senthilkumaran B., 2005. Ontogenic expression patterns of several nuclear receptors and cytochrome P450 aromatases in brain and gonads of the Nile tilapia, *Oreochromis niloticus* suggests their involvement in sex differentiation. Fish Physiol. Biochem., 31:129–135.

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

Tan-fermin J.D., Garcia L.M.B., Castillo Jr., A.R., 1994. Induction of sex inversion in juvenile grouper, *Epinephelus suillus* (Valenciennes) by injections of 17α-methytestosterone. Jpn. J. Ichthyol., 40:413-420.

Trant, J.M., Gavassa, S., Ackers, J., Chung, B.-C., Place, A.R., 2001. Developmental expression of cytochrome P450 aromatase genes (*cyp19a* and *cyp19b*) in zebrafish fry (*Danio rerio*). J. Exp. Zool., 290:475–483.

Van Vuren J.H.J., Soley J.T., 1990. Some ultra structural observations of Leydig and Sertoli cells in the testis of *Tilapia rendalli* following induced testicular recrudescence. J. Morphol., 206:57-64.

Valenzuela N., 2008. Sexual development and the evolution of sex determination. Sex Dev., 2:64-72.

Volff J-N., 2005. Genome evolution and biodiversity in teleost fish. Heredity, 94: 280-294.

Volff J.N., Nanda I., Schmid M., Schartl M.A., 2007. Governing sex determination in fish: regulatory putsches and ephemeral dictators. Sex Develop., 1:85-99.

Vizziano D., Randuineau G., Baron D., Cauty C., Guiguen Y., 2007. Characterization of early molecular sex differentiation in rainbow trout, *Oncorhynchus mykiss*. Dev. Dyn., 236: 2198-2206.

Vizziano D., Randuineau G., Baron D., Cauty C., Guiguen Y., 2008. Comparison of gonadal gene expression patterns after masculinization of female rainbow trout with an androgen or an aromatase inhibitor. Cybium, 32:83-85.

Wang D.S., Zhou L.-Y., Kobayashi T., Matsuda M., Shibata Y., Sakai F., Nagahama Y., 2009. Doublesex-and Mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favouring the male pathway in tilapia. Endocrinology, doi:10.1210/en.2009-0999.

Wang D.S., Kobayashi T., Zhou L.Y., Paul-Prasanth B., Ijiri S., Sakai F., Okubo K., Morohashi K., Nagahama Y., 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor1. Mol. Endocrinol., 21:712-725.

Wei G., Mahowald A.P., 1994. The germline: Familiar and newly uncovered properties. Annu. Rev. Genet., 28:309-324.

Yamamoto T., 1969. Sex differentiation. In: Fish Physiology, vol. 3., (W.S. Hoar and D.J. Randall, Eds.), Academic Press, New York, pp.117–175.

Young G., Kagawa H., Nagahama Y., 1982. Oocyte maturation in the amago salmon (*Oncorhynchus rhodurus*) *in vitro* effects of salmon gonadotropin, steroids and cyanoketone (an inhibitor of 3β-hyroxysteroid dehydrogenase). J. Exp. Zool., 224:265-275.

Young G., Ueda H., Nagahama Y., 1983. Estradiol-17β and 17α, 20β-dihydroxy-4-pregnen-3-one production by isolated ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) in response to mammalian pituitary and placental hormones and salmon gonadotropin. Gen. Comp. Endocrinol., 52: 329-335.

Zhang W-L., Zhou L-Y., Senthilkumaran B., Huang B-F., Sudhakumari C.C., Kobayashi T., Nagahama Y., Wang D-S., 2010. Molecular cloning of two isoforms of 11β-hydroxylase and their expressions in the Nile tilapia. Gen. Comp. Endocrinol., 165:34-41.

# *Chapter 1*

## Molecular cloning, expression and enzyme activity of ovarian aromatase (*cyp19a1*) during ovarian development and oogenesis in air-breathing catfish *Clarias gariepinus* and *in vivo* hCG-modulation of *cyp19a1* during female reproductive cycle

## Chapter 1: Cloning, expression and activity analysis of *cyp19a1* in catfish

**Abstract:**

To investigate the specific role of cytochrome P450 ovarian aromatase *(cyp19a1*) during ovarian development and annual reproduction in air-breathing catfish *C. gariepinus* we initially cloned full length cDNA of *cyp19a1* from the catfish ovarian tissue containing 1551 bp of open reading frame (ORF), which displayed 79% homology with channel catfish *cyp19a1*. Characterization of the encoded protein in non–steroidogenic COS-7 cells illustrated that *cyp19a1* ORF could efficiently catalyze the aromatization reaction by producing estradiol-17β ($E_2$) from testosterone. Tissue distribution pattern revealed the predominance of ovarian form in the ovary with trace amount being detected in other tissues including brain. The seasonal expression profile of *cyp19a1* measured using relative real-time PCR in connection with the different ovarian follicular stages revealed, high expression in the prespawning phase when compared to spawning, preparatory and regressed phases which corroborated with the measured serum $E_2$ levels. The enzymatic activity assessed by means of a sensitive radiometric assay in ovarian tissues collected during different phases recorded results consistent with the expression levels. Ontogeny results displayed sexual dimorphism, with relatively early expression of ovarian form in ovary than the testis clearly indicating its role in female sex differentiation. Further to understand the role of gonadotropins in modulating the *cyp19a1* transcripts and enzyme activity, we performed an *in vivo* study by injecting human chorionic gonadotropin (hCG) in adult catfish at three phases and observed phase-dependent stimulatory effect of hCG in the preparatory and prespawning phases. However, in spawning phase induction of transcripts was not sustained suggesting that the stage of oocyte during ovarian cycle was crucial and gonadotropins could not override the induction of *cyp19a1* transcripts once the

follicles have undergone or undergoing meiotic maturation. These results demonstrates specific role of *cyp19a1* during ovarian differentiation by displaying dimorphic expression during the period of gonadal differentiation in catfish, and a distinct expression pattern and activity during ovarian cycle, indicating its involvement in maintaining the periodicity of the ovarian cycle by modulating the levels of $E_2$.

**Introduction**

Cytochrome P450 aromatase (*cyp19a1)*, an endoplasmic reticulum terminal steroidogenic enzyme, catalyzing the production of estradiol-17β ($E_2$) is the prime gene associated in sex differentiation, oogenesis, sexual behavior and sex change in teleosts (Yamamoto, 1969; Devlin and Nagahama, 2002; Guiguen *et al*., 2010). Varied experimental methods from fishes belonging to diverse reproductive strata have lucidly illustrated *cyp19a1* role in feminizing the bipotential gonad and in folliculogenesis (Kagawa *et al*., 1984; Donaldson 1996; Chang *et al*., 1997; Trant, 1994; Choi *et al*., 2005; Luckenbach *et al*., 2005; Matsuoka *et al*., 2006; Sawyer *et al*., 2006; Nunez and Applebaum, 2006; Karube *et al*., 2007; Barney *et al*., 2008). Not only in teleosts but also in higher vertebrates $E_2$ is required during ovarian differentiation and reproduction and its deficiency or depletion leads to maleness or sterility, ascertaining *cyp19a1* role in diverse vertebrate form in maintaining the androgen- estrogen homeostasis (Wilson, 1994; Hayes, 1998; Miyata and Kubo, 2000; Pieau and Dorizzi, 2004). This enzyme is encoded by a single gene *cyp19* (aromatase) with multiple tissue specific promoters in both steroidogenic and non-steroidogenic tissues in higher vertebrates (Simpson *et al*., 2002). On the contrary in teleosts, existence of two isoforms of aromatase, ovarian and brain is demonstrated in numerous fish species (Gelinas *et al*., 1998; Tchoudakova and Callard, 1998; Kishida and Callard, 2001; Trant *et*

*al*., 2001; Goto-Kazeto *et al*., 2004; Esterhuyse *et al*., 2008) formed as a consequence of genome duplication in teleosts (Simpson *et al*., 1994; Callard *et al*., 2001). The ovarian form, *cyp19a1*, is implicated a crucial role during sex determination/differentiation, (Kwon *et al*., 2001; Matsuda, 2003; Nagahama, 2005; Blazquez *et al*., 2008; Ijiri *et al*., 2008;) in gametogenesis (Ijiri *et al*., 2003; Kobayashi *et al*., 2004) and in the regulation of vitellogenesis during reproductive cycle (Nagahama *et al*., 1994). This makes it all the more important to study thoroughly the mode of action of $E_2$ in different tissues and gain a molecular insight of *cyp19a1*, by studying its spatiotemporal expression, its transcript level/enzyme activity during reproductive cycle and gonadal differentiation. In addition, we also intended to probe whether *cyp19a1* could be used as a candidate marker for ovarian differentiation in catfish, *Clarias gariepinus*. The study initially started with cloning of *cyp19a1* from catfish, then characterizing the enzyme (recombinant protein) and studying its expression profiles during gonadogenesis and reproductive cycle in ovarian tissue. A positive correlation was demonstrated between *cyp19a1* expression, activity and follicular stages, which were further substantiated by *in vivo* hCG responsiveness of expression and activity during preparatory, prespawning and spawning phases in female catfish. The expression study and enzyme activity was performed using quantitative real-time RT-PCR and tritiated radiometric assay, respectively, thus establishing the role of $E_2$ during female sex differentiation and recrudescence in the annual breeder *C. gariepinus*.

**Materials and Methods**

**Procuring of male and female catfish and rearing conditions**

Male and female larvae of *C. gariepinus* belonging to different age groups were obtained from our laboratory aquaculture facility by breeding and rearing these fish. The hatchlings

were kept in the glass water tanks supplied with circulating filtered water under normal photoperiod and ambient temperature. They were fed with live feed i.e. tubeworms *ad libitum* twice daily till they grew to fingerling size and later they were fed with goat liver. The various phases of female reproductive cycle namely preparatory, prespawning, spawning, resting were closely followed by catfishes (adult) maintained more than a year in outdoor tanks under natural conditions. Maintenance of adult fish and its annual reproductive cycle were described earlier (Swapna *et al*., 2006; Raghuveer and Senthilkumaran, 2009). Ovaries from various age group fish as well as from adult fish at different phase were collected by sacrificing them after immersing in ice-cold water. Before sacrificing the adult fish blood was collected by caudal puncture, for serum $E_2$ estimation by using Cayman $E_2$-EIA-kit as described later. The tissues and serum samples were stored in -80ºC before being used for various assays.

**Total RNA isolation and cDNA synthesis**

Total RNA from the ovary of adult catfish was isolated using 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 were reverse transcribed to cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA) and oligod$T_{18}$ primers following the manufacturer's instructions. The efficiency of the transcription was checked by performing a PCR for *β-actin,* a constitutively expressed gene. For ontogeny studies, ovaries from 10-20 larvae were dissected at 45, 60, 75, 100 and 150 days post hatch (dph) and pooled to have 5 biological samples (n=5) for total RNA preparation. Similarly to study expression

pattern at different phases of reproductive cycle the ovarian tissues were collected from five fishes. The pooled tissues were divided into five samples for further analysis (n=5).

**Molecular cloning of catfish *cyp19a1***

A set of degenerate primers were designed from conserved region by aligning the existing sequences of teleost *cyp19a1* using Lasergene software, (release 3.05; DNASTAR, Madison, WI, USA) which yielded partial cDNA fragments of 264 bp from ovary. The partial cDNA was cloned in pGEMT-easy vector (Promega, Madison, WI, USA) and sequence was determined. The identity of amplified partial cDNA was analyzed by NCBI-BLAST.

**cDNA library construction and screening of *cyp19a1* gene**

A cDNA library was constructed from ovary of catfish as per the method described earlier (Senthilkumaran *et al*., 2002). Briefly total RNA from ovary of catfish was prepared using TRI-reagent. Using 1.5mg of total RNA, mRNA was prepared with oligotex-mRNA kit (Qiagen, GmbH, Germany). Using 5µg of mRNA, first strand cDNA synthesis was carried out using stratagene cDNA synthesis kit. 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 vector and packaged into lambda phage heads using Giga pack III–gold packaging extract (Stratagene, Cedar Creek, TX, USA). Screening of the cDNA library for *cyp19a1* was performed using RT-PCR amplified cDNA fragment as probe, homologous to *cyp19a1*, which was radiolabelled with $^{32}$P-dCTP using random primer labeling kit (Perkin Elmer, Boston, MA,

USA) After three rounds of screening, 2 positive clones were isolated after excision. The pBluescript phagemids were then sequenced bi-directionally.

**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 truncated *cyp19a1* cDNA obtained after library screening. 5' and 3' RACE templates and RT-PCR were performed using gene-specific primers designed from truncated *cyp19a1* clone as per the manufacturer's protocol. The sequences of gene specific primers used for 5' and 3' RACE are listed in table 1 .The RACE products obtained were cloned into pGEMT-easy vector , subsequently sequenced and their identity were analyzed by NCBI- BLAST search.

**Table 1. Primers for cloning and real-time RT-PCR expression analysis of *cyp19a1***

| Sl. NO. | Primer | Sequence 5' to 3' | Purpose |
|---|---|---|---|
| 1. | DF1 | **5' TGGWYKGGNATHGGBACDGC 3'** | To amplify a cDNA fragment |
| 2. | DR1 | **5'GGVCCDGTBARVGCTTTRG 3'** | To amplify a cDNA fragment |
| 3. | 3P | **5'GCACTCTCATCTCCCTCAGCTCACGTT 3'** | 3'primary RACE primer for *cyp19a1* |
| 4. | 3N | **5' GCATGCGCAGCGCTGACGATGATGTC 3'** | 3'nested RACE primer for *cyp19a1* |
| 5. | 5P | **5' CTCCTCCCCACAGATCCACACTCGCA 3'** | 5'primary RACE primer for *cyp19a1* |
| 6. | 5N | **5'AGACACTGCAGCCCGACTTTACTCCCG 3'** | 5' nested RACE primer for *cyp19a1* |
| 7. | RTF | **5' AGGTCCCTGGTTTTGTCTG 3'** | Real-time RT-PCR |
| 8. | RTR | **5' TGCAGATGGCCTGCTGAGG 3'** | Real-time RT-PCR |
| 9. | *β-actin* | **5' ACCGAAGTCCATCACAATACCAGT 3'** | Real-time RT- |

| | | | |
|---|---|---|---|
| | fw | | PCR |
| 10. | *β-actin Rv* | **5' GAGCTGCGTGTTGCCCCTGAG 3'** | Real-time RT-PCR |

**Functional characterization of catfish *cyp19a1* expressed in mammalian COS-7 cells**

Functional characterization of *cyp19a1* in COS-7 cells was performed following the method described earlier (Chang *et al*., 2005). 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 *cyp19a1* ORF in pCDNA3.1$^+$ TOPOV5-His mammalian expression vector (Invitrogen) 1-1.5µg of recombinant plasmid DNA was transfected in COS-7 cells using Tfx20 (Promega). Mock pcDNA3.1 (ORF insert locked in reverse orientation) recombinant plasmid was used as negative control. After 24h incubation, 30ng of testosterone was added as substrate to the cells. Subsequently after 24h, the culture medium was separated from cells by centrifugation at 1000 rpm, 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). Entire protocol was repeated thrice with three replicates each time. The $E_2$ produced in the culture medium was measured using estradiol-17β enzyme linked Immunoassay kit (Cayman) according to the manufacturer's protocol. A recovery of 93-95% was obtained by the extraction procedure described in $E_2$- EIA- kit. The minimal detection threshold was 20pg/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 variation was 1.36 ± 0.04% and 1.75 ± 0.06% for $E_2$ produced from *cyp19a1* transfected plate. Results were expressed as means ± 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 $P< 0.05$.

**Real-time qRT-PCR for *cyp19a1***

Expression of *cyp19a1* was analyzed by qRT-PCR. Gene-specific primers were designed for *cyp19a1* 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 between 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* sequence obtained from catfish which is listed in table1. Primer specificity for 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) using SYBR Green 1 (Applied Biosystems). Each sample (n=5) was run in triplicates with a final volume of 25µl containing 0.3µl of cDNA representing the four different phases of the testis, 10 pmol of each primer, and 12.5µl of SYBR Green PCR master mix (Applied Biosystems). 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 145 Biosystems) was used to compile data from all plates and compare expression levels. Transcript abundance of *cyp19a1* gene was 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. For each PCR run, a non-template control was

included to ensure the absence of contamination. Data analysis was carried out using one-way ANOVA followed by Tukey's test. Significance was accepted at $P < 0.05$.

**Changes in aromatase activity in ovary at different phases of female reproductive cycle**

The aromatase activity in ovarian tissues collected from five fishes each in different seasons was assayed as described by Tsai *et al*., (2000) with minor modifications. Briefly, microsomes from the ovary were prepared by homogenizing 500 mg of tissue in 3 ml of 0.1M potassium phosphate ($KPO_4$) buffer pH7.4 followed by centrifugation at 9000Xg for 20 min at 4ºC to clear debris, and a final spin at 105,000Xg for 1h at 4ºC. The microsomal pellet was then rinsed with 0.1M $KPO_4$, pH 7.4 buffer containing 0.1mM EDTA and resuspended in 500µl of $KPO_4$ buffer, 0.1mM EDTA and 20% v/v glycerol. *cyp19a1* activity was measured by incubating 300 µg of microsomal protein and 0.6 µM of 1β-[$^3$H] androstenedione in 500µl of $KPO_4$, 1mM EDTA, 1mM β-$NADP^+$, 5 mM glucose-6-phosphate and 10 unit of glucose-6-phosphate dehydrogenase at 37°C for 1h in a water shaker. The reaction was stopped by adding iced 10% tricholoacetic acid containing 20 mg charcoal/ml. After centrifugation at 1000Xg for 10min at 4ºC the supernatant was collected in scintillation vials and 3ml of cocktail-T was added (SRL chemicals, Mumbai). The radioactivity was measured using liquid scintillation counter. Data analysis was carried out using one-way ANOVA followed by Tukey's test. Significance was accepted at $P < 0.05$.

**Expression profiles of *cyp19a1* and aromatase activity in gonads and brain of developing catfish**

Expression pattern of *cyp19a1* was assessed in developing male and female groups by real-time PCR. Tissue samples were collected from developing fish larvae by carefully

dissecting with fine forceps under stereozoom microscope (Leica, Wetzlar, Germany), after morphologically (histologically) identifying them as testis and ovary at 45, 60, 75, 100 and 150 dph, under sterile conditions. The tissue samples were then immediately snap-frozen in liquid $N_2$ and kept at -80ºC for subsequent analysis. For expression study, gonads and brain from 10-20 fishes were pooled and for enzymatic analysis 20-25 (ovaries) and 10-12 (brains) were collected to have five biological samples (n=5) as per their age. All the assays were repeated thrice. From histological observation it was evident that the critical period of sex determination was around 35-50 dph when the gonads get morphologically distinguishable in air-breathing catfish *C. gariepinus* (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 larvae for expression and enzyme activity analysis of aromatase. Aromatase enzyme activity in testicular tissue was not performed in developing fish as the testes weighed less (in µg) till 75 dph in catfish. Further pooling of samples was also difficult, as we need to sacrifice large number of male larvae having similar stage of testicular development. Data analysis was carried out using one-way ANOVA followed by Tukey's test. Significance was accepted at $P < 0.05$ for the various stages of gonad development.

**RT-PCR analysis of tissue distribution pattern of *cyp 19a1***

5µg of total RNA was isolated from different tissues of female catfish (preparatory phase) 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') 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, Foster, CA, USA).

**hCG-induced *in vivo* study of expression and activity of *cyp19a1* gene**

Fishes were collected during different phases i.e., February (preparatory), May (prespawning), September (spawning) and injected with hCG (1000 IU/kg body weight), intraperitoneally. Ovarian tissue at every 4h up to 12h was collected by sacrificing five fishes after exposing it to ice cold water. Expression and activity analyses of *cyp19a1* following hCG induction were performed as elucidated above. Present experiment was conducted thrice using different batches of female fish (n=5). All the data were expressed as mean ± SEM of three replicates. Significance among groups was tested by ANOVA followed by Tukey's test. Difference among groups were considered significant at $P<0.05$.

**Immunocytochemistry of *cyp19a1***

Immunocytochemistry was carried out using heterologus primary antibody (antibody was raised against ovarian aromatase form of the Nile tilapia) on vitellogenic catfish ovary and during early ovarian development 30, 50 and 72 dph. Avidin-biotin peroxidase method as described by Swapna *et al*. (2006) was followed for cellular localization of *cyp19a1*. In brief, 5µm thick ovarian paraffin sections were cut using rotatory microtome, deparaffinized and blocked using 10% normal goat serum for 1h at room temperature. Then primary antibody was added (1:750 times dilution) and sections were incubated at 4ºC for 24h in humid chamber. Subsequently, they were incubated with HRP labeled secondary antibody (Bangalore Genei) 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 inbuilt camera.

## Results

### Molecular cloning of catfish *cyp19a1*

A set of degenerate primers yielded a partial cDNA of 268 bp (ovary) whose sequence identity was confirmed by NCBI-BLAST. Full-length cDNA of *cyp19a1* was obtained through 5', 3' RACE strategy using gene specific primers designed from partial cDNA fragment. 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, 1551 bp ORF encoding 517 amino acid with ATAAA as polyadenylation signal that is 47 bp upstream of poly-A tail (Fig. 1 & 2). The sequence data of *cyp19a1* has been submitted to GenBank (Accession Number: **GU220075**). ClustalW multiple alignments revealed the presences of conserved signature domains which include the heme binding region, the I-helix region and the aromatase specific sequence (Fig. 3). *cyp19a1* gene of catfish specifically displayed high homology in the signature domains regions with aromatases cloned from other teleosts. Phylogenetic analysis revealed that, catfish *cyp19a1* has about 73-86% homology with other piscine ovarian form sharing highest identity with channel catfish (Fig. 4).

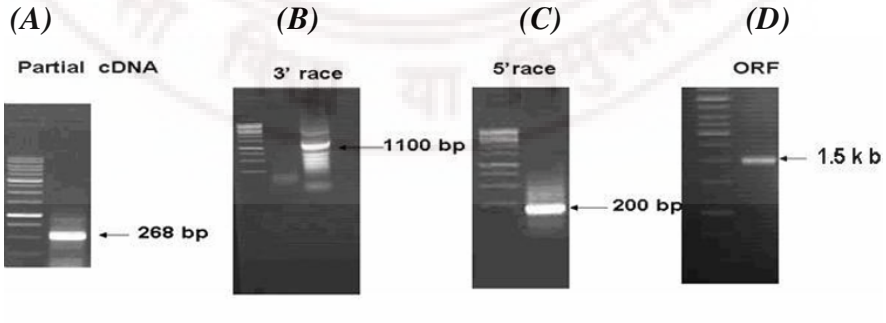



***Figure 1. 1% Agarose gels showing RT-PCR products (A) partial cyp19a1 cDNA fragment, (B), (C), (D) 3', 5' RACE fragment and ORF of cyp19a1.***

```
0001 GAATCGAGCTCGTACTCCCGAATGCATCTAAATAAGCAGGGGTATCAACGCAGAGTACGCGGGGCAGTGAGA
0073 GACAGAACCCCCTCTCACGTCCC
0096 Atggcagcacacgtgttaccagtgtgtgagtgcggtgtgaagccggcgcgtttcagccagacggtcatggag
       M  A  A  H  V  L  P  V  C  E  C  G  V  K  P  A  R  F  S  Q  T  V  M  E
0168 attttactgcacggggctcggaacgggacgagccctcagcaggagaacccgcgtgggattacactcgtactg
       I  L  L  H  G  A  R  N  G  T  S  P  Q  Q  E  N  P  R  G  I  T  L  V  L
0240 ctgctgctgtgtctggttctgctgctcgctgtgtggaaccgcaacgagaagaagtgctgcattccaggtccc
       L  L  L  C  L  V  L  L  L  A  V  W  N  R  N  E  K  K  C  C  I  P  G  P
0312 tggttttgtctgggcttgggtcccctgttgtcctacctcaggtttatctggatgggaatcgggacagcgagt
       W  F  C  L  G  L  G  P  L  L  S  Y  L  R  F  I  W  M  G  I  G  T  A  S
0384 aattactacaatgagaagtacggagacatggtgcgagtgtggatctgtggggaggagacgcttgtcctcagc
       N  Y  Y  N  E  K  Y  G  D  M  V  R  V  W  I  C  G  E  E  T  L  V  L  S
0456 aggccatctgcagtttatcacgtgctgaaacactcccagtacacgtccaggttcgggagtaaagtcgggctg
      R  P  S  A  V  Y  H  V  L  K  H  S  Q  Y  T   S  R  F  G  S  K  V  G  L
0528 cagtgtctcgggatgcacgagcagggaatcatcttcaactccaacgtcgccctctggaagaaagtgcgcagc
       Q  C  L  G  M  H  E  Q  G  I  I  F  N  S  N  V  A  L  W  K  K  V  R  S
0600 tacttcgctaaagctctaactggtccaggtctgcagaggacgctaaaaatctgcaccacatccgcaaacaca
       Y  F  A  K  A  L  T  G  P  G  L  Q  R  T  L  K  I  C  T  T  S  A  N  T
0672cacacctggatgatctgtctcagctgtggacgcgcagggacaggtgaacgtcctgaacctgctgcgctgcat
      H  T  W  M  I  C  L  S  C  G  R  A  G  T  G  E  R  P  E  P  A  A  L   H
0744 tgtggtggacatctccaaccgcctgttcctgggtgtgccactaaacgaacaaaacctgctgtccaaaatcca
       C  G  G  H  L  Q  P  P  V  P  G  C  A  T  K  R  T  K  P  A  V  Q  N  P
0816 caggtacttcgacacgtggcagacggtcttgatcaaaccggacatttatttcaggctgaagtggctccacga
       Q  V  L  R  H  V  A  D  G  L  D  Q  T  G  H  L  F  Q  A  E  V  A  P  R
0888 caaacacaagcacgctgctcaggaacttcacgacgcctttgtgatctgttgaacagaaacgaactgaactgc
       Q  T  Q  A  R  C  S  G  T  S  R  R  L  C  D  L  L  N  R  N  E  L  N  C
0960 agcaggctgaaaaactcgacaacctgctttactgaggaactgatatttgcccagagtcacggtgagctgacg
       S  R  L  K  N  S  T  T  C  F  T  E  E  L  I  F  A  Q  S  H  G  E  L  T
1032 gcggagaacgtggggcagtgtgtgttggagatggtgatcgcggctccggacacgctgtccatcagtgtgttc
       A  E  N  V  G  Q  C  V  L  E  M  V  I  A  A  P  D  T  L  S  I  S  V  F
1104 ttcatgctgctgctgctgaaacaggacacggaggtggagagacgcatcctgactgagatacacactgtgctg
       F  M  L  L  L  L  K  Q  D  T  E  V  E  R  R  I  L  T  E  I  H  T  V  L
1176 ggtgaagctgagctgcagcactctcatctccctcagctcacgttctggagtgtttcattatggctctgcgct
       G  E  A  E  L  Q   H  S  H  L  P  Q  L  T  F  W  S  V  S  L  W  L  C  A
1248 ttcaccgtggtggactcagcatgcgcagcgctgacgatgatgtcattgagggctacaggtaccgagagggac
       F  T  V  V  D  S  A  C  A  A  L  T  M  M  S  L  R  A  T  G  T  E  R  D
1320 aaacatcatcctgacgtgggccgaatgcaccgctccgagttcttcccaaaccctcagagttcagcctcgaac
       K  H  H  P  D  V  G  R  M  H  R  S  E  F  F  P  N  P  Q  S  S  A  S  N
1392 aacttcaacaaactggtccccagtcgtttcttccagccctttggctctggtcctcgtttcctgtgttggtac
       N  F  N  K  L  V  P  S  R  F  F  Q  P  F  G  S  G  P  R  F  L  C  W  Y
1464 acatcgccatggtgtgatgaaggccatcttggtgtcggtgttgtctcgtttctccgtgtgtcctgggaaagc
       T  S  P  W  C  D  E  G  H  L  G  V  G  V  V  S  F  L  R  V  S  W  E  S
1536 tgcactgtggagaacatcgctcacaccaacgacctctcacagcaacctgtggaggacacactgagtgtacgc
       C  T  V  E  N  I  A  H  T  N  D  L  S  Q  Q  P  V  E  D  T  L  S  V  R
1608 ttcatcccgcgaaacacacacaccagaacccaagcgtga          AGGCCATCTTGGTGTCGGTGTT
       F  I  P  R  N  T  H  T  R  T  Q  A  *
1669 GTCTCGTTTCTCCGTGTGTCCTGGGAAAGCTGCACTGTGGAGAACATCGCTCACACCAACGACCTCTCACAGC
1742 AACCTGTGGAGGACACACTGAGTGTACGCTTCATCCCGCGAAACACACACACCAGAACCCAAGCGTGAACTCC
1816 TCTGTATTACACGCAGCTGGAACCCTTACAGCTTTCCCTCTGACTGTTATAAAGCTCTGACACTCGAGACTCC
1888 TTCCACTGATGTACTGCAAAGCCTCACAAAAAAAAAAAAAAAAAAAAAAAA
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***Figure 2. Nucleotide and deduced amino acid sequences of catfish cyp19a1. UTRs (5' 1-97 bp, 3' 1648-1941 bp) are boldfaced and the first polyadenylation signal is underlined.***

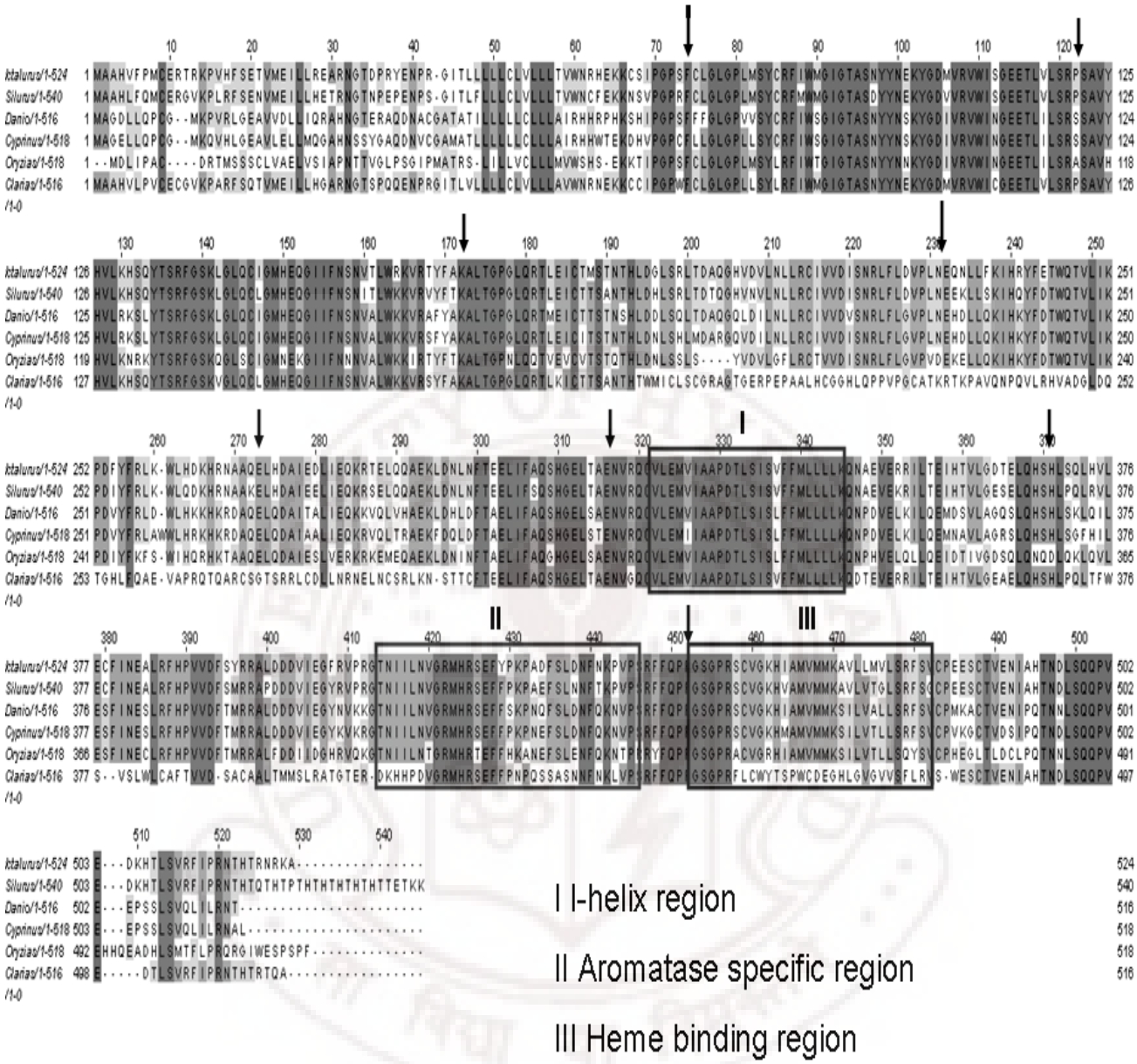


***Figure 3. ClustalW multiple alignment of catfish cyp19a1 with other vertebrate counterparts. The conserved domains are the I-helix region, aromatase specific site and the heme binding region which are indicated by roman numbers and shown in rectangle. The intron positions are marked by arrows. Ictalurus punctatus S75715; Silurus meridonalis AY325908; Danio rerio AF226620; Cyprinus carpio EU375455; Oryzias latipes D82968 and Clarias gariepinus GU220075.***

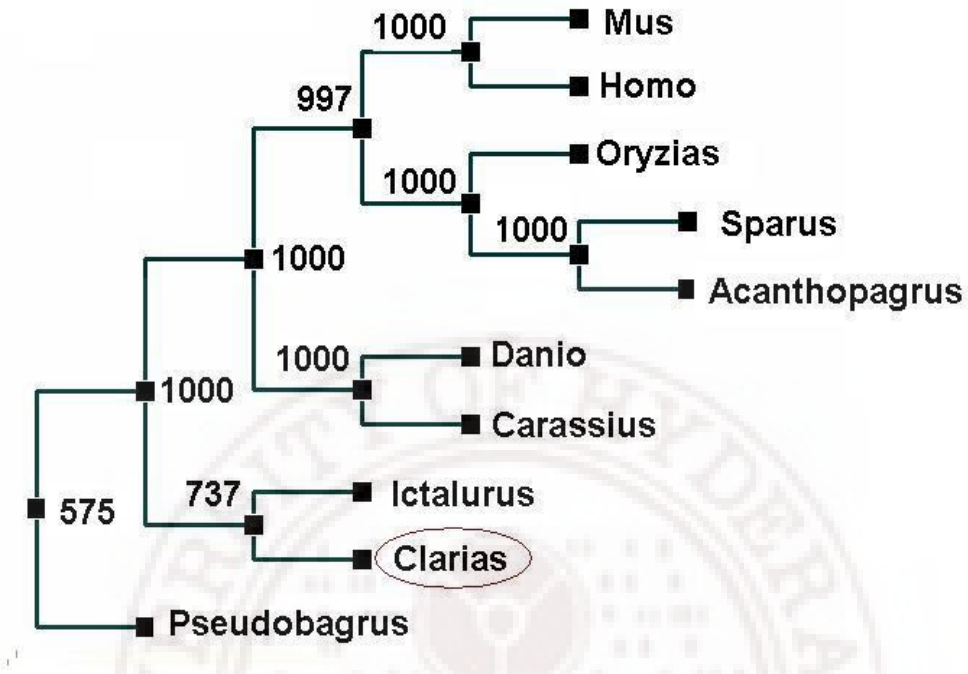


***Figure 4. Phylogenetic tree showing the evolutionary relationship of catfish cyp19a1. POWER tool (www.power.nhri.org) with 1000 bootstrap trial was used to construct the unrooted tree. Branch length is proportional to the distance between each protein. Bootstrap values are the number of trials that this cluster was found in 1000 trials. The GenBank accession numbers for the teleostian cyp19a1 are given in fig.3. Accession no.: Pseudobagrus fulvidraco AY871802; Carassius auratus AB009336; Sparus aurata AF399824; Acanthopagrus schlegeii AY273211; Homo sapien NM_000103; Mus musculus NP031836 and Clarias gariepinus GU220075.***

**Functional characterization in COS-7 cells**

The transiently expressed *cyp19a1* ORF in COS-7 cells after transfection was able to transform the substrate testosterone to $E_2$ demonstrating that the inserted cDNA in mammalian expression vector pcDNA3.1 were indeed functional, possessing the property to aromatize C19 androgens to C18 estrogens ($E_2$). 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 % by *cyp19a1* (Fig. 5).

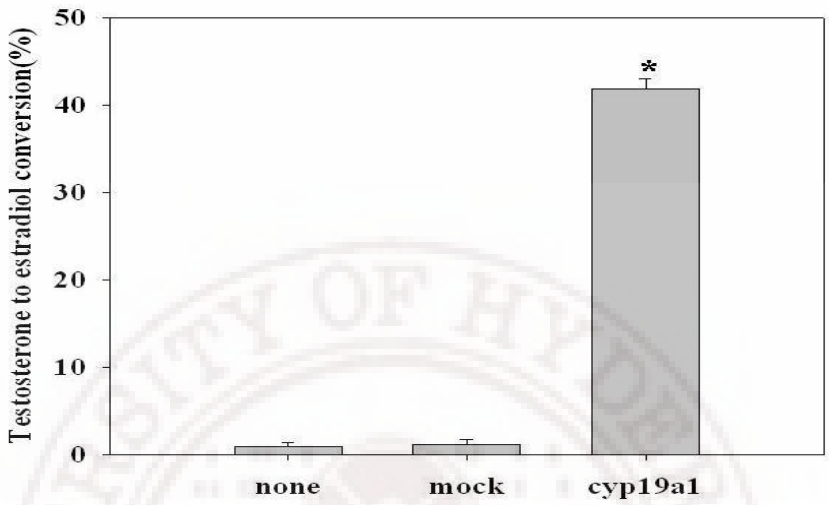


***Figure 5. Representative histogram showing the percentage conversion of testosterone to estradiol-17β by recombinant cyp19a1 protein transiently expressed in COS-7 cells (n=3 different transfection with triplicate assays at each time), Data from percentage conversion of testosterone to estradiol were expressed as mean ± SEM. Significance between groups were tested by ANOVA followed by Dunnett's test using Graph pad prism 5 software, (P<0.05),* indicates the significance.***

**Phase-dependent expression and enzyme activity of *cyp19a1* in ovary**

Phase-dependent expression of ovarian aromatase revealed *cyp19a1* mRNA was abundantly expressed in prespawning phase followed by a sharp decline in spawning and regressed phases (Fig.6 A). Analyses of ovarian aromatase activity during different phases of ovarian cycle were in accordance with the mRNA levels (Fig.6 B). Serum $E_2$ level followed seasonal variation peaking in the prespawning phase (Fig.6 C), further supporting the data of *cyp19a1* transcript and aromatase activity during different phases of ovarian cycle.

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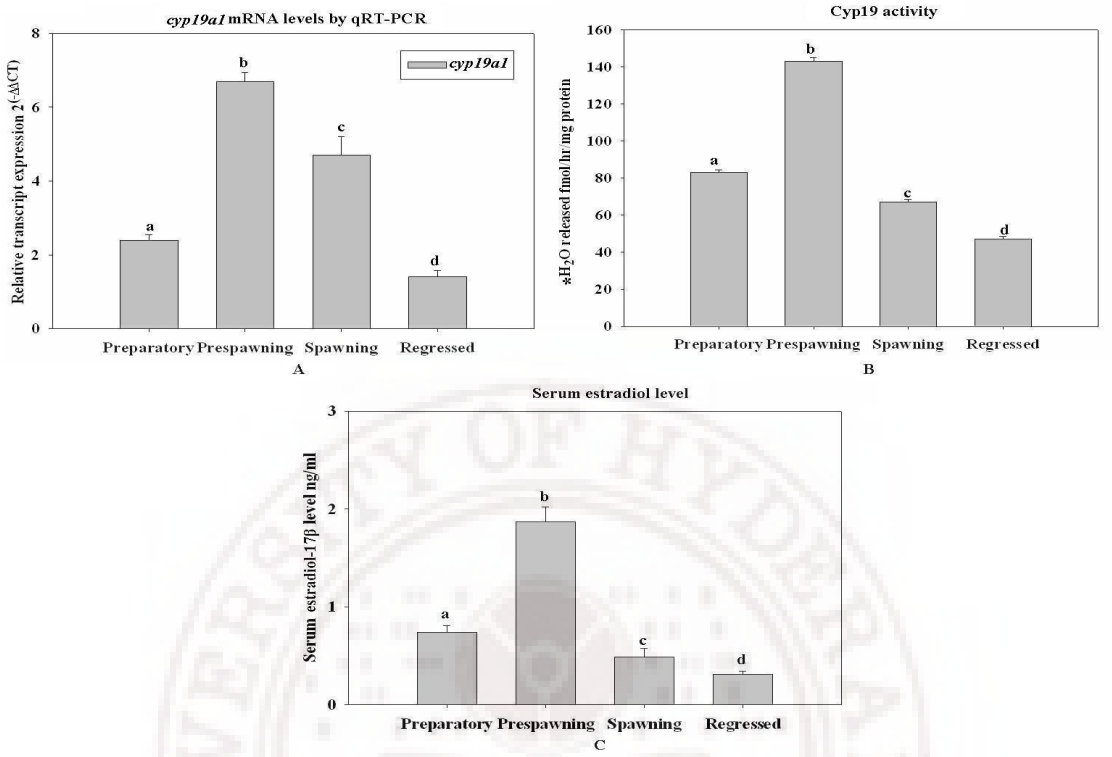



***Figure 6. Graphical representation of changes in A) transcript level of cyp19a1 by real-time PCR, B) aromatase (cyp19) activity at different phases of ovary and C) serum estradiol-17β levels from female fish in preparatory, prespawning, spawning, and regressed phase. Data were expressed as mean ± SEM. Significance between groups were tested by ANOVA followed by Tukey-Kramer's test using Graph pad prism 5 software. Means with different alphabets differ significantly, P<0.05.***

**Ontogeny of *cyp19a1* and aromatase activity**

Expression of *cyp19a1* was prominent in ovary and female brain (Fig. 7A). In male brain relatively low levels of *cyp19a1* was detected from 75 dph and in testis from 60 dph. The enzyme activity analysis demonstrated elevation in ovarian aromatase activity in age-dependent manner (Fig. 7B). In female brain, the aromatase activity was high (Fig. 7C) when compared to male brain during early development in catfish. It also showed an increase in age-dependent manner.

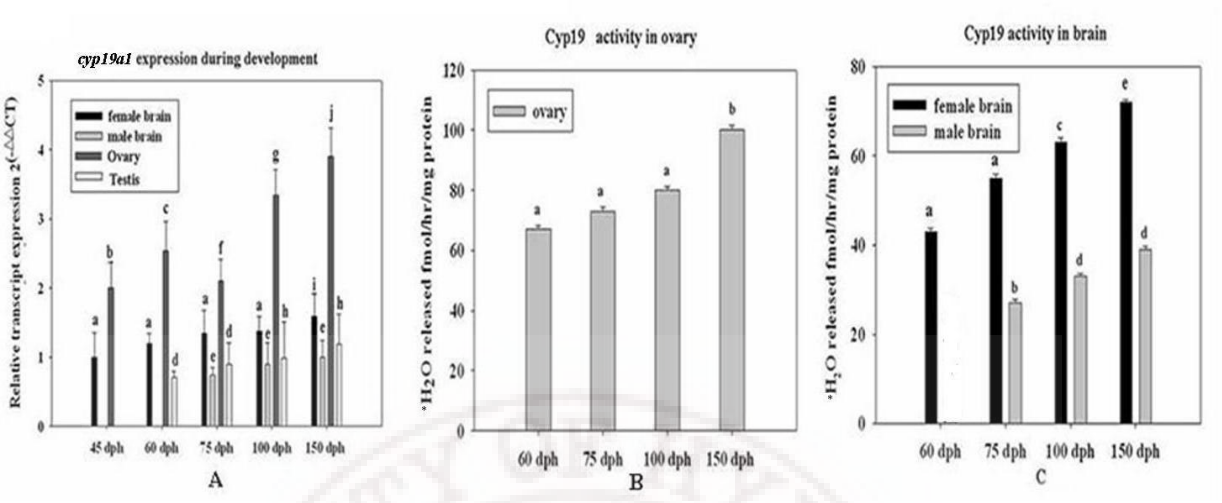



***Figure 7 Graphical representation of changes in A) temporal expression pattern of cyp19a1 in larvae at 45, 60, 75, 100 and 150 dph in gonad and brain of male and female catfish by real-time RT-PCR, B) enzyme activity of aromatase (cyp19) at 60, 75, 100 and 150 dph in ovarian tissue and C) enzyme activity of aromatase (cyp19) at 60, 75, 100 and 150 dph in brain. Data from real-time PCR were expressed as mean ± SEM. Significance between groups were tested by ANOVA followed by Tukey-Kramer's test using Graph pad prism 5 software. Means with different alphabets differ significantly. Common alphabets indicate means that are not significantly different (P<0.05).***

**Tissue distribution**

Using RT-PCR analysis, *cyp19a1* expression was detected in several tissues other than gonads including brain, spleen, liver, intestine, kidney, heart and muscle. The expression was predominant in ovary, intestine, muscle and spleen (Fig. 8).

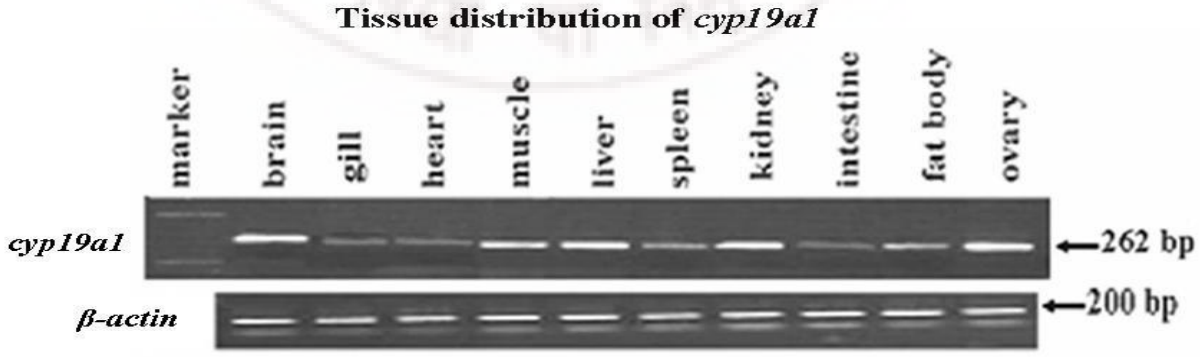


***Figure 8. Representative gel showing spatial expression pattern of cyp19a1 in different female tissues by semi-quantitative RT-PCR analysis.***

**Expression and activity of *cyp19a1* in female catfish during preparatory, pre-spawning and spawning phase after *in vivo* induction with hCG**

*In vivo* hCG treatment during preparatory phase resulted in significant increase of *cyp19a1* transcripts and enzyme activity from 8h with subsequent rise measured up till 24h, when compared to control ovarian samples (Fig. 9A & B). In the pre-spawning phase, induction with hCG demonstrated a rapid increase in *cyp19a1* transcript levels and aromatase activity right from 4h reaching a maximum value at 8h followed by drop at later time points (Fig. 10A & B). There was no significant change in the transcripts and enzyme activity of *cyp19a1* after hCG induction in the spawning phase ovary (Fig. 11 A & B).

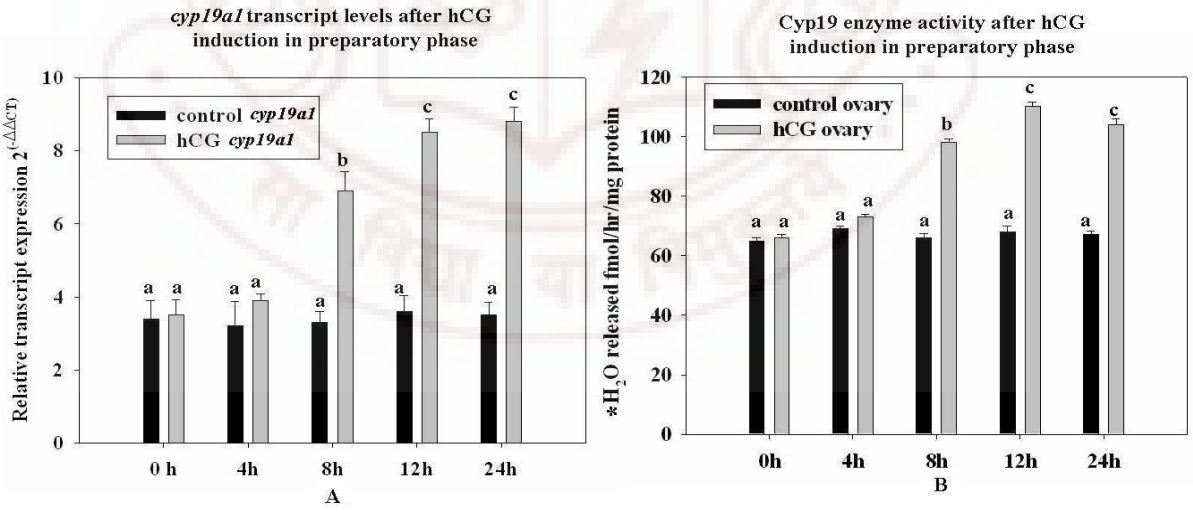



***Figure 9. Graphical representation of the changes in A) the expression pattern of cyp19a1 by real-time PCR and B) enzyme activity of aromatase (cyp19), compared to control after hCG-induction in the preparatory phase catfish. X-axis represents hours after treatment. Statistical analysis was done by one way ANOVA followed by Dunnett's test using Graph pad prism 5 software. Values are mean ± SEM, n=5, common***

***alphabets indicate means that are not significantly different. Means with different alphabets differ significantly (P<0.05).***

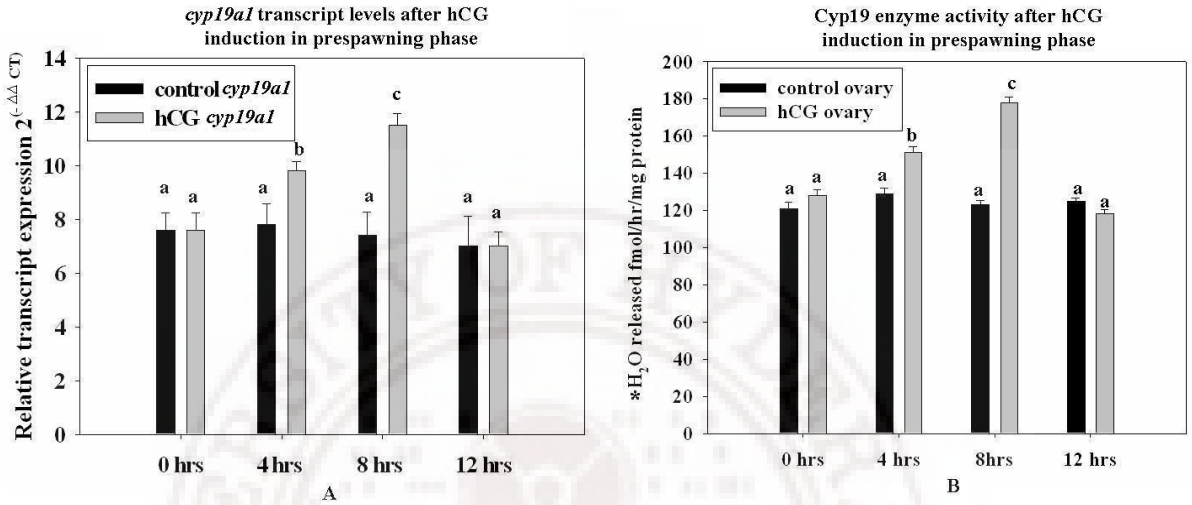



***Figure 10. Graphical representation of the changes in A) the expression pattern of cyp19a1 by real-time PCR and B) enzyme activity of aromatase (cyp19), compared to control after hCG-induction in prespawning phase catfish. X-axis represents hours after treatment. Statistical analysis was done by one way ANOVA followed by Dunnett's test using Graph pad prism 5 software. Values are mean ± SEM, n=5, common alphabets indicate means that are not significantly different. Means with different alphabets differ significantly (P<0.05).***

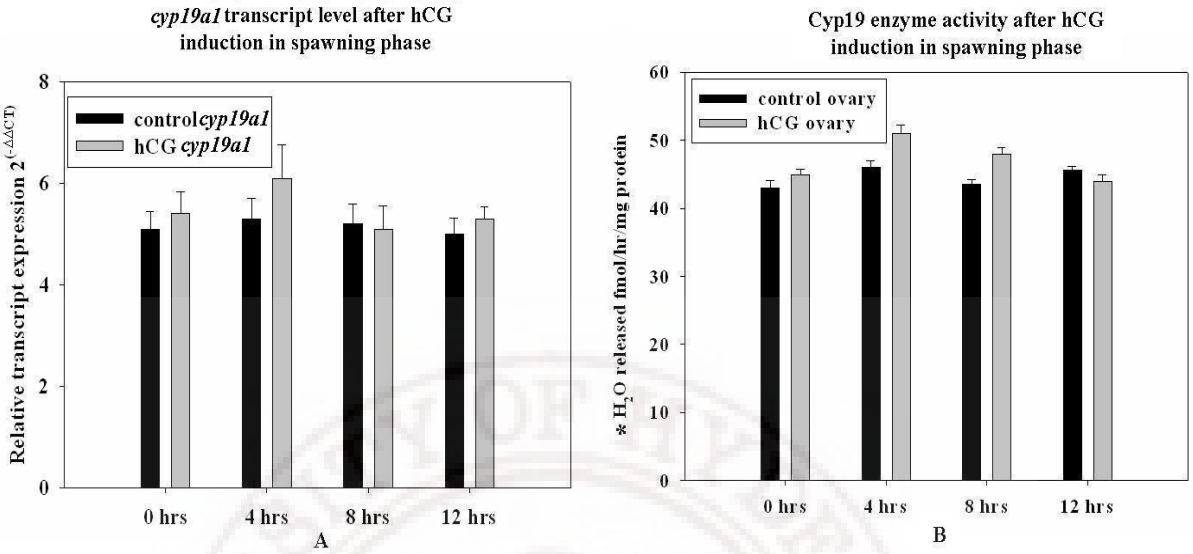
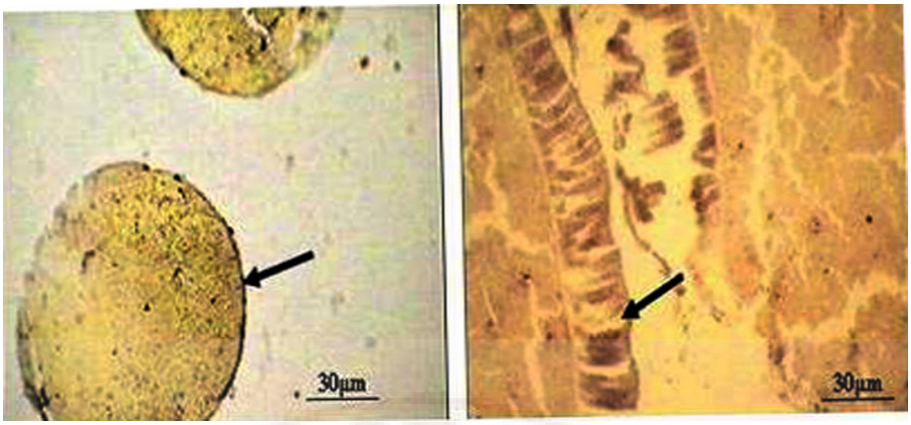


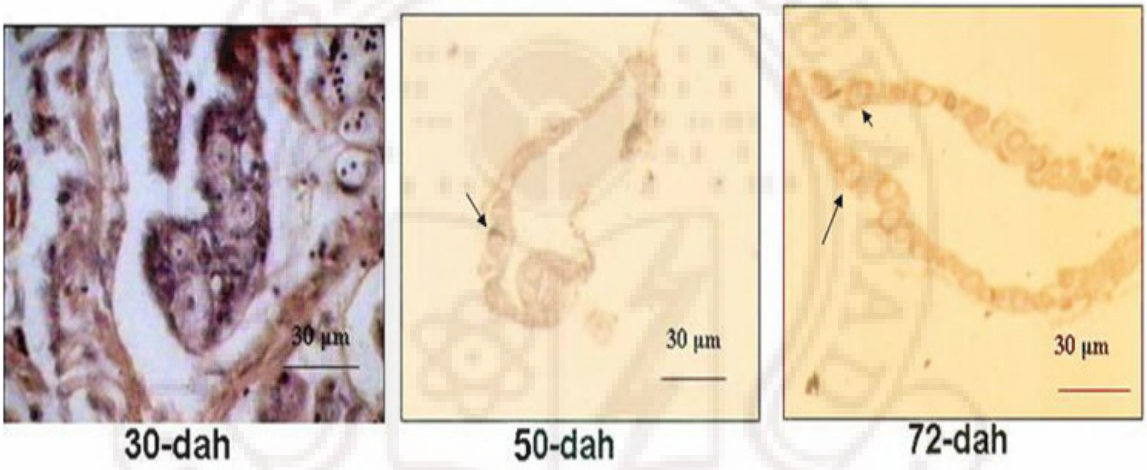

***Figure 11. Graphical representation of the changes in A) the expression pattern of cyp19a1 by real-time PCR and B) enzyme activity of aromatase (cyp19), compared to control after hCG-induction in spawning phase catfish. X-axis represents hours after treatment. Statistical analysis was done by one way ANOVA followed by Dunnett's test using Graph pad prism 5 software. Values are mean ± SEM, n=5, P<0.05.***

**Immunocytochemistry of *cyp19a1***

Immunolocalization of ovarian form using heterologous (the Nile tilapia) aromatase antibody gave positive immunoreactivity in follicular layers surrounding the oocytes. The diaminobenzidine staining deepened in the layer surrounding the early postvitellogenic phase (Fig. 12A), confirming the abundance of *cyp19a1* protein in the course of oocyte growth and development during catfish reproductive cycle. Ontogeny study using aromatase antibody from 30 dph up to 72 dph in juvenile catfish clearly demonstrated the appearance of *cyp19a1* protein from the inception of ovary formation at around 50 dph (Fig.12B) implying a definite role of aromatase during female sex differentiation in *C. gariepinus*.




A




B

***Figure 12. Immunolocalization of cyp19a1 in A) vitellogenic ovary and B) at 30, 50 and 72 days after hatch (dah) in catfish.***

**Discussion**

Various approaches had been attempted to validate the significance of $E_2$ and *cyp19a1* during ovarian differentiation and oogenesis across vertebrate phyla including teleosts (Simposon *et al.*, 1994; Hayes, 1998; Piferrer and Blazquez, 2005; Guiguen *et al.*, 2010). Previous independent studies from teleosts displaying varied reproductive strategies, i.e., gonochoristic, sequential hermaphrodites and true hermaphrodite, have furnished

information on the structure of *cyp19a1* genes, its transcript levels and/or their enzyme activity during embryogenesis, gonad development and female reproductive cycle to discern *cyp19a1* specific contribution (Donaldson 1996; Nagahama, 2005; see Piferrer and Blazquez, 2005; see Guiguen *et al.*, 2010). Nevertheless, a comprehensive approach on *cyp19a1* has not been attempted so far in a fresh water annual breeder, *C. gariepinus*, which exhibited distinct reproductive phases. Hence in the present study, we focused on elucidating the contributions of the ovarian form of aromatase during ovarian differentiation and gametogenesis. This was accomplished by initially analyzing the transcript and/or activity of *cyp19a1* isoforms in brain, ovary and testis of juvenile catfish from 45 dph up till catfish reached puberty (150 dph) followed by analysis of transcript levels (by real-time PCR) and aromatase activity (by radiometric assay) from the ovarian tissues during different phases. Furthermore, radiometric assay adopted in the present study is better when compared to Chourasia and Joy (2008), where the limitation of assay was end product $E_2$ measurement which may not take into the account of the loss of $E_2$ conversion if any, by $E_2$ converting enzymes (estrogen-2/4-hydroxylases). The transcripts and activity data corroborated well with the measured serum $E_2$ levels.

Finally, we also tried to study the responsiveness of *cyp19a1* expression and enzyme activity after hCG induction at different phases of ovarian cycle, to substantiate our findings that the production of $E_2$ might be under the control of gonadotropins. Hence, the present work examines all the parameters (mRNA, terminal enzyme activity and its modulation by gonadotropins) that lead to the synthesis of effecter steroid hormone $E_2$ by *cyp19a1,* to support our hypothesis whether *cyp19a1* could serve as a candidate marker

gene during ovarian development in catfish and its role in maintaining the reproductive cycle in female catfish.

*Cloning, phylogenetic analysis and functional characterization of cloned cDNA*

The inception of the study was by cloning the cDNA of ovarian aromatase yielding 1551bp ORF encoding deduced protein of 516 amino acid residues whereas the channel catfish (Trant, 1994), and tilapia (Chang *et al*., 1997) *cyp19a1* are composed of 524 and 522 residues, respectively. The catfish form thus being slightly shorter than other teleost. The predicted amino acid sequence of catfish displayed 67% homology to channel catfish 60-57 % identity to other teleost and less than 50% homology with other vertebrate species. Phylogenetic analysis revealed that the catfish *cyp19a1* forms a separate branch within the siluriforms clade in the distance tree by Neighbour-joining method. Transient expression of *cyp19a1* protein in non-steroidogenic mammalian COS-7 cells catalyzed the aromatization of testosterone to $E_2$ indicating that the cloned cDNA indeed coded for functional aromatase and the catalyzing efficiency was comparable to the reported *cyp19a1* recombinant protein from the Nile tilapia (Chang *et al*., 1997).

*Stage dependent expression and enzyme activity of cyp19a1 in ovary*

In *C. gariepinus* expression of *cyp19a1* transcript correlated well with its activity and plasma $E_2$ levels similar to findings from channel catfish and rainbow trout where the plasma $E_2$ levels paralleled *cyp19a1* expression (Kumar *et al*., 2000). The transcript levels and enzyme activity precipitously increased during ovarian recrudescence, i.e. from preparatory phase to the 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. Similar results of seasonal variation in *cyp19a1* transcript and/or activity peaking just prior to oocyte maturation was observed in divergent group of fish species: the Nile tilapia, killifish and blue gourami (Chang *et al*., 1997; Goto-Kazeto *et al*., 2004; Wu Dong *et al*., 2008; Meital *et al*., 2008). Furthermore alike catfish, fathead minnow and goby displayed significant correlations between *cyp19a1* transcript levels and aromatase activity in ovary (Villenevue *et al*., 2006; Kobayashi *et al*., 2004). The fall in enzyme activity of aromatase in *C. gariepinus* during the regressed phase was less pronounced than their northern counterparts (*Clarias batrachus*). Same is true for serum $E_2$ level. This may be essentially due to extended spawning phase in *C. gariepinus* followed by a shorter regression phase as compared to other catfish which inhabits northern part of India. Changes in the months of seasonal phases of catfish reproductive cycle inhabiting northern and southern India may be attributed to explain this phenomenon broadly.

*Ontogeny and immunolocalization*

Non availability of genetic sex population in air breathing catfish impeded us to study the expression of *cyp19a1* before 45 dph and also difficulty in obtaining sufficient tissues constrained us to perform the enzymatic analysis of aromatase in gonad and brain tissues before 45 dph. Therefore, we investigated the relative change in transcript levels from 45 dph onwards and enzymatic analysis after phenotypic sex differentiation, i.e., from 60 dph. Present study revealed dimorphic expression pattern of *cyp19a1* from 45 dph with early expression of brain and gonadal form in female larvae suggesting a crucial role of $E_2$ in maintenance of ovarian differentiation and development. Further detection of relatively low levels of *cyp19a1* expression in the testis implied that estrogens may not be required during early testicular differentiation, reinforcing the validity of the gynoinductor

hypothesis of $E_2$. 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 crucial role at the time of ovarian differentiation and oocyte growth. Aromatase gene expression during larval development is used as proxies for sex differentiation in increasing number of teleost fish, such as zebrafish (Trant et al., 2001; Goto-Kazeto et al., 2004; Sawyer et al., 2006), the Atlantic halibut (Matsuoka et al., 2006), European sea bass (Blazquez and Piferrer 2004), the Nile tilapia (Chang et al., 2005), protogynous wrasse (Choi et al., 2005), southern flounder (Luckenbach et al., 2005) and common carp (Barney et al., 2008). Furthermore, Chiang *et al*., (2001) emphasized that *cyp19a1* but not *cyp19a2* (brain isoform) is a strong candidate involved in ovarian differentiation by the existence of binding regions for sex determining transcription factors in the 5' flanking region of Nile tilapia, zebrafish and goldfish *cyp19a1* gene. Supporting histochemical data showed positive immunoreactivity around the primary follicles found at the periphery of the ovarian cavity a characteristic feature of differentiating ovary corroborating the early detection of aromatase transcript from 45 dah.

*Tissue distribution*

Catfish aromatases are predominantly expressed in ovary (*cyp19a1*) with co-expression of ovarian isoforms in brain. Similar results of overlapping expression of *cyp19a1* isoforms were previously reported in killifish and zebrafish (Trant *et al*., 2001, Chiang *et al*., 2001; Greytak *et al*., 2005). On the contrary in the Nile tilapia and southern catfish, *cyp19a1* was exclusively found in ovary (Chang *et al*., 2005; Liu *et al*., 2007) but the exact role of this overlapping expression is not yet clear. Tissue specific expression revealed presence of

*cyp19a1* isoforms in almost all tissues studied (Patil and Gunasekera, 2008). Expression of *cyp19a1* was also detected in steroidogenic tissues like the testis and anterior kidney but at lower levels concomitant to the report from sea bass (Dalla valle *et al*., 2002). Discrepancy among fishes in the spatial expression of *cyp19a1* may be explained as a consequence of species specific gene specialization that befits their mode of reproduction, sexual and courtship behavior and their habitat.

*Phase-dependent expression and activity of both the isoforms after in vivo hCG treatment*

By using hCG, we intended 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 aromatase activity and transcript levels of *cyp19a1* after hCG induction in the prespawning and preparatory phases while no response was seen in the spawning phase. Induction by hCG in the preparatory and prespawning phases but not in the spawning phase could be explained by the reported shift in the steroidogenesis which occurs in oocytes undergoing full-grown immature stage to reach meiotic maturation (Senthilkumaran *et al*., 2004). 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). In the Nile tilapia hCG stimulated mRNA levels of *cyp19a1* and *Ad4BP1/SF1* in full-grown ovarian follicles suggesting that gonadotropin regulated *cyp19a1* expression via modulation of *Ad4BP1/SF-1* (Yoshiura *et al*., 2003). The mechanism of action of hCG as already proposed might be through transcription effectors *CREB* and *Ad4BP1/*SF-1whose presence needs to be probed in our model as well. However, gonadotropins had no influence on oocytes that were undergoing

final oocyte maturation (Yoshiura *et al*., 2003; Senthilkumaran *et al*., 2004). Present study is first of its kind to simultaneously correlate transcript and enzyme activity changes in ovarian tissues after hCG treatment.

**Conclusion**

In summary, full-length cDNA of *cyp19a1* was cloned from ovary of catfish which exhibited high homology with other siluriforms. Purified recombinant protein catalyzed the aromatization of testosterone to $E_2$. Present study demonstrated the stage-dependent expression and activity of *cyp19a1* in ovary during reproductive cycle. Further present study confirms the specific role of ovarian form of aromatase during ovarian differentiation justifies *cyp19a1* could as a candidate marker gene for ovarian differentiation in *C. gariepinus*. Present study also report differential responsiveness of *cyp19a1* to hCG induction during different phases of reproductive cycle.

**References**

Blazquez M., Piferrer F., 2004. Cloning, sequence analysis, tissue distribution, and sex-specific expression of the neural form of P450 aromatase in juvenile sea bass (*Dicentrarchus labrax*). Mol. Cell. Endocrinol., 219:83–94.

Blazquez M., Gonzalez A., Papadaki M., Mylonas C., Piferrer F., 2008. Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol., 158:95–101.

Barney M.L., Patil J.G., Gunasekera R.M., Carter C.G., 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenic expression. Gen. Comp. Endocrinol., 156: 499–508.

Callard G.V., Tchoudakova A.V., Kishida M., Wood E., 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J .Steroid Biochem. Mol. Biol., 79: 305–314.

Chang X.T., Kobayashi T., Kajiura H., Nakamura M., Nagahama Y., 1997. Isolation and characterization of the cDNA encoding the tilapia (*Oreochromis niloticus*) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein and enzyme activity in ovarian follicles during oogenesis. J. Mol. Endocrinol., 18: 57-66.

Chang X.T., Kobayashi T., Senthilkumaran B., Kobayashi-Kajura H., Sudhakumari C.C., Nagahama Y., 2005. Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol., 141: 101-115.

Chiang E.F.L., Yan Y.L., Guiguen Y., Postlethwait J., Chung B., 2001. Two cyp19 (P450 aromatase) genes on duplicated zebrafish chromosomes are expressed in ovary or brain. Mol. Biol. Evol., 18:542–550.

Choi J., Park J., Jeong H., Lee Y., Takemura A., Kim S., 2005. Molecular cloning of cytochrome P450 aromatases in the protogynous wrasse, *Halichoeres tenuispinis*. Comp. Biochem. Physiol., 141B: 49–59.

Chourasia T.K., Joy K.P., 2008. Ovarian P450 aromatase activity in the catfish, *Heteropneustes fossilis*: Seasonal changes and effects of catecholestrogens. Gen. Comp. Endocrinol., 156: 537–543.

Dalla Valle L., Ramina A., Vianello S., Belvedere P., Colombo L., 2002. Cloning of two mRNA variants of brain aromatase cytochrome P450 in rainbow trout (*Oncorhynchus mykiss* Walbaum) J. Steroid Biochem. Mol. Biol., 82:19–32.

Devlin R.H., Nagahama Y., 2002. Sex determination and sex differentiation in fish: an overview of the genetic, physiological, and environmental influences. Aquaculture 208:191–364.

Donaldson E.M., 1996. Manipulation of reproduction in farmed fish. Anim. Reprod. Sci., 42: 381–392.

Esterhuyse M.M., Helbing C.C., van Wyk, J.H., 2008. Temporal expression of two cytochrome P450 aromatase isoforms during development in *Oreochromis mossambicus*, in association with histological development. Comp. Biochem. Physiol., 3D: 297–306.

Gelinas D., Pitoc G.A., Callard G.V., 1998. Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment1. Mol. Cell. Endocrinol., 138: 81–93.

Goto-Kazeto R., Kight K.E., Zohar Y., Place A.R., Trant J. M, 2004. Localization and expression of aromatase mRNA in adult zebrafish. Gen. Comp. Endocrinol., 139: 72–84.

Greytak S.R., Champlin D., Callard G.V., 2005. Isolation and characterisation of two cytochrome P450 aromatase forms in killifish (*Fundulus heteroclitus*): differential expression in fish from polluted and unpolluted environments. Aquat. Toxicol., 71: 371-389.

Guiguen Y., Fostier A., Piferrer F., Chang C.F. 2010. Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol., 165: 352-366.

Hayes T.B., 1998. Sex determination and primary sex differentiation in amphibians: Genetic and developmental mechanisms. J. Exp. Zool. 281: 373–399.

Ijiri S., Kazeto Y., Lokman, P.M., Adachi, S., Yamauchi, K., 2003. Characterization of a cDNA encoding P-450 aromatase (CYP19) from Japanese eel ovary and its expression in ovarian follicles during induced ovarian development. Gen. Comp. Endocrinol., 130:193–203.

Ijiri S., Kaneko H., Kobayashi T., Wang D.S., Sakai F., Paul-Prasanth B., Nakamura M., Nagahama Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia (*Oreochromis niloticus)*. Biol. Reprod., 78:333–341.

.

Kagawa H., Young G., Nagahama Y., 1984. *In vitro* estradiol-17β and testosterone production by ovarian follicles of the goldfish, *Carassius auratus*. Gen. Comp. Endocrinol., 54:139-143.

Kagawa H., Gen K., Okuzawa K., Tanaka H., 2003. Effects of luteinizing hormone and follicle-stimulating hormone and insulin-like growth factor-1 on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, *Pagrus major*. Biol. Reprod., 68:1562-1568.

Karube M., Fernandino J. I., Strobl-Mazzulla P., Strussmann C. A., Yoshizaki G., Somoza G. M., Patino R., 2007. Characterization and expression profile of the ovarian cytochrome P-450 aromatase (*cyp19A1*) gene during thermolabile sex determination in pejerrey, *Odontesthes bonariensis*. J. Exp. Zool., 307A, 625–636.

Kishida M., Callard G. V., 2001. Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology, 142: 740–750.

Kobayashi Y., Kobayashi T., Nakamura M., Sunobe T., Morrey C.E., Suzuki N., Nagahama Y., 2004. Characterization of two types of cytochrome P450 aromatase in the serial-sex changing gobiid fish, *Trimma okinawae*. Zool. Sci., 21: 417-425.

Kumar R.S., Ijiri S., Trant J.M., 2000. Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a reproductive cycle. Biol. Reprod., 63: 1676-1682.

Kwon J.Y., Mc Andrew B. J., Penman D. J., 2001. Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia (*Oreochromis niloticus*). Mol. Reprod. Dev., 59: 359–370.

Liu Z., Wu F., Jiao B., Zhang X., Hu C., Huang B., Zhou L., Huang X., Wang Z., Zhang Y., Nagahama Y., Cheng C.H., Wang D.S., 2007. Molecular cloning of double sex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in southern catfish and their possible roles in sex differentiation. J. Endocrinol., 194: 223-241.

Luckenbach J., Early L., Rowe A., Borski R., Daniels H., Godwin J., 2005. Aromatase cytochrome P450: cloning, intron variation, and ontogeny of gene expression in southern flounder (*Paralichthys lethostigma*). J. Exp. Zool., 303A: 643-656.

Matsuda M., 2003. Sex Determination in Fish: Lessons from the sex-determining gene of the teleost medaka, *Oryzias latipes*. Dev. Growth Diff., 45:397-403.

Matsuoka M.P., van Nes S., Andersen O., Benfey T. J., Reith M., 2006. Real-time PCR analysis of ovary- and brain-type aromatase gene expression during Atlantic halibut (*Hippoglossus hippoglossus*) development. Comp. Biochem. Physiol., 144B: 128–135.

Meital, E., Svetlana, Y., Doron, G., Karen, J., Berta, L., Gad, D., 2008. Expression of the two cytochrome P450 aromatase genes in the male and female blue gourami (Trichogaster trichopterus) during the reproductive cycle. Gen. Comp. Endocrinol., 159:208-213.

Miyata S., Kubo T., 2000. *In vitro* effects of estradiol and aromatase inhibitor treatment on sex differentiation in *Xenopus laevis* gonads. Gen. Comp. Endocrinol., 119: 105–110.

Nagahama Y., Matsuhisa A., Iwamatsu T., Sakai N., Fukada S., 1991. A mechanism for the action of pregnant mare serum gonadotropin on aromatase activity in the ovarian follicle of the medaka, *Oryzias latipes*. J. Exp. Zool., 259: 53-58.

Nagahama Y., Yamashita M., Tokumoto T. 1994. Regulation of oocyte maturation in fish. Curr. Topics Dev. Biol., 30:103–145.

Nagahama Y., 2005. Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol. Biochem., 31:105–109.

Nunez B.S., Applebaum S.L., 2006. Tissue- and sex-specific regulation of *CYP19A1* expression in the Atlantic croaker (*Micropogonias undulatus*). Gen. Comp. Endocrinol., 149:205–216.

Patil, J.G., Gunasekera, R.M., 2008. Tissue and sexually dimorphic expression of ovarian and brain aromatase mRNA in the Japanese medaka (*Oryzias latipes*): Implications for their preferential roles in ovarian and neural differentiation and development. Gen. Comp. Endocrinol., 158:131-137.

Piferrer F., Blazquez M., 2005. Aromatase distribution and regulation in fish. Fish Physiol. Biochem., 31: 215–226.

Pieau C., Dorizzi M., 2004. Oestrogens and temperature-dependent sex determination in reptiles: all is in the gonads. J. Endocrinol., 181:367–377.

Raghuveer K., Senthilkumaran B. 2009. Identification of multiple *dmrt1s* in catfish: localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment. J. Mol. Endocrinol., 42: 437–448.

Sawyer S.J., Gerstner K.A., Callard G.V., 2006. Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: gene specific tissue distribution, sex differences, developmental programming and estrogen regulation. Gen. Comp. Endocrinol., 147:108–117.

Senthilkumaran B., Sudhakumari C.C., Chang X.T., Kobayashi T., Oba Y., Guan G., Yoshiura Y., Yoshikuni M., Nagahama Y., 2002. Ovarian carbonyl reductase-like 20β-hydroxysteroid dehydrogenase shows distinct surge in messenger RNA expression during natural and gonadotropin-induced meiotic maturation in Nile tilapia. Biol. Reprod., 67:1080-1086.

Senthilkumaran B., Yoshikuni M., Nagahama Y., 2004. A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. Mol. Cell. Endocrinol., 215:11–18.

Simpson E.R., Mahendroo M.S., Means G.D., Kilgore M.W., Hinshelwood M.M., Graham-Lorence S., Amarneh B., Ito Y., Fisher C.R., Michael M.D., 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev., 15:342–355.

Simpson E.R., Clyne C., Rubin G., Boon W.C., Robertson K., Britt K., Speed C., Jones M., 2002. Aromatase - a brief overview. Annu. Rev. Physiol., 64: 93–127.

Smith C.A., Sinclair A.H., 2004. Sex determination: insights from the chicken. Bio Essays, 26:120–132.

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

Tchoudakova A., Callard G.V., 1998. Identification of multiple *CYP19* genes encoding different cytochrome P450 aromatase isozymes in brain and ovary. Endocrinology, 139:2179–2189.

Trant J.M., 1994. Isolation and characterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450arom. Gen. Comp. Endocrinol., 95:155-168.

Trant J.M., Gavassa S., Ackers J., Chung B.-C., Place A.R., 2001. Developmental expression of cytochrome P450 aromatase genes (*cyp19a* and *cyp19b*) in zebrafish fry (*Danio rerio*). J. Exp. Zool., 290: 475–483.

Tsai C.L., Wang L.H., Chang C.F., Kao C.C., 2000. Effects of gonadal steroids on brain steroidogenic and aromatase activity during the critical period of sexual differentiation in Tilapia, *Oreochromis mosambicus*. J. Neuroendocrinol., 12:894- 898.

Villenevue D., Iris K., Michael D., Kathleen M., Hammermeister D., Katie J., Lindsey S., Gerald T., 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (*Pimephales promelas*). Aquat. Toxicol., 76: 353-368.

Wilson J., 1994. Translating gonadal sex into phenotypic sex. In the differences between the sexes. Cambridge University Press 1st edn., pp. 203–212.

Wu Dong., Kristine, L.W., 2008. Local expression of *CYP19A1*1 and *CYP19A1*2 in developing and adult killifish *(Fundulus heteroclitus)*. Gen Comp Endocrinol. 155: 307–317.

Yamamoto T., 1969. Sex differentiation. In: Fish Physiology, Vol. 3, (W.S. Hoar and D.J. Randall, Eds.), Academic Press, New York, pp.117–175.

Yoshiura Y., Senthilkumaran B., Watanabe M., Oba Y., Kobayashi T., Nagahama Y., 2003. Synergistic expression of Ad4BP/SF-1 and cytochrome P-450 aromatase (ovarian type) in the ovary of Nile tilapia, *Oreochromis niloticus*, during vitellogenesis suggests transcriptional interaction. Biol. Reprod., 68: 1545–1553.

# *Chapter 2*

## Cloning, expression and enzyme activity analysis of testicular 11β-hydroxysteroid dehydrogenase during seasonal cycle and after hCG-induction in air-breathing catfish *Clarias gariepinus*

## Abstract

A full length cDNA encoding 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2*) was cloned from testis of air-breathing catfish, *C. gariepinus* which showed high sequence homology to zebrafish and eel. *11β-HSD2* ORF was then transfected to COS-7 cells. The transfected cells converted 11β-hydroxytestosterone (11-OHT) to 11-ketotestosterone (11-KT) at a considerable rate than mock transfected cells. Tissue distribution analysis by RT-PCR revealed prominent expression in testis, anterior kidney and liver. Expression of *11β-HSD2* in the testes was assayed by real-time PCR during four testicular phases (preparatory, prespawning, spawning and resting phases) and was found to peak during the prespawning phase and gradually decline during the spawning and resting phases. With $NAD^+$, testicular microsomes oxidized 11-OHT with apparent $K_m$ of 56 ± 4 nM and $V_{max}$ of 55 ± 6 pmol/h/mg-protein, respectively. Seasonal *11β-HSD2* dehydrogenase activity in testicular tissues revealed highest production of 11-KT during the prespawning phase. Serum 11-KT levels corroborated well with the levels of transcripts and activity of 11β-HSD2. *In vivo* hCG administration enhanced *11β-HSD2* expression in the testis, especially during the prespawning phase, at 4, 8, 12 and 24h after induction. It also augmented 11-KT production by testicular microsomes at 8 and 24h. Ontogeny study indicated that this enzyme is expressed after the fate of gonad is determined. However, levels of *11β-HSD2* transcripts were significant during testicular differentiation. Thus the spatiotemporal expression results supported with dehydrogenase activity and circulating 11-KT. Based on these observations, present study clearly indicated a major role for *11β-HSD2* during testicular differentiation and seasonal testicular cycle in catfish.

## Introduction

It is widely accepted that sex steroids are involved during the process of sex differentiation, gametogenesis and sex reversal in fish (Devlin and Nagahama 2002). Role of estradiol-17β (the steroid hormone produced by *cyp19a1*) in ovarian differentiation, oogenesis and as a feminizing agent is well documented in many fishes (Baroiller *et al*., 1999; Andersen *et al*., 2003; Miura *et al*., 2007). The role of 11-oxygenated androgen, 11-ketotestosterone (11-KT) in teleost reproduction and testis formation is in its primitive stage with few studies suggesting its role, during testis formation and differentiation, (Liu *et al*., 2000; Rougeot *et al*., 2007) during sex change in sequential hermaphrodites (Miura *et al*., 2008) and during spermatogenesis and sperm maturation (Miura *et al*., 1991a; Miura *et al*., 2003). On the contrary, there are also report which states that 11β-hydroxylase, a penultimate steroidogenic enzyme involved in the synthesis of 11-KT, is not expressed at early stages or during male sex determination (Baron *et al*., 2005; Wang and Orban, 2007). Judging from the role of 11-KT, the expression of 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2*) and activity (the enzyme involved in 11-KT production) might be important for testicular differentiation (Miura *et al*., 2008). Thus, the involvement of *11β-HSD2* as a marker for testis determination in teleost is a contentious topic and needs further investigation. We have chosen an air-breathing, gonochoristic, male heterogametic annual breeding catfish, *Clarias gariepinus* having lobular testis with synchronous developing cyst as our experimental model because of the ease in breeding, rearing and maintaining them in laboratory/natural conditions. These features allow us to obtain catfish larvae from day one till they mature, to perform ontogeny study and to carry out seasonal expression and activity study on *11β-HSD2*. Production of 11-KT can also be influenced by peripheral

conversion more specifically from liver and anterior kidney. However, the contribution from testis cannot be ruled out as studies from our laboratory showed the presence of 11-KT in testes which underwent changes during thyroid depletion (Chapter 4; Swapna *et al*., 2006) leading to impairment of testicular recrudescence. Further judging from the presence of *11β-HSD2* transcript and activity analyses, 11-KT production in testes might be essential for testicular function (Jiang *et al*., 2003, Kusakabe *et al*., 2003). This gene is primarily implicated in initiating and maintaining the spermatogenic cycle. Previous reports in catfish have emphasized the role of 11-KT in promoting and modulating puberty in juveniles, during spermatogenesis and in the development of secondary sexual characteristics (Cavaco *et al*., 1997a). Interestingly, testosterone (T) is also required for stimulating the hypothalamo-hypophyseal axis vis-à-vis the release of lutenizing hormone (Cavaco *et al*., 2001a) nevertheless excess T inhibited the production of 11-KT (Cavaco *et al*., 2001b). Identification of two different androgen receptors with different ligand binding specificity in teleosts (Ikeuchi *et al*., 1999) also provides evidence that T and 11-KT are required for the normal functioning of the testis and development of secondary sexual characters in males. There are vast number of reports that illustrates the serum profile of 11-KT corresponding to the varied testicular phases in different fishes (Scott *et al*., 1980; Cavaco *et al*., 1997a; 2001a; Koya *et al*., 2002). However, a comprehensive analysis of expression, enzyme activity backed with $K_m$ and $V_{max}$ value of *11β-HSD2* for 11-OHT and serum profile of 11-KT has not been carried out in a lower vertebrate so far to understand the role of *11β-HSD2* in testis. In addition no report exists, to substantiate the regulatory role of gonadotropins on *11β-HSD2* expression and activity after *in vivo* induction using hCG, a hormone that emulates the action of gonadotropins. Hence, the present study was

aimed to clone *11β-HSD2*, analyze its expression pattern, and measure 11-KT levels and enzyme activity to decipher specific role of *11β-HSD2* during gametogenesis including the seasonal testicular cycle and after hCG-induction.

## 2. Materials and Methods

### Animals

Air-breathing catfish, *C. gariepinus* were procured from local fish markets in and around Hyderabad at different phases of seasonal cycle. They were acclimated for 2-3 weeks by maintaining in aquarium tanks filled with filtered tap water under natural photoperiod and ambient water temperature conditions. Seasonal changes in catfish testis were described earlier by Swapna *et al.* (2006). Catfish were fed with live tube worms/minced goat liver/pelleted food in *ad libitum* during acclimation and experimentation. We also bred and reared the catfish in order to obtain fish at different time points for ontogeny studies.

### RT-PCR amplification of partial cDNA homologous to *11β-HSD2*

Degenerate primers were designed by aligning the existing sequences of vertebrate 11β-hydroxysteroid dehydrogenase type 2 obtained from DDBJ/EMBL/GenBank databases, to clone partial cDNA fragment of *11β-HSD2* from the testis of catfish. Using these degenerate primers, sense DF1, 5' GCG GTS YTC ATC ACM GGY TGT GA 3'and antisense DR1, 5'GCT GCY TTS GAG GYY CCA TA 3', a cDNA fragment of 464 bp homologous to *11β-HSD2* was amplified by RT-PCR and cloned in pGEM-T-easy vector (Promega, Madison, WI, USA).

### cDNA library construction and screening

cDNA library from testis of catfish was constructed using UNI-ZAP cDNA library synthesis kit (Stratagene). Total RNA from testis was prepared using TRI-reagent (Sigma).

Using 2 mg of total RNA, mRNA was prepared by oligotex-mRNA kit (QIAGEN). Then 5 µg of mRNA was taken to synthesize cDNA using stratagene cDNA synthesis kit. The purified cDNA was ligated and packaged into UNI-ZAP-XR system using Gigapack II Gold packaging extract kit (Stratagene). Screening of the testis cDNA library for *11β-HSD2* was performed by using 464 bp cDNA fragments as probe, obtained by RT-PCR, which shared 68% homology with other teleost *11β-HSD* type 2 genes. The probe was radiolabelled with $^{32}$P-dCTP using random primer labeling kit (Perkin Elmer). Single clone excision was performed for positive clones to obtain plasmid DNA for bidirectional nucleotide sequencing. Wherever necessary, we also performed 5'RACE as per the method described earlier (Raghuveer and Senthilkumaran, 2009).

**Capacity of 11β-HSD2 to produce 11-KT from 11-OHT in COS-7 cells and determination of apparent $K_m$ and $V_{max}$ values of *11β-HSD2***

Analysis of putative 11β-dehydrogenase activity of recombinant protein was performed as described in previous studies with few modifications (Kusakabe *et al*., 2003). Briefly, the deduced complete open reading frame (ORF) of *11β-HSD2* was inserted into the pCDNA3.1$^+$ TOPOV5-His mammalian expression vector (Invitrogen). The sequence integrity of the insert was verified by nucleotide sequence analysis. Approximately $3\times10^5$ COS-7 cells were laid onto a 6-cm tissue culture plate containing 4ml of DMEM with or without (during transfection) 10% (v/v) fetal calf serum. The cells were cultured at 37°C in 5% $CO_2$ until confluent. Then 1–2 µg of recombinant plasmids, mock (insert locked in reverse direction) and control vector (without insert) were transiently transfected into COS-7 cells using Tfx-20 (Promega) according to the supplier's protocol. 24h after transfection, the COS-7 cells were incubated with 30 ng of 11β-OHT (Sigma). 24h after

incubation with substrate the culture medium was collected from each well by centrifugation at 1000 rpm, extracted twice with diethyl ether followed by evaporation in a vacuum centrifuge. The steroids were reconstituted in 100µl EIA buffer supplied in the 11-ketotestosterone enzyme linked immunoassay (EIA) kit (Cayman). Entire protocol was repeated thrice with three replicates each time to get concomitant values. The 11-KT produced in the culture medium was measured using 11-KT- EIA kit according to the manufacturer's protocol. Cross-reactivity of the antibody against 11-KT to 11β-OHT was 1.7%, the minimal detection thresholds was 1.3 pg/ml for 11-KT. After measurements, the conversion rates were calculated and the values of cross-reactivity were subtracted. Results were expressed as mean ± SEM of three replicates. Data analysis was carried out using one-way ANOVA followed by Dunnett's test. Significance was accepted at $P < 0.05$. Next we studied the affinity and capacity of the enzyme *11β-HSD2* to oxidize 11-OHT with $NAD^+$ as cosubstrate. Kinetic study was performed following procedure described by Stewart *et al*. (1994) with few alterations. After preliminary experiments on fractional conversion of 11-OHT versus time and protein concentration, testes microsomes (250ug/ml of protein from pellet obtained at 1,05000g after differential centrifugation) in 0.1M potassium phosphate buffer, pH 7.4 ($KPO_4$) were incubated with various concentrations of 11-OHT (0.005-5µmol/L) and 100 pmol/L NAD for 15, 30, 45 and 60 min in a shaking water bath at 37ºC. This was performed on microsomes obtained from five separate testis of prespawning phase male. The reaction volume was 500 µL and the experiment was terminated placing the tube on ice. Steroids were extracted with diethyl ether (thrice the incubation volume), dried, dissolved in EIA buffer, and 11-KT levels estimated using 11-KT- EIA kit. The percentage conversion of 11-OHT to 11-KT was

calculated. For the kinetic studies, the reaction rate (V), expressed as picomoles of 11-KT formed per h/mg-.protein, was linear, for each substrate concentration (S). From a Lineweaver-Burk plot of l/V vs. l/[S], the apparent $K_m$, and maximum velocity ($V_{max}$) was calculated. All incubates were analyzed in triplicate. Data analysis and Lineweaver-Burk plot was drawn using Graph Pad Prism 5 software (San Diego, California, USA).

**Real-time RT-PCR**

The relative expression of the steroidogenic enzyme gene *11β-HSD2* in testicular samples was studied by real-time PCR using *β-actin* (sense: 5'-ACC GAA TGC CAT CAC AAT ACC AGT-3'; antisense: 5'-GAG CTG CGT GTT GCC CCT GAG-3') as endogenous control at four phases of the reproductive cycle i.e. preparatory, prespawning, spawning and resting. Gene specific primers were designed at the intron–exon boundaries by comparing the ORF with already available *11β-HSD2* sequences in GenBank. With respect to *11β-HSD2* the sense primer was located between exon 1 and exon 2, 5'ATC ACA GGG TGCGAC TCG GGT TTC GGG 3' whereas the antisense primer was located in exon 2, 5'CGG CTG AGT GAT GTC CAC CTG A 3', which amplified 168 bp fragments. Real-time PCR was carried out in a 7500 Fast thermocycler (Applied Biosystems) at 95°C denaturing temperature and 60°C annealing temperature for 40 cycles according to the manufacturer's recommendations. During PCR, fluorescence accumulation resulting from DNA amplification was recorded using the sequence detector software (Applied Biosystems). Comparative $C_T$ method was used to quantify the target gene abundance. Each sample (n=5) was run in triplicates with a final volume of 25 µl containing 0.3 µl of cDNA representing the four different phases of the testis, 10 pmol of each primer, and 12.5 µl of SYBR Green PCR master mix (Applied Biosystems). A non-template control was

included as negative control. Analysis was done by using the RQ Manager to compare expression levels among genes. The RQ (relative quantification) was carried out using preparatory phase expression as calibrator. The amount of target normalized to an endogenous control and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$. Data analysis was carried out using one-way ANOVA followed by Tukey-Kramer's multiple comparison test. Significance was accepted at $P < 0.05$.

**Rate of production of 11–KT by testicular fragments at four testicular phases**

The testicular tissues that were collected from five catfishes each in different seasons to monitor expression level at four different testicular phases were simultaneously used to study the enzyme activity of *11β-HSD2*. The conversion of 11-OHT to 11-KT was carried out as described by Stewart *et al.* (1994) and Hu *et al.* (2008) using cold steroids with few modifications. Testicular microsomes were prepared by homogenizing 500 mg of tissue in 3ml of 0.1M $KPO_4$ buffer pH 7.4, clearing debris at 9,000xg for 20 min, and centrifuging at 1,05,000xg for 1h. The microsomal pellet was washed with 0.1M $KPO_4$ pH 7.4 buffer containing 0.1mM EDTA, resuspended in 500 µl of 0.1M $KPO_4$ buffer, 0.1mM EDTA and 20% v/v glycerol. To 1ml of assay medium, 300 µg of testicular microsome, 50nm 11β-OHT, 100µM NAD was added and incubated in a water-bath with shaker at 37ºC for 60 min. The reaction was stopped by adding ice-cold diethyl ether. The steroids were extracted with diethyl ether and the organic layer was dried under $N_2$ gas and dissolved in 100µl of EIA buffer (Cayman). The amount of 11-KT formed was detected by using Cayman 11-KT-EIA kit as per the method described above. Data analysis was carried out using one-way ANOVA followed by Kruskal-Wallis' test. Significance was accepted at $P < 0.05$ for the testicular fragments of four different phases. Negative (heat-denatured

microsome) and positive (recombinant *11β-HSD2*) controls were used to check assay validity.

**Measurement of 11-KT levels in catfish**

Blood was collected by caudal puncture from five male catfishes each at different phases of testicular cycle before sacrificing the fish. It was then allowed to coagulate, centrifuged at 1,500xg for 10 min to collect the serum. The 11-KT levels in the serum were measured using the 11-KT-EIA kit as described previously. Results were expressed as mean ± SEM of five samples that were done in three replicates each.

**Effect of *in vivo* hCG treatment on *11β-HSD2* expression and 11-KT production**

To study the seasonal effect of gonadotropins on the expression of *11β-HSD2* transcript and 11-KT production, especially during late testicular recrudescence (May) and quiescent (December) phases, five catfishes weighing about 400-500 gm were injected intraperitoneally with single dose of hCG (1000 IU/Kg body weight) after standardizing the dosage in our laboratory. Control fish were injected with fish physiological saline. Further, at an interval of every four hours up to 24h, fishes were sacrificed after immersing in ice cold water, to collect testes. This procedure was repeated thrice with different batch of fish (n=5). The testicular samples were snap-frozen in liquid nitrogen and stored in -80°C until assay. Total RNA was then prepared using Tri-reagent (Sigma) as per the manufacturer's protocol, followed by 1$^{st}$ strand cDNA synthesis using random primer-Superscript III (Invitrogen). To study the changes in the expression level of the *11β-HSD2* transcript, semi-quantitative RT-PCR was performed using specific primers and the intensity of the gel bands was analyzed by densitometric method using Bio-Rad Gel Documentation 1000 system and multi-analyst software program (Bio-Rad, CA, USA). To

measure the rate of production of 11-KT by putative dehydrogenase activity of 11β-*HSD2* at different time points, microsomes were prepared from the testicular tissue and activity measured as per the method described above.

**Tissue distribution of *11β-HSD2* in catfish by RT-PCR**

Total RNA was prepared from various tissues of adult male catfish (prespawning phase) using Tri-reagent (Sigma) as per the manufacturer's protocol. First strand cDNA was then synthesized using oligod$T_{18}$-Superscript III (Invitrogen) and semi-quantitative RT-PCR was performed to study the spatial expression of *11β-HSD2* in various tissues. The PCR cycle employed for analyzing expression was 94°C-2 min followed by 30 cycles at 94°C-45 (sec), 58°C-30 (sec), 72°C-1 (min) followed by final extension at 72°C-10 (min), specific primers were designed for this purpose (sense 5'-TAC CTG CTC TCC TCG CTT CAC CTT 3' and antisense primer 5'-GCT GTT CAC CTG ACG GAC TGG AGA 3') which amplified 296 bp fragments. A no template control was included as negative control.

**Ontogeny expression study of *11β-HSD2***

Earlier finding (Raghuveer and Senthilkumaran 2009) from our laboratory reported that the morphological signs of sex differentiation in catfish were evident around the period of 40-50 days post hatch (dph). To study the temporal expression of *11β-HSD2*, catfish larvae were collected at 45, 55, 75, 90, 150 and 260 dph. 15-20 larvae were dissected for each time period under dissection microscope (Carl-Zeiss, Germany) and the gonads were pooled to have five biological samples (n=5) for total RNA preparation in sterile condition, snap-frozen using liquid nitrogen and stored in -80°C for later use. Total RNA was prepared using Tri-reagent (Sigma) as per the manufacturer's protocol. 2µg RNA was reverse transcribed using random primer and Superscript III (Invitrogen). Subsequently

real-time PCR was performed as described for stage-dependent *11β-HSD2* expression study using 45 dph expression as calibrator.

**Results**

**Molecular cloning of *11β-HSD2* from testis of catfish**

A 464 bp partial cDNA fragment homologous to *11β-HSD2* was obtained from catfish testis by RT-PCR. This was used as a probe to screen approximately 7.5 x $10^5$ recombinant phages from testis library. After three rounds of screening, five positive clones were obtained and they were sequenced from both the ends. Four of them were 5' truncated while one clone had full length sequence. The full length sequence was also confirmed by performing 5'RACE with the sequence data of 5' truncated clones. The testicular *11β-HSD2* was 2172 bp long with 21 bp 5' untranslated region (UTR) and 918 bp 3' (UTR). The ORF encoded a protein of 410 amino acids with four ATTAAA as poly-adenylation signals which are 636, 598, 60 and 11 bp apart from the 21 bp poly (A) tail (Fig.1). The sequence data of *11β-HSD2* has been submitted to GenBank (Accession Number: **GU220074**). The clone exhibited conserved NAD binding domain typical of type-2 *11β-HSD*, and the presence of characteristic five amino acid residues (MEVNF) common for both type 1 and 2 *11β-HSD*. The signature domains typical of short-chain dehydrogenase reductase (SDR) superfamily, which included the Rossmann fold and the catalytic domains, were clearly found in the catfish testicular *11β-HSD2* gene. ClustalW multiple alignment analysis demonstrated that these regions were highly conserved among vertebrates (Fig. 2). Phylogenetic analysis constructed using POWER program showed three distinct clades, the mammalian*11β-HSD2*, the teleost*11β-HSD2*, and non-vertebrate *11β-HSD* clade. Catfish *11β-HSD2* grouped in the teleost clade shared high homology with

that of zebrafish followed by eel, rainbow trout and the Nile tilapia (68%-63%), whereas *Ciona intestinalis-11β-HSD3* and *Caenorhabditis elegans*-short chain dehydrogenase used as an out group branched together in a separate clade (Fig. 3).

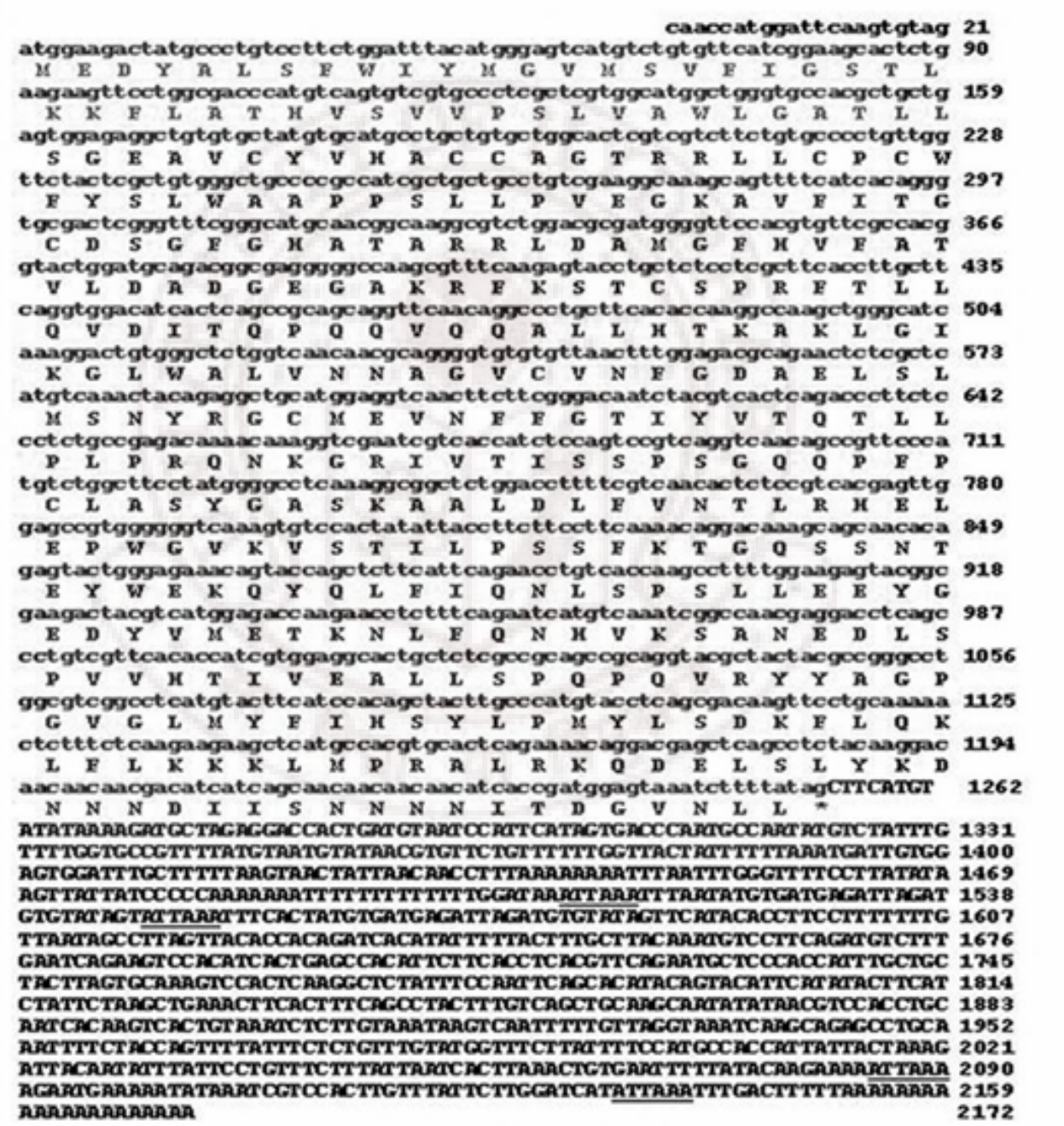


**Fig. 1**. ***Nucleotide and deduced amino acid sequence of catfish testis 11β-HSD2. UTRs, (5' 1-21 bp, 3' 1254-2142 bp) are boldfaced and the polyadenylation signals are underlined.***

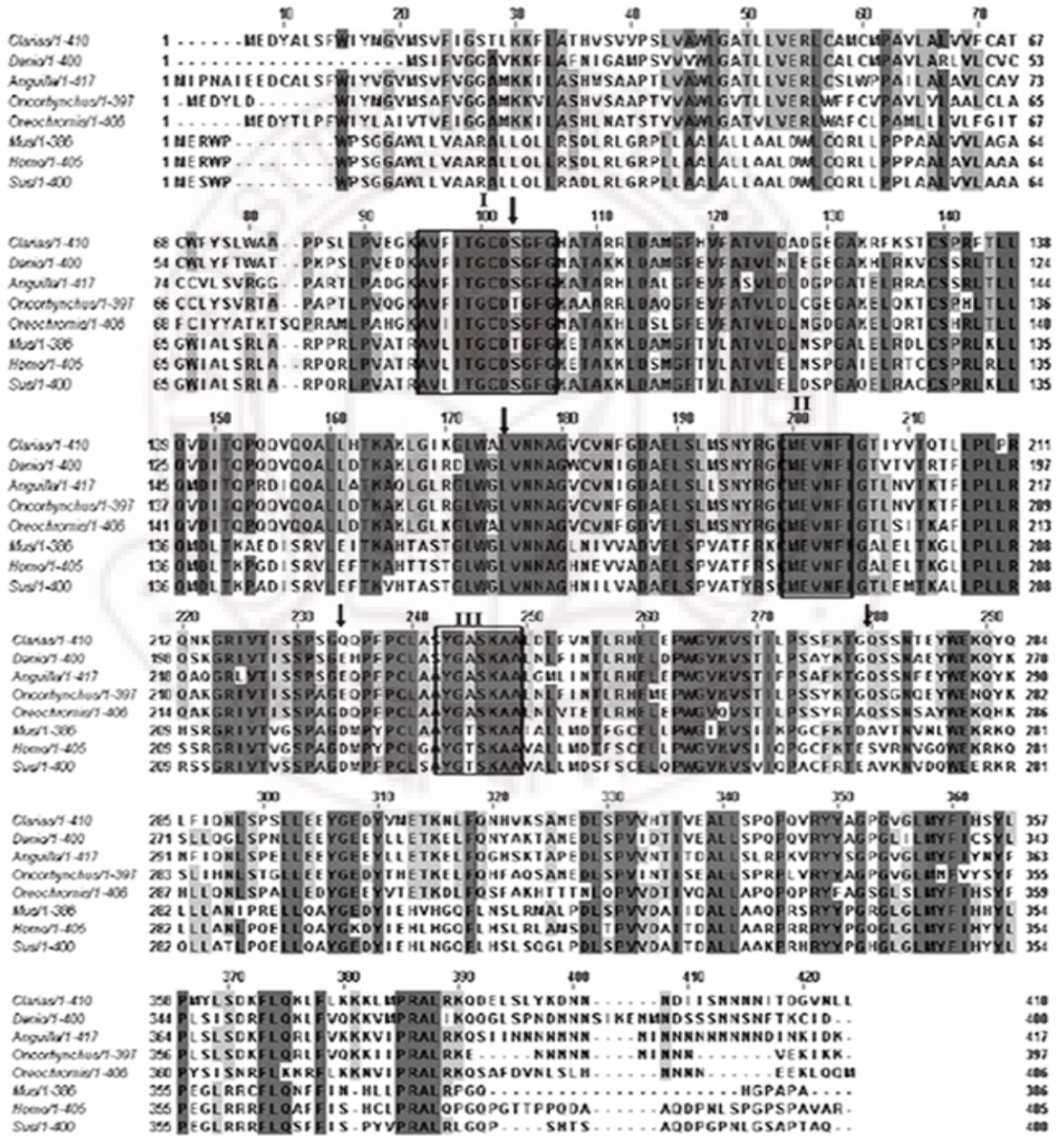


**Fig. 2**. ***Alignment of deduced amino acid sequences of catfish 11β-HSD2 (GU220074) with that of other teleosts 11β-HSD2 using ClustalW multiple alignment tool. Conserved domains are shown in rectangles. I: NAD-binding domain, II: 11β-HSD2 conserved sequence, III: catalytic site. Highly conserved regions are shaded. The four intron positions are marked by arrows. Clarias: Clarias gariepinus, Danio: Danio rerio, Anguilla: Anguilla japonica, Oncorhynchus: Oncorhynchus mykiss, Oreochromis: Oreochromis niloticus, Mus: Mus musculus, Homo: Homo sapiens, Sus: Sus sacrofa. The GenBank accession numbers for the teleostean and mammalian 11β-HSD2 are given in fig. 3.***

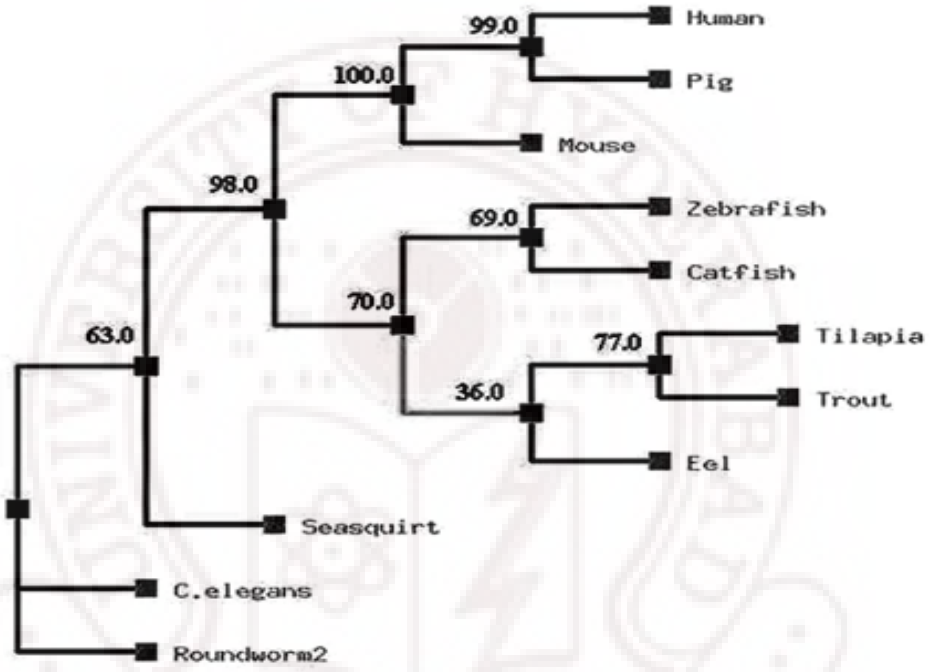



**Fig. 3.** ***Phylogenetic analysis of vertebrate 11β-HSD2 showing evolutionary relationship. POWER tool (www.power.nhri.org) with 100 bootstrap trial was used to construct the Phylogenetic tree. C. elegans short chain dehydrogenase protein, belonging to SDR family, was used as outgroup. Branch length is proportional to the distance between each protein. Bootstrap values are the number of trials that this cluster was found in 100 trials. Accession No.: Human BC036780; Mouse BC066209; Pig NM213913; Tilapia DQ991146; Trout AB104415; Eel AB252646; Zebrafish NM212720; seasquirt: Ciona intestinalis AK116129; C. elegans: Caenorhabditis elegans AF022968; Roundwom2: Caenorhabditis elegans AF00310 and catfish: Clarias gariepinus GU220074***

**Transient transfection of *11β-HSD2* in COS-7 cells and apparent $K_m$ and $V_{max}$ values of 11β-HSD2**

The result of transient expression study in non-steroidogenic COS-7 cells transfected with pCDNA3.1$^{+}$ vector harboring putative ORF of *11β-HSD2*, was expressed as, percentage conversion of 11β-OHT (substrate) to 11-KT (product). *11β-HSD2* showed about 38% conversion of 11β-OHT to 11-KT ($P<0.05$), compared to blank (only vector) and mock transfection (the ORF locked in reverse direction) Fig. 4A. Kinetic analysis of testicular microsomes incubated with increasing concentrations of 11-OHT, revealed a high affinity for 11β-hydroxy testosterone with apparent affinity value, $K_m$ 56 ± 4 nmol/L and maximum velocity $V_{max}$ 55 ± 6 pmol/h /mg microsomal protein (Fig. 4B).

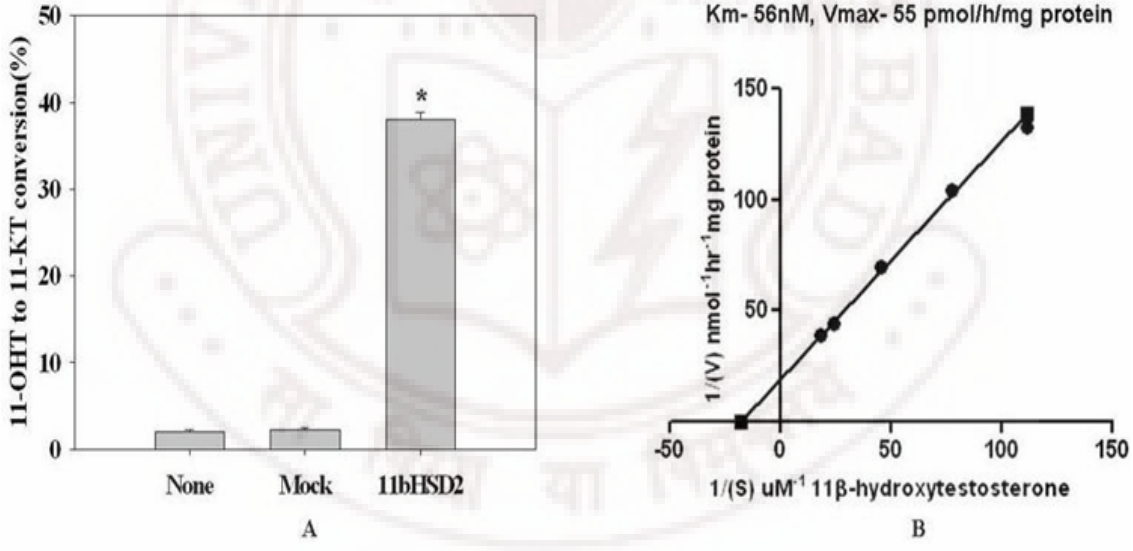



**Fig. 4.** ***(A) Representative histogram showing the percentage (%) conversion of 11β-hydroxytestosterone to 11-ketotestosterone by recombinant 11β-HSD2 protein transiently expressed in COS-7 cells (n=3 different transfection with triplicate assays at each time), * indicates the significance (B) 11β-HSD2 activity in catfish testis microsomes depicting apparent Km and $V_{max}$ for 11-OHT. Each point represents the mean of three separate experiments [$K_m$ 56 ± 4 (±SE) nM and $V_{max}$ 55 ± 6 pmol/h/mg protein].***

**Phase-dependent expression and activity of *11β-HSD2* in testis**

Real-time RT-PCR analysis demonstrated seasonal fluctuation in the *11β-HSD2* transcripts with relatively high mRNA levels in preparatory phase, which peaked in the prespawning phase followed by a drop in spawning and regressed phases (Fig. 5A) The putative dehydrogenase activity of *11β-HSD2* (Fig. 5B) and serum 11-KT levels (Fig. 5C) measured in four different phases showed positive correlation with the transcript levels, displaying maximum 11-KT levels in the prespawning phase.

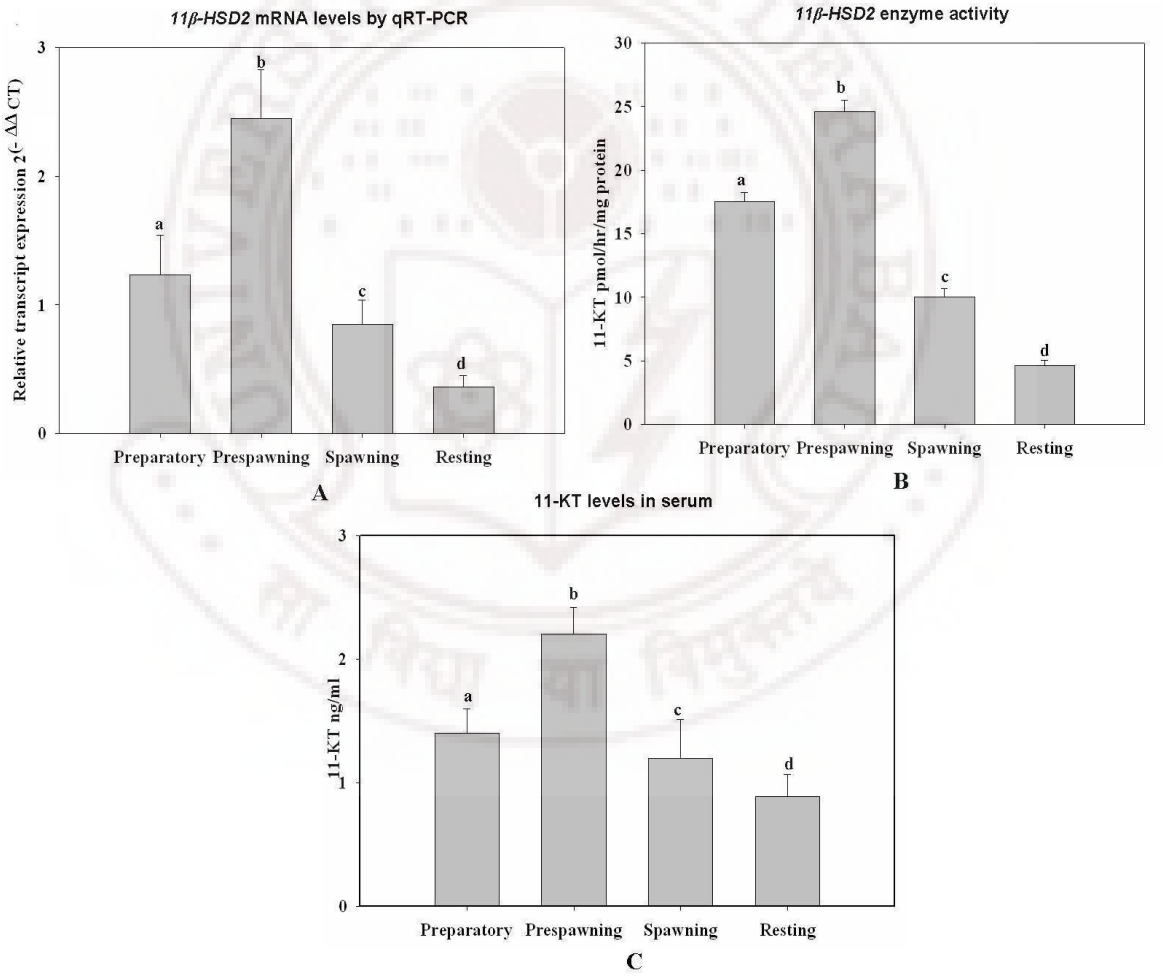



**Fig. 5.** ***(A) Real-time RT-PCR analysis of 11β-HSD2 expression, (B) change in the rate of production of 11-KT and (C) 11-ketotestosterone levels in the serum during catfish testicular cycle. Means with different letters differ significantly, (P<0.05, ANOVA).***

### *11β-HSD2* expression and rate of 11-KT production after *in vivo* hCG induction

The hCG injection in the prespawning phase significantly enhanced *11β-HSD2* expression and activity when compared to saline treated group. The sustained rise in *11β-HSD2* transcript and enzyme activity at different time points was evident from 4h after induction with a maximum at 24h (Fig. 6A-C). On the other hand in the resting phase, fishes responded with an initial spurt in *11β-HSD2* mRNA levels and protein dehydrogenase activity which later dwindled at 8h and was further maintained in line with the control group (Fig. 7A-C).

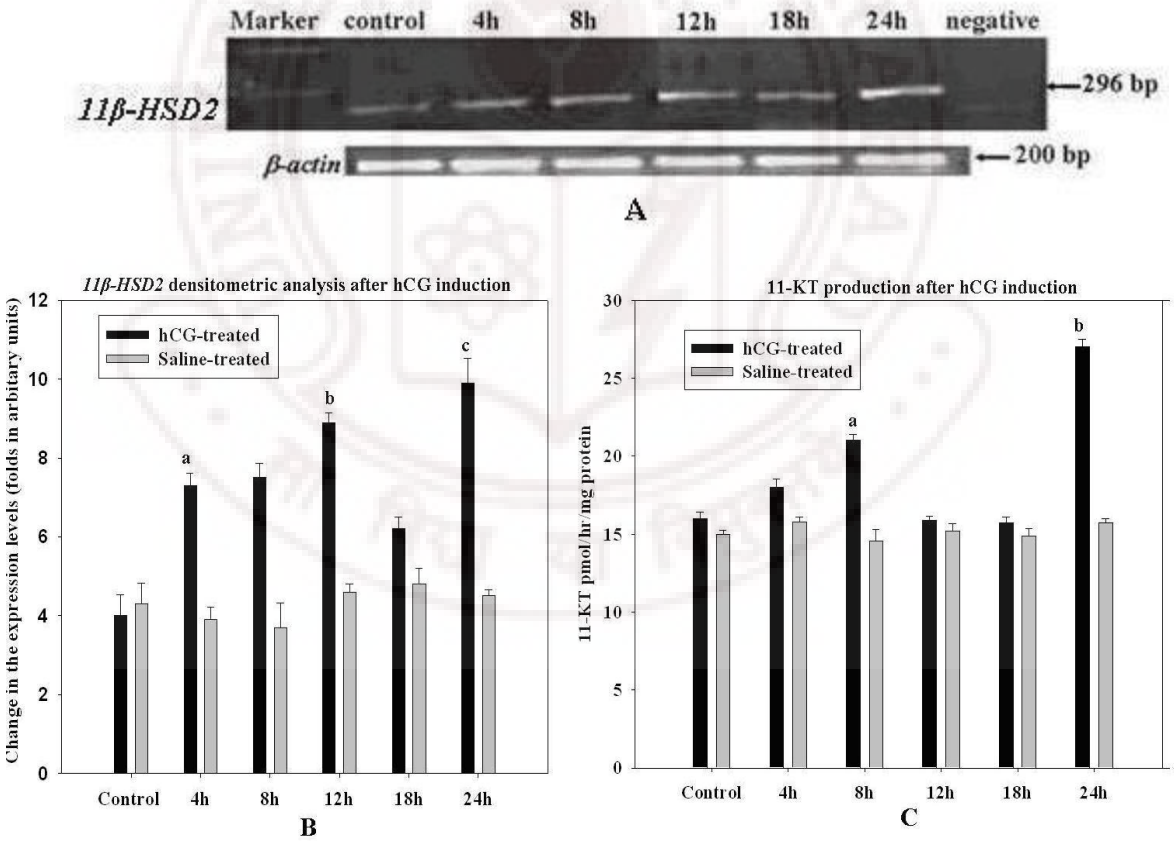



**Fig. 6.** ***Semi-quantitative RT-PCR analysis of 11β-HSD2 (A) expression (B) densitometric analysis of expression, small letter over bars a, b and c represent significant change compared to control and (C) rate of production of 11-KT in testis,***

***after hCG induction in the prespawning phase. X-axis represents hours after treatment, (P<0.05, ANOVA).***

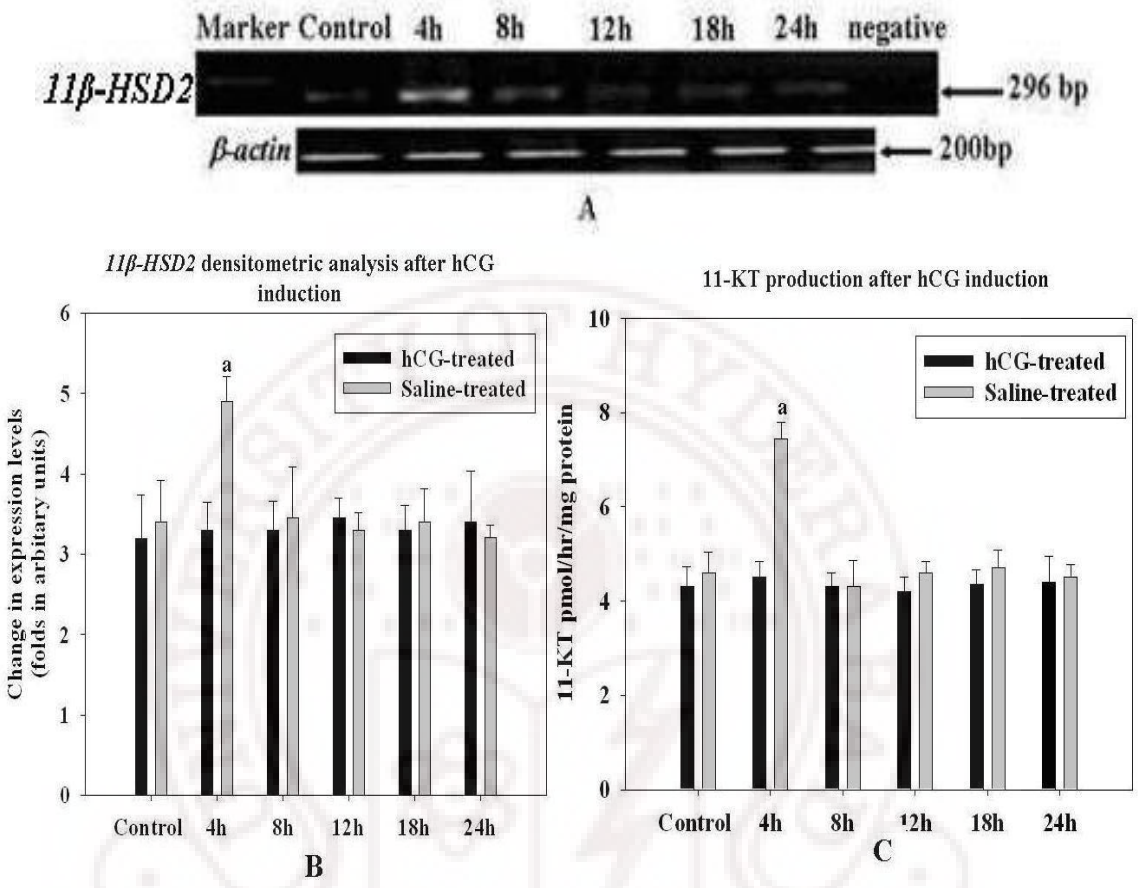



**Fig. 7**. ***Semi-quantitative RT-PCR analysis of 11β-HSD2 (A) expression (B) densitometric analysis of changes in the expression and (C) rate of production of 11-KT in testis, after hCG induction in the resting phase. X-axis represents hours after treatment, (P<0.05, ANOVA).***

**Tissue distribution of *11β-HSD2***

Semi-quantitative RT-PCR analysis detected *11β-HSD2* expression in several tissues other than testis including brain, gills, heart, muscle, spleen, liver, kidney and ovary. However, the expression was prominent in testis, liver, kidney and gills (Fig. 8).

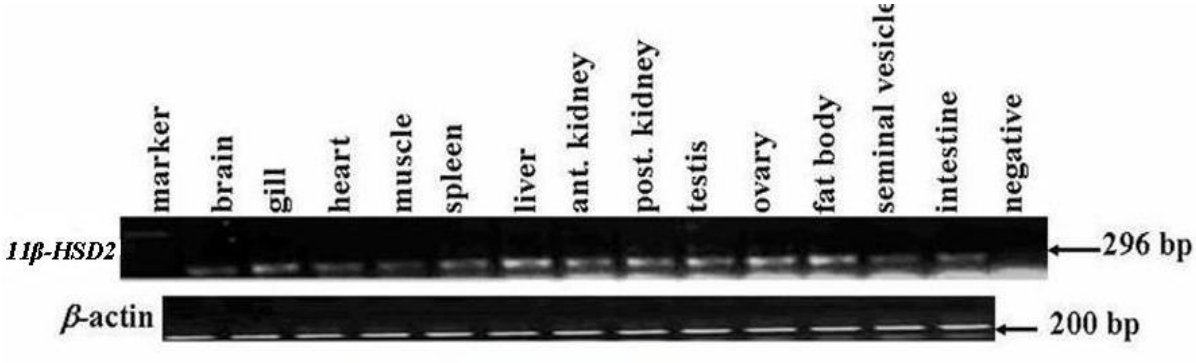



**Fig. 8.** ***Semi-quantitative RT-PCR analysis of spatial expression pattern of catfish 11β-HSD2 in different tissues. Negative control contains no cDNA template.***

**Ontogeny of *11β-HSD2***

Temporal expression of *11β-HSD2* by real-time PCR was performed from 45 dph up to 260 dph to study its role during testicular growth and differentiation. In the testes of 45 dph group, no amplification of *11β-HSD2* transcript was observed. The transcript was first detected at 55 dph with subsequent rise in expression measured up till 260 dph (Fig. 9).

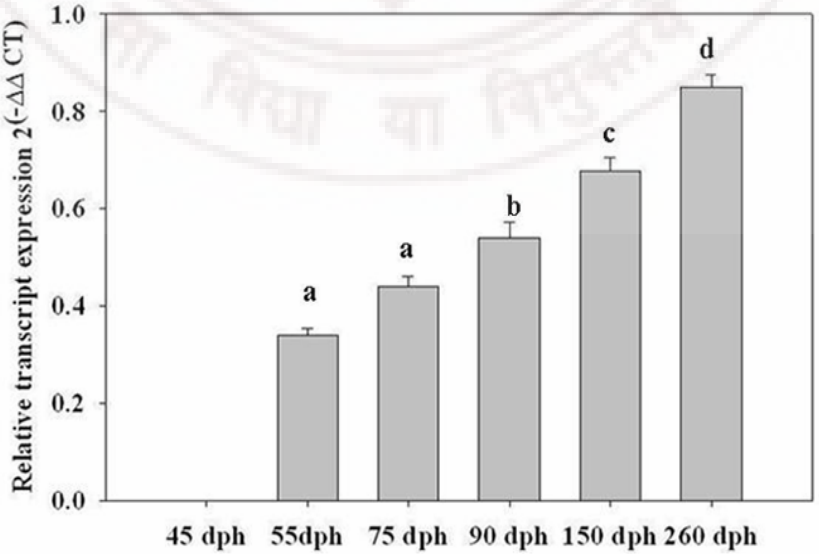


**Fig.9.** ***Real-time RT-PCR analysis of temporal expression pattern of catfish 11β-HSD2 in developing larvae at 45, 55, 75, 90, 150 and 260 dph. Means with different alphabets differ significantly (P<0.05, ANOVA). No detection was seen in 45 dph***

## Discussion

The role of *11β-HSD2* during testicular differentiation and in the maintenance of reproductive cycle in the gonochoristic male catfish was demonstrated in the present study by a comprehensive analysis of *11β-HSD2* expression pattern, its putative steroidogenic capacity to produce 11-KT and subsequently correlating with the circulating 11-KT levels during various phases of testicular development and recrudescence. Present work also demonstrated modulation in steroidogenic capacity and *11β-HSD2* transcript expression in testis of prespawning and resting phase by hCG administration. Further we reported for the first time in teleost the capacity and affinity of the enzyme *11β-HSD2* for 11–OHT. The apparent Km value of *11β-HSD2* for 11-OHT was in range with Km value obtained for glucocorticoids in higher vertebrates (Katz *et al*., 2008), however, the capacity of this enzyme was low when compared to data from avian and mammalian *11β-HSD2* kinetic study. Likewise transfection study also showed considerable synthesis of 11-KT by recombinant 11β-*HSD2* and testicular fragments using 11-OHT and $NAD^+$ as substrate similar to the finding by Kusakabe *et al*. (2003) and Ozaki *et al*. (2006). Homology study of catfish *11β-HSD2* with Japanese eel showed 65% sequence identity suggesting common function of the gene. Further eel *11β-HSD2* converted cortisol to cortisone but cortisone to cortisol conversion was negligible (Ozaki *et al.,* 2006). However, this was not probed in the present study as we focused on *11β-HSD2* and 11-KT. It would also be inadequate to overlook the role of *11β-HSD3* (an isoform of *11β-HSD2*) in contributing to the

production of 11-KT from 11-OHT (if any) which is also a unidirectional oxidative enzyme with proven dehydrogenase activity in pig, chicken and humans using $NADP^+$ as cosubstrate (Katz *et al*., 2008, Robinzon and Prough *et al*., 2005; 2009). Furthermore Baker (2004) reported the existence of *11β-HSD3* isoform in medaka, zebrafish and fugu. He also confirmed the absence of *11β-HSD1* in the genome of these fishes proposing that *11β-HSD3* may be the ancestral form of *11β-HSD1* that arose in terrestrial forms after the divergence of ray-finned and lobed-finned fishes. At the same time no report exists from teleost that could accounts for either the involvement or up regulation of *11β-HSD3* isoform during spermatogenesis. BLAST search of catfish *11β-HSD2* showed high identity with $C_{11}$ and $C_{17}$ hydroxysteroid dehydrogenase type 2 genes. Part of the sequence also matched with 3-hydroxyl butyrate dehydrogenase type 1 and retinol dehydrogenase gene which is in concurrent with Baker's (1998) findings on hydroxysteroid dehydrogenase evolution in animal kingdom. Although data on 11-KT are available in catfish to support its role in spermatogenesis by hormone implantation studies in juveniles and by measurement of plasma and tissue levels of steroids along with *in vitro* and *in vivo* bioconversion of precursor steroids by testicular fragment at the time of puberty (Cavaco *et al*., 1997a, Schulz *et al*., 2008). Nonetheless there exist neither any reports on early expression of *11β-HSD2* during gonad development nor any reports on stage-wise expression pattern and activity during testicular cycle, to implicate a specific role to *11β-HSD2* during testis formation and development in catfish. To start with, we cloned *11β-HSD2* cDNA encoding 410 amino acid residues that displayed conserved catalytic and characteristic GlyXXXGlyXGly regions, which are hallmarks of the SDR super family. Studies pertaining to structure function relationship utilizing site-directed mutagenesis and

X-ray crystallography demonstrated that the co-factor binding domain (NAD-binding), Rossmann fold and active site motif are crucial (Daux and Ghosh, 1997). The SDR super-family is one of the biggest families with more than 2000 known primary structures (Kallberg *et al*., 2002). In spite of highly divergent primary structures in this family, they all have super-imposable tertiary structures, highly conserved signature domains and these motifs are well conserved in catfish *11β-HSD2*.

Real-time PCR analysis demonstrated a steady elevation in the *11β-HSD2* transcripts during the proliferation of spermatogonial cells followed by a gradual decline during maturation and spermiation in catfish. These results corroborate well with the seasonal pattern of plasma 11-KT levels measured in the present study and by Cavaco *et al.* (1997a) in the same species during puberty. The pattern of seasonal change of *11β-HSD2* expression and 11-KT production by testis clearly reflected the testicular phases. In concurrent with our findings, expression data from rainbow trout, Pacific herring, and sea bass also reported similar seasonal fluctuation of genes involved in 11-KT production (Liu *et al*., 2000; Kusakabe *et al*., 2003; Koya *et al*., 2002; Vinas and Piferrer, 2008). However in salmonids, plasma 11-KT levels and expression of steroidogenic genes implicated in 11-KT production were low during early spermatogenesis yet peaked at spermiation (Borg 1994; Maugars and Schimtz 2008). The presence of high expression of *11β-HSD2* in preparatory/prespawning testes is also in agreement with previous findings in eel, which proved an induction of *11β-HSD2* transcript by hCG treatment in immature testes (Jiang *et al*., 2003; Miura *et al*., 1991b) leading to initiation of the spermatogenic cycle and production of spermatocytes, spermatozoa and spermatids. The events that ensued after induction of *11β-HSD2* transcript by gonadotropins were activation of Sertoli cells, which

in turn produced activin B and proteins involved in initiation of mitotic cycle (Nagahama, 1994). The waning of *11β-HSD2* expression in spawning and resting phase is also in accordance with the existing data on eel's steroid profile which testifies a shift in steroid from 11-KT to 17α-20β-dihydroxy-4-pregnen-3-one (17α-20β-DP) with the onset of spawning season (Sakai *et al*., 1989). On the other hand, (Borg, 1994; Ozaki *et al*., 2006; Maugar and Schmitz, 2008) reported the requirement of both 11-KT and 17α-20β-DP at the time of sperm maturation and spermiation. Analysis of putative *11β-HSD2* oxidation activity in the present study using testicular explants from different reproductive phases showed similar results with the dehydrogenase activity peaking in the prespawning phase, might be due to abundant number of spermatogonial cells present after its proliferation, which along with interstitial cells, expresses steroidogenic genes (Vinas and Piferrer, 2008) required for the synthesis of 11-oxygenated androgens. Further, the expression of *11β-HSD2* and activity pattern of 11-oxo-androgen production (11-KT) studied at different time points after administration of hCG in the prespawning phase revealed steady increase in *11β-HSD2* transcript levels and 11-KT production up to 24h accompanied by induction of spermatogenesis. However, results obtained in the resting phase, where hCG administration could not induce sustained elevation in the expression of *11β-HSD2*, indicates that gonadotropin input alone cannot trigger 11-KT production vis-à-vis spermatogenic cycle during testicular quiescence. These results are in agreement with the previous hCG induction studies performed on eel testes belonging to various developmental stages where hCG promoted spermatogenesis and increased the milt volume in developed testis but could not sustain completion of spermatogenic cycle in the quiescent testis but for initiating few mitotic division in spermatogonia (Miura *et al*.,

1997). These findings together with the present study suggests that various factors, for example sex steroids, androgen receptors, environmental cues and signals from the hypothalamo-hypophyseal axis may act collectively in a complex coordinated manner to initiate the spermatogenic cycle after testicular quiescence. Nevertheless, judging from changes in *11β-HSD2* during seasonal cycle and after hCG-induction in prespawning phase in the present study, it is plausible to infer that gonadotropins targets up regulation of *11β-HSD2* at the level of testis to promote testicular recrudescence. This may be one of the mechanisms to entrain testicular cycle.

Spatial expression pattern of catfish *11β-HSD2* by semi-quantitative RT-PCR demonstrated ubiquitous expression with predominant expression in testis, gill, anterior kidney and liver. The occurrence of extra testicular expression is in agreement with reports in the Nile tilapia, eel and rainbow trout, (Jiang *et al*., 2003; Kusakabe *et al*., 2003). The presence of *11β-HSD2* in gill suggests a role in osmoregulation. In kidney, *11β-HSD2* might play a protective role as that of mammalian *11β-HSD* type-2 gene, where it is involved in the protection of mineralocorticoid receptor from over stimulation by excess corticosteroid and also in the prevention of inhibitory action of cortisol on androgen synthesis (Monder *et al*., 1994; Bambino and Hsueh, 1981). Earlier reports in teleosts ascertained the existence of genes coding for enzymes involved in corticosteroid synthesis pathway and mineralocorticoid receptor in kidney, reconfirming the protective role of *11β-HSD2* from cortisol, the main corticosteroid in teleosts (Colombe *et al*., 2000). The presence of abundant expression in liver is in agreement with the previous report in this species where they indicated, extra testicular conversion of T to 11-KT by liver that contributed to the 11-KT level measured in plasma (Cavaco *et al*., 1997b). Biological role

of *11β-HSD2* has been implicated in teleost reproduction but the presence of *11β-HSD2* transcripts in non-steroidogenic tissues such as heart and muscle is unclear. Expression of *11β-HSD2* in brain is not unusual since steroidogenic enzyme genes are often detected in brain (Tomy *et al*., 2007). In rainbow trout, *in situ* hybridization with *11β-HSD2* mRNA yielded positive signals in the thecal layer of the ovarian follicle, which supports the occurrence of *11β-HSD2* expression in catfish ovary, assigning it a protective role in ovary from the excessive circulatory cortisol, as suggested in rainbow trout and carp (Kusakabe *et al*., 2003; Nematollahi *et al*., 2009).

Ontogeny study was undertaken to confirm *11β-HSD2* role during testicular differentiation in catfish which displayed *11β-HSD2* transcripts in testis from 55 dph onwards followed by stability in transcript levels at 75, 90, 150 and 260 dph catfish larvae strongly emphasizing its role at least in testicular development.

In summary, a full-length cDNA for *11β-HSD2* was cloned from testis of catfish. Catfish *11β-HSD2* cDNA showed high homology to that of zebrafish followed by eel. Dehydrogenase capacity of the recombinant *11β-HSD2* protein was demonstrated in COS-7 cells. We also studied the affinity and capacity of testicular *11β-HSD2* enzyme towards 11-OHT. Present study provided substantial evidence on stage-dependent expression of *11β-HSD2* and 11–KT production in maintaining the testicular cycle. Further, we demonstrate the responsiveness of testis to hCG induction, *in vivo* at recrudescence but not in quiescent phase to validate our hypothesis that gonadotropins might regulate *11β-HSD2* vis-à-vis 11-KT to entrain testicular cycle. It is apparent from the ontogeny expression study in catfish that *11β-HSD2* might be required only during late stages of testicular differentiation or development. Based on our comprehensive study, it is possible to

implicate an important role for *11β-HSD2* during testicular development and recrudescence in catfish.

**References**

Andersen L., Holbech H., Gessbo A., Norrgren L., Petersen G.I., 2003. Effects of exposure to 17α-ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). Comp. Biochem. Physiol., 134C: 365-374.

Baker M.E., 2004. Evolutionary analysis of 11β-hydroxysteroid dehydrogenase-type 1, -type 2, -type 3 and 17β-hydroxysteroid dehydrogenase-type 2 in fish. FEBS Letters 574:167-170.

Baker M.E., 1998. Evolution of mammalian 11β-and 17β-hydroxysteroid dehydrogenases-type 2 and retinol dehydrogenases from ancestors in *Caenorhabditis elegans* and evidence for horizontal transfer of a eukaryote dehydrogenase to *E. coli*. J. Steroid Biochem. Mol. Biol., 66: 355-363.

Bambino T.H., Hsueh A.J., 1981. Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis *in vivo* and *in vitro*. Endocrinology, 108:2142–2148.

Baroiller J.F., Guiguen Y., Fostier A., 1999. Endocrine and environmental aspects of sex differentiation in fish. Cell Mol. Life Sci., 55:910–931.

Baron D., Houlgatte R., Fostier A., Guiguen Y., 2005. Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol. Reprod., 73:959-966.

Borg B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol., 109C: 219–245.

Cavaco J.E.B., Lambert J.G.D., Schulz R.W., Goos H.J.Th., 1997a. Pubertal development of male African catfish, *Clarias gariepinus*: *In vitro* steroidogenesis by testis and interrenal tissue and plasma levels of sexual steroids. Fish Physiol. Biochem. 16:129-138.

Cavaco J.E.B., Vischer H.F., Lambert J.G.D., Goos H.J.Th., Schulz R.W., 1997b. Mismatch between patterns of circulating and testicular androgens in African catfish (*Clarias gariepinus*). Fish Physiol. Biochem. 17:155–162.

Cavaco J.E.B., van Baal J., Van Dijk W., Hassing G.A.M., Goos H.J.Th., Schulz R.W., 2001a. Steroid hormones stimulate gonadotrophs in juvenile male African catfish (*Clarias gariepinus*). Biol. Reprod., 64:1358-1365.

Cavaco J.E.B., Bogerd J., Goos H.J.Th., Schulz R.W., 2001b. Testosterone inhibits 11-ketotestosterone-induced spermatogenesis in African catfish (*Clarias gariepinus*). Biol. Reprod., 65:1807-1812.

Colombe L., Fostier A., Bury N., Pakdel F., Guiguen Y., 2000. A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: Cloning and characterization of its steroid binding domain. Steroids 65: 319-328.

Daux W.L., Ghosh D., 1997. Structure and function of steroid dehydrogenase involved in hypertension, fertility, and cancer. Steroids, 62:95-100.

Devlin R.H., Nagahama Y., 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. Aquaculture, 208:191–364.

Hu G.X., Lin H., Sottas C.M., Morris D.J., Hardy M.P., Ge R.-S., 2008. Inhibition of 11β-hydroxysteroid dehydrogenase enzymatic activities by glycyrrhetinic acid *in vivo* supports

direct glucocorticoid-mediated suppression of steroidogenesis in Leydig cells. J. Androl., 29:345-351.

Ikeuchi T., Todo T., Kobayashi T., Nagahama Y., 1999. cDNA cloning of a novel androgen receptor subtype. J. Biol. Chem., 274:25205-25209.

Jiang J.Q., Wang D.S., Senthilkumaran B., Kobayashi T., Kobayashi H.K., Yamaguchi A.,Ge W., Young G., Nagahama Y., 2003. Isolation, characterization and expression of 11β-hydroxysteroid dehydrogenase type 2 cDNAs from the testes of Japanese eel (*Anguilla japonica*) and Nile tilapia (*Oreochromis niloticus*). J. Mol. Endocrinol., 31:305–315.

Kallberg Y., Oppermann U., Jornvall H., Persson B., 2002. Short-chain dehydrogenase reductase (SDR) relationships: A large family with eight clusters common to human, animal, and plant genomes. Protein Sci. 11: 636-641.

Katz A., Heiblum R., Meidan R., Robinzon B., 2008. Corticosterone oxidative neutralization by 11β-hydroxysteroid dehydrogenases in kidney and colon of the domestic fowl. Gen. Comp. Endocrinol., 155:814-820.

Koya Y., Soyano K., Yamamoto K., Obana H., Matsubara T., 2002. Testicular development and serum profiles of steroid hormone levels in captive male Pacific herring *Clupea pallasii* during their first maturational cycle. Fish. Sci., 68:1099-1105.

Kusakabe M., Nakamura I., Young G., 2003.Enzymatic activity of 11β-hydroxysteroid dehydrogenase in rainbow trout *Oncorhynchus mykiss*. Fish Physiol. Biochem., 28:197-198.

Liu S., Govoroun M., D'Cotta H., Ricordel M.J., Lareyre J.J., Mc Meel O.M., Smith T., Nagahama Y., Guiguen Y., 2000. Expression of cytochrome P45011β (11β–hydroxylase)

gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. J. Steroid Biochem. Mol. Biol., 75:291-298.

Maugars G., Schmitz M., 2008. Gene expression profiling during spermatogenesis in early maturing male Atlantic salmon parr testes, Gen. Comp. Endocrinol., 159: 178–187.

Miura T., Yamauchi K., Takahashi H., Nagahama Y., 1991a. Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). Proc. Natl. Acad. Sci., USA, 88:5774–5778.

Miura T., Yamauchi K., Nagahama Y., Takahashi H., 1991b. Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool. Sci., 8: 63–73.

Miura T., Kawamura S., Miura C., Yamauchi K., 1997. Impaired spermatogenesis in the Japanese eel, *Anguilla japonica*: Possibility of the existence of factors that regulate entry of germ cells into meiosis. Dev. Growth Differ., 39: 685-691.

Miura T., Miura C.I., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiol. Biochem., 28:181-186.

Miura C., Higashino T., Miura T., 2007. A progestin and an estrogen regulate early stages of oogenesis in fish. Biol. Reprod., 77:822–828.

Miura S., Horiguchi R., Nakamura M., 2008. Immunohistochemical evidence for 11β-hydroxylase (P45011β) and androgen production in the gonad during sex differentiation and in adults in the protandrous anemone fish *Amphiprion clarkii*. Zool. Sci., 25: 212-219.

Monder C., Miroff Y., Marandici A., Hardy M.P., 1994. 11β-hydroxsteroid dehydrogenase alleviates glucocorticoid-mediated inhibition of steroidogenesis in rat leydig cells. Endocrinology, 134:1199-1204.

Nagahama Y., 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol., 38:217–229.

Nematollahi M.A., van Pelt-Heerschap H., Komen J., 2009. Transcript levels of five enzymes involved in cortisol synthesis and regulation during the stress response in common carp: Relationship with cortisol. Gen. Comp. Endocrinol. 164: 85-90.

Ozaki Y., Higuchi M., Miura C., Yamaguchi S., Tozawa Y., Miura T., 2006. Roles of 11β-hydroxysteroid dehydrogenase in fish spermatogenesis. Endocrinology, 147: 5139–5146.

Raghuveer K., Senthilkumaran B., 2009. Identification of multiple *dmrt1s* in catfish: Localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment. J. Mol. Endocrinol., 42: 437-448.

Robinzon B., Prough R.A., 2009. A novel $NADP^+$-dependent dehydrogenase activity for 7α/β and 11β-hydroxysteroids in human liver nuclei: A third 11β-hydroxysteroid dehydrogenase. Arch. Biochem. Biophys., 486:170-176.

Robinzon B., Prough R.A., 2005. Interactions between dehydroepiandrosterone and glucocorticoid metabolism in pig kidney: Nuclear and microsomal 11β-hydroxysteroid dehydrogenases. Arch. Biochem. Biophys., 442:33-40.

Rougeot C., Krim A., Mandiki S., Kestemont P., Mélard C., 2007. Sex steroid dynamics during embryogenesis and sexual differentiation in Eurasian perch, *Perca fluviatilis*. Theriogenology, 67:1046-1052.

Sakai N., Ueda H., Suzuki N., Nagahama Y., 1989. Steroid production by amago salmon (*Oncorhynchus rhodurus*) testes at different development stages. Gen. Comp. Endocrinol., 75: 231-240.

Schulz R.W., Liemburg M., García-López A., Van Dijk W., Bogerd J., 2008. Androgens modulate testicular androgen production in African catfish (*Clarias gariepinus*) depending on the stage of maturity and type of androgen. Gen. Comp. Endocrinol., 156:154-163.

Scott A.P., Bye V.J., Baynes S.M., Springate J.R., 1980. Seasonal variation in plasma concentrations of 11-ketotestosterone and testosterone in male rainbow trout, *Salmo gairdneri* Richardson. J. Fish Biol., 17: 495-505.

Stewart P.M., Murry B.A., Mason J.I., 1994. Human kidney 11β-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type 1 isoform. J. Clin. Endocrinol. Metab., 79: 480-484.

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

Tomy S., Wu G-C., Huang H-R., Dufour S., Chang C-F., 2007. Developmental expression of key steroidogenic enzymes in the brain of protandrous black porgy fish, *Acanthopagrus schlegeli*. J. Neuroendocrinol., 19: 643-656.

Viñas J., Piferrer F., 2008. Stage-specific gene expression during fish spermatogenesis as determined by laser-capture micro dissection and quantitative-PCR in sea bass (*Dicentrarchus labrax*) gonads. Biol. Reprod., 79: 738-747.

Wang X.G., Orban L., 2007. Anti-Mullerian hormone and 11β-ydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. Dev. Dyn., 236:1329–1338.

# *Chapter 3*

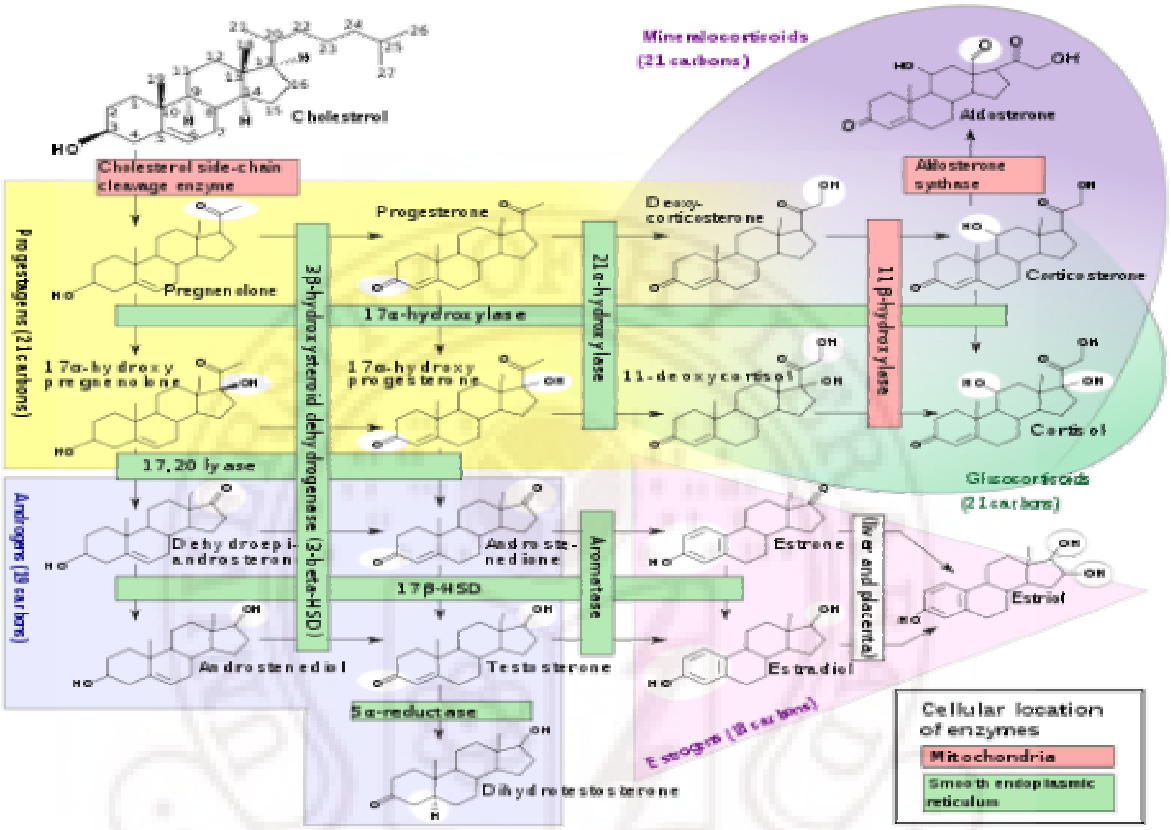
## Cloning and expression analysis of testicular 11β-hydroxylase during seasonal cycle and after hCG-induction in air-breathing catfish *Clarias gariepinus*

**Abstract:**

Cytochrome P450 11β-hydroxylase (*11β-H)* gene is involved in production of 11β-hydroxytestosterone (11β-OHT) the precursor for the synthesis of 11-ketotestosterone (11-KT), a potent androgen for several male fishes. In the present study, a partial *11β-H* cDNA sequence (768bp) was cloned from air-breathing catfish *Clarias gariepinus* which shared high homology with zebrafish (76%) and rainbow trout (72%). Tissue distribution analysis revealed expression of *11β-H* in most of the tissues with predominance in testis, anterior kidney, liver and gills. Ontogeny study by real-time PCR from 45 days post hatch (dph) till 260 dph in male and female catfish larvae, detected the expression of *11β-H* from 55 dph suggesting that 11-KT might not be required during testis formation but subsequent increase in transcript levels clearly indicated a crucial role of *11β-H* in advancing testicular growth and development. Bleak expression was detected in female catfish larvae from 150 dph. Expression of *11β-H* correlated well with testicular recrudescence, displaying maximum expression in the prespawning phase. Administration of hCG elevated *11β-H* mRNA levels from 4h in the prespawning testis while priming was ineffective in the resting phase. These results tend to suggest that onset of spermatogenesis during testicular recrudescence is marked by corresponding increase in the mRNA levels of steroidogenic enzymes *11β-H* and *11β-HSD2* (Chapter 2).

***Note: 11β-Hydroxylase is abbreviated as cyp11b1 in some research reports. However, in this thesis it has been abbreviated as 11β-H in this chapter.***

**Introduction:**




**Adopted from Wikipedia: (http://en.wikipedia.org/wiki/Steroid_11-beta-hydroxylase)**

**Introduction**

Cytochrome P450 11β-hydroxylase (*11β-H)* is a mitochondrial protein involved in the synthesis of cortisol (interrenal cells of kidney) and 11−oxo androgens (testis) in teleosts (Borg, 1994; Jiang *et al*., 1998). Labiality of sex-determination in teleosts (Baroiller and Guiguen, 2001; Devlin and Nagahama, 2002) and possibility of masculinization of adult and juvenile fish by exogenous androgens:17α-methyltestosterone and 11-oxo androgens (Cardwell and Liley, 1991; Pandian and Sheela, 1995; Govoroun *et al*., 2001; Raghuveer

*et al*., 2005; Baron *et al*., 2008; Raghuveer and Senthilkumaran, 2009) prompted researchers to speculate whether like estradiol (whose presence in juvenile fish hints towards ovarian development) 11-ketotestosterone (11-KT), a potent fish androgen, could pilot testis formation from bipotential gonad during critical period of sex differentiation. In this direction attempts have been made, by performing expression studies during larval development for the steroidogenic enzyme genes: cholesterol side chain cleavage, 17-hydroxylase/lyase, 3β-hydroxysteroid dehydrogenase and *11β-H* that lead to the production of 11-KT (Govoroun *et al*., 2001; Baron *et al*., 2005) and metabolite analysis from monosex male population (Baroiller *et al*., 1998). In particular, *11β-H* and 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2*) expression and localization in gonadal tissues were investigated in sex changing hermaphrodite fishes (Liu *et al*., 2009; Miura *et al*., 2008) along with measurement of plasma 11-KT levels during male to female sex conversion (Alam *et al*., 2006). Although *11β-H* and *11β-HSD2* role in sex inversion in hermaphroditic fishes are proved, it is difficult to assert these genes with the role of testis inductor until the factor that governs their expression is analyzed. Ontogeny study for *11β-H* from gonochoristic fish species like sea bass, the Nile tilapia, rainbow trout and zebrafish (Liu *et al*., 2000; Socorro *et al*., 2007; Wang and Orban, 2007; Ijiri *et al*., 2008) provided ambiguous results related to its contribution in testis formation. Therefore, we wanted to examine *11β-H* expression pattern during larval development in air-breathing catfish and study the feasibility of using this gene as early indicator of testis formation in catfish where monosex population is not available. To begin with, we attempted to clone full length or partial cDNA of *11β-H* from catfish testis and then studied the developmental expression pattern by designing gene specific primers.

Role of 11-KT in spermatogenesis and male reproductive cycle via *11β-H* and/or *11β-HSD2* expression/activity (Miura *et al*., 1991a; Yokota *et al*., 2005; Maugars and Schimtz, 2008) in different fish species including catfish (Chapter 2) has been investigated. Further to gain an insight whether *11β-H* mRNA also displayed similar seasonal fluctuation like *11β-HSD2* (Chapter 2) or whether 11β-hydroxytestosterone (11β-OHT) produced by *11β-H* had any directional inhibitory role on *11β-HSD2* dehydrogenase activity as observed in rat Leydig cells (Wang *et al*., 2002). Till date an orthologus *11β-HSD1* gene has not been detected in teleosts. However, no report exists from teleosts that provide evidence for the inhibitory role of excess 11β-OHT on 11-KT production but for excess testosterone (Cavaco *et al*., 2001; Schulz *et al*., 2008) and cortisol levels (Shankar and Kulkarni, 2000) impeding spermatogenesis. Reports from eel (Miura *et al*., 1991a) and catfish (Chapter 2) demonstrate initiation of spermatogenesis on administration of human chorionic gonadotropin (hCG). This was mediated through induction of *11β-HSD2* transcripts, (Miura *et al*., 1991b; Chapter 2) enzyme activity (Chapter 2) and simultaneous alteration in the levels of plasma follicle stimulating hormone (FSH)/lutenizing hormone LH (Penaranda *et al*., 2009) but no report exists to account for changes in the *11β-H* mRNA levels after induction, which produces the precursor 11β-OHT required by *11β-HSD2* to synthesize 11-KT. Hence priming the fish with hCG was undertaken at prespawning and resting phases to monitor *11β-H* transcript response.

## Materials and Methods

### Animals

Maintenance, breeding and rearing of catfish from larva to adult was elaborately mentioned in Chapters 1 and 2. The experimental strategy and fish sacrifice for the present chapter was followed as per Chapter 2.

### Cloning of catfish *11β-H*

A set of degenerate primers were designed by aligning the existing sequences of vertebrate *11β-H,* to clone a cDNA fragment from the testis of catfish. Using these degenerate primers, a cDNA of 404 bp was amplified by RT-PCR from catfish testicular tissue and cloned in pGEMT-easy vector (Promega, Madison, WI, USA) and subsequently sequence was determined. The identity of amplified partial cDNA was analyzed by BLAST search. Several attempts were made to get the full length sequence from the catfish testis cDNA library (Chapter 2) using the partial radiolabelled cDNA as probe but ended up getting non-specific clone. Subsequently RNA-Ligase mediated RACE system (Invitrogen, Carlsbad, CA, USA) was used to clone the 5' and 3' end sequences of the catfish *11β-H*. The gene specific primers for 5' and 3' RACE are listed in table 1. Preparation of 5' and 3' cDNA (RACE) templates and RT-PCR amplifications were done as per the manufacturer's instructions and different sized PCR amplicons were cloned into pGEM-T vector and subsequently sequence was determined through ABI PRISM sequencer (Applied Biosystems, Foster, CA, USA).

**Table 1: List of primers used for cloning and expression studies**

| Sl. No | Primer name | Sequence | Purpose |
|---|---|---|---|
| 1 | DF1 | 5′CCTNGGSCCCATWTACAGGSAG 3′ | cloning |
| 2 | DR1 | 5′GTCGTGTCCACYSCYCCBGCCAT 3′ | cloning |
| 3 | 3P | 5′GGAGCGAATGCTGAACACA 3′ | 3' RACE |
| 4 | 3N | 5′GGGACCACATCTTCAGTCACGCTGATGAG 3′ | 3' RACE |
| 5 | 5P | 5′ CCACAGCACCCCGGGGAACCTTGAG 3′ | 5' RACE |
| 6 | 5N | 5′ GAGCAGGGGAGGCGTTGTGTTCAGCA 3′ | 5' RACE |
| 7 | Spfw | 5′GGCAGTGGAGCGAATGCTGAA 3′ | tissue distribution |
| 8 | Sprv | 5′ GCACCCCGGGGAACCAGC 3′ | tissue distribution |
| 9 | *β-actin*-Fw | 5′ ACCGAAGTCCATCACAATACCAGT 3′ | Real-time RT-PCR |
| 10 | *β-actin*-Rv | 5′ GAGCTGCGTGTTGCCCCTGAG 3′ | Real-time RT-PCR |

**Real-time RT-PCR**

Expression of *11β-H* was analyzed by real-time RT-PCR as per the method described in chapter 2 using *11β-H* specific primer pair listed in table1. Each sample (n=5) was run in triplicates with a final volume of 25 µl containing 0.3 µl of cDNA representing the four different phases of the testis, 10 pmol of each primer and 12.5 µl of SYBR Green PCR master mix (Applied Biosystems). Transcript abundance of *11β-H* was normalized to that of *β-actin* and reported as fold change in abundance relative to the values obtained for

preparatory phase using the formula $2^{-\Delta\Delta CT}$. Data analysis was carried out using one-way ANOVA followed by Tukey-Kramer's test. Significance was accepted at $P < 0.05$.

**RT-PCR analysis of tissue distribution pattern of catfish *11β-H***

2.5 µg of total RNA extracted from different tissues of male catfish (in preparatory phase of testicular cycle) was reverse transcribed to first-strand cDNA using Superscript III (Invitrogen) reverse transcriptase. PCR reaction was performed at 94°C for 1min, 60°C for 30sec and 72° for 1 min for 30 cycles using a dual-block thermal cycler ABI9700 (Applied Biosystems, Foster, CA, USA). Specific primers were designed for this purpose and the amplicon size and identity confirmed by sub-cloning in pGEM-T Easy vector (Promega) and subsequently sequencing and BLAST search.

**Effect of hCG on *11β-H* expression**

For *in vivo* hCG induction studies, five male catfishes in prespawning and resting phases were injected with hCG (1000 IU/kg body weight) or saline (0.15M) intraperitoneally and sacrificed at different time points to collect and store the testicular tissue at -80°C for later analyses. To study the changes in the expression level of the *11β-H* transcript, semi-quantitative RT-PCR was performed using same primers designed for tissue distribution. This procedure was repeated thrice with different batch of fish (n=5). Data analysis was carried out using one-way ANOVA followed by Dunnett's test. Significance was accepted at $P < 0.05$.

**Ontogeny study of *11β-H* to study the expression**

Critical window for sex differentiation in catfish was around 35-49 days post hatch (dph; Raghuveer and Senthilkumaran, 2009) which made it feasible to study the expression of *11β-H* from 45 dph without any ambiguity because by that time the ovary and testis were

morphologically distinguishable. Therefore, for temporal expression study of *11β-H*, about 15-20 male and female catfish larvae were dissected at 45, 55, 75, 90, 150 and 260 dph under dissection microscope (Carl-Zeiss, Germany) and the gonads pooled to have 5 biological samples (n=5) for total RNA preparation under sterile condition, then snap-frozen in liquid nitrogen and stored in -80$^{o}$C for later use. This procedure was repeated thrice and the results were expressed as mean ± SEM of three replicates. Reverse transcription was carried out with Superscript III (Invitrogen) using 2.5µg RNA. *11β-H* transcripts were detected by real-time PCR using specific primers. Data analysis was carried out using one-way ANOVA followed by Tukey-Kramer's test. Significance was accepted at $P < 0.05$. All the data analysis was done using Graph Pad Prism 5 software (San Diego, California, USA).

**Results**

**Cloning of catfish testicular *11β-H***

Using a set of degenerate primers, a partial cDNA of 404 bp was isolated by RT-PCR and sequence identity was confirmed by BLAST search. Further 5' and 3' RACE strategy using gene specific primers designed from partial cDNA fragment yielded 768 bp cDNA (Fig. 1 & 2). Though we obtained partial cDNA, ClustalW multiple alignments demonstrated the presence of signature steroid binding domain showing high homology to that of *11β-H* of other teleosts (Fig. 3). Phylogenetic analysis by Neighbour-Joining revealed that catfish *11β-H* has about 76-65% homology with other teleost and shares highest percent identity with zebrafish (76%) and rainbow trout (72%) *11β-H*. In addition it showed 40-48% identity to aldosterone synthase (*cyp11b2)* from rat, mouse and human (Fig. 4). Our repeated attempts, both 5' and 3' did not yield full length transcripts. The

difficulty in obtaining 5' RACE fragment may be attributed to high GC content. The reason for non-amplification of 3' RACE may be due to the length. It may also be possible that the number of full-length transcripts might not be so high during the stage of RACE template cDNA preparation.

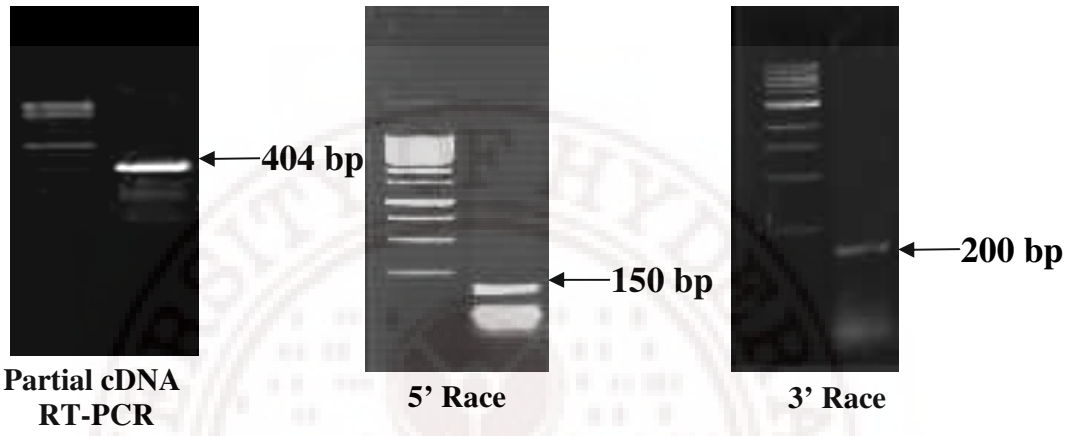



***Figure 1. 1% Agarose gels showing RT-PCR products using degenerate primers for amplification of partial 11β-H cDNA fragment, (A) using degenerate primer and (B), (C) 5', 3' RACE products using gene specific primers.***

**5'…….ATTTACAGGCAGTACTTGGGCTCTCAGTGCAGTGTGAACATCAT**
**TCTCCCGGTGGATATCGCCGAACTCTTCCACTCTGAGGGACTGAATCCAC**
**GCCGTATGGCGCTGCAGCCCTGGAACACACACCGTGAAACACGCAAACAC**
**AGCAAGGGAGTCTTCCTCAAAAATGGACCGGAGTGGCGCTCTGACCGCTT**
**GCTGTTGAACCGTGAGGTGATGTTGAGCTCGGCCGTACGCCGGTTCCTGC**
**CCCTGCTGGACGAGGTGGCTCGGGATTTTTCACATGTCCTGCAGCATAAA**
**GTCCACACTAAGGGACAGGTGAAGAATGGCACACATACACTGACCTTTGA**
**CCCGAGCCCTGAGCTTTTCCGCTTTGCTTTAGAAGCAAGTTGTCATGTCCT**
**GTACAGCGAGCGCATCGGTCTCTTCTCCTCCAAGCCGTCTCAGGAGTCAG**
**AGCGCTTTATTCTGGCAGTGGAGCGAATGCTGAACACAACGCCTCCCCTG**
**CTCTATTTAGTGCCCCGCCCACCACTGCGGTTCTACACACCCCTGTGGAC**
**TCACCATGCGACTGCGTGGGACCACATCTTCAGTCACGCTGATGAGGAAG**
**CACAGCGTGTGTATAAGGGAGGGCGGCTCCAGGGTCGGGGGGGCAGTGA**
**GGAGGCAGCAGACAGCTGGTTCCCCGGGGTGCTGTGGCAGCTGATGGAG**
**AGAGGACAGCTTCCGCTGGAGGTCATCAAGGCTAACATCACTGAACTAAT**
**GGCAGGAGGAGTGGACACGAC…….3'**

**5'…SQCSVNIILPVDIAELFHSEGLNPRRMALQPWNTHRETRKHSKGVFLKNG**
**PEWRSDRLLLNREVMLSSAVRRFLPLLDEVARDFSHVLQHKVHTKGQVKNG**

**THTLTFDPSPELFRFALEASCHVLYSERIGLFSSKPSQESERFILAVERMLNTTP PLLYLVPRPPLRFYTPLWTHHATAWDHIFSHADEEAQRVYKGGRLQGRGGS EEAADSWFPGVLWQLMERGQLPLEVIKANITELMAGGVDT…3'**

***Figure 2. Partial nucleotide and deduced protein sequences of catfish 11β-H by RACE strategy.***

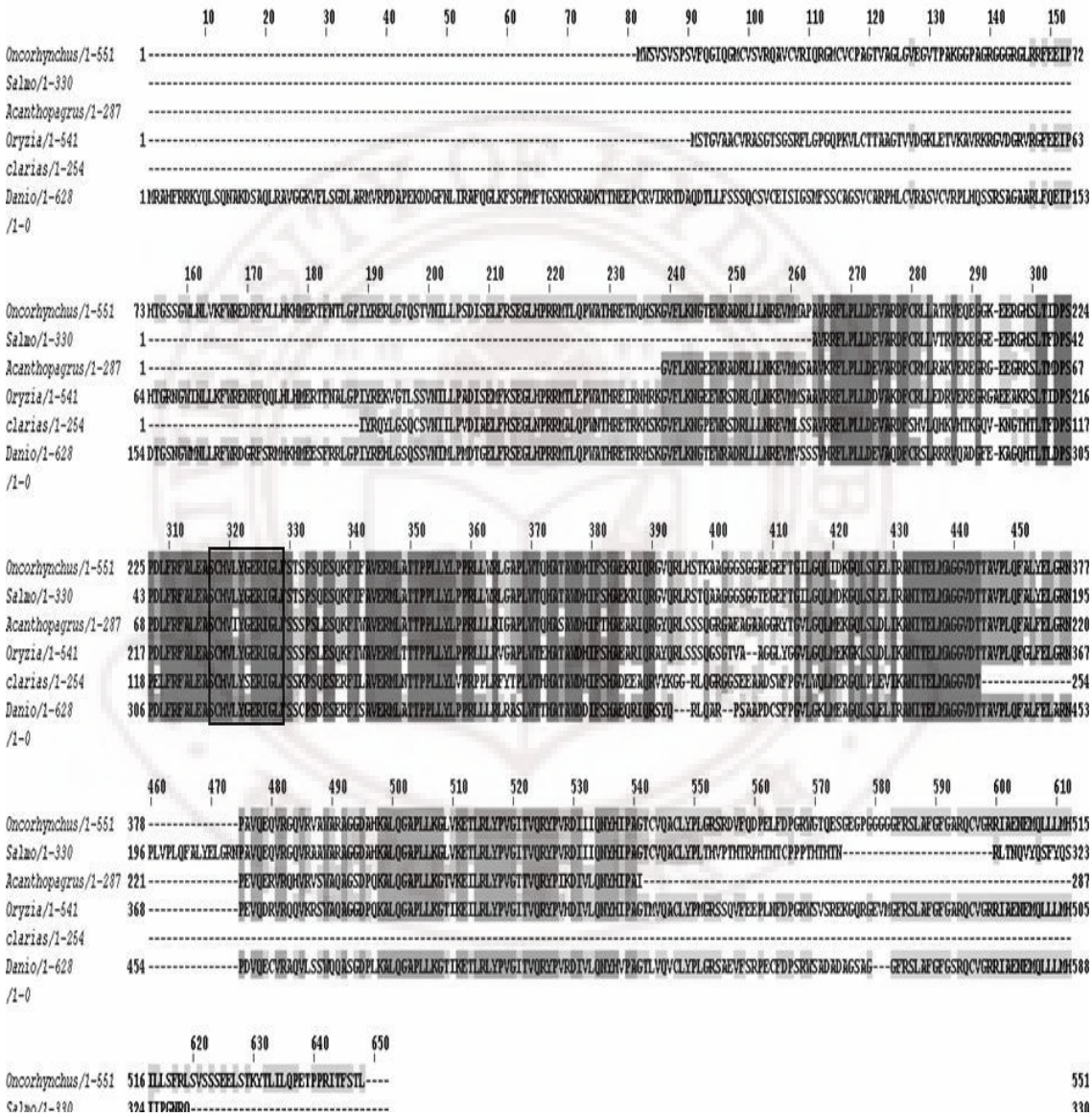



***Figure 3. ClustalW multiple alignment of catfish 11β-H with other teleost counterparts. The only conserved domain that is present in the partial sequence is the steroid binding region shown in rectangle.***

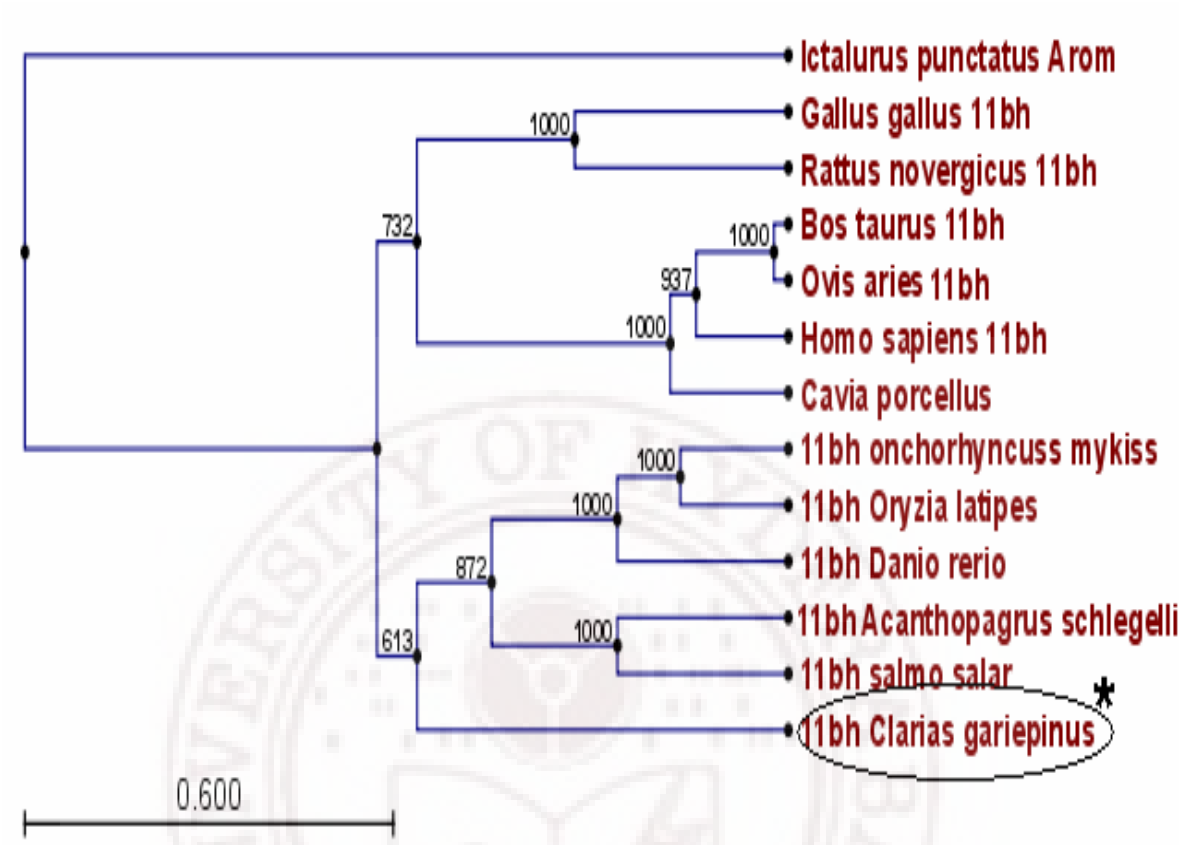



***Fig. 4. Phylogenetic tree showing the evolutionary relationship of catfish 11β-H. Scale bar indicates an evolutionary distance of 0.6 amino acid substitution per position in the sequence. Phylogenetic analysis was done using CLC bio: CLC main workbench software. Bootstrap analysis with 1000 replicates was used to asses the strength of nodes in the tree. The sequences of 11β-H were extracted from GenBank database with their Accession no.: Acanthopagrus schlegelli* EF423618; Oryzia latipes EF025509; Oncorhynchus mykiss AF179894; Salmo salar* DQ352841; Danio rerio BC155806; Cavia porcellus AF018569; Ovis aries U78478; Rattus novergicus BC089100; Homo sapiens X54741; Xenopus laevis AF449175; Gallus gallus NM001756; Bos taurus M17843 and (out grouped with aromatase gene from Ictalurus punctatus) Ictalurus punctatus S75715.* indicates partial sequence.***

**Phase-dependent expression of *11β-H* in testis**

Real-time RT-PCR analysis demonstrated an increase in *11β-H* transcripts during early stages of spermatogenesis followed by a decrease in the expression level as the process of spermiogenesis commenced (Fig. 5). These results paralleled the serum 11-KT levels (Chapter 2) measured during the different phases of testicular cycle thereby suggesting that the levels of 11-KT required during spermatogenesis are modulated by the changes in the transcript levels of the enzymes producing it (*11β-H* and *11β-HSD2*).

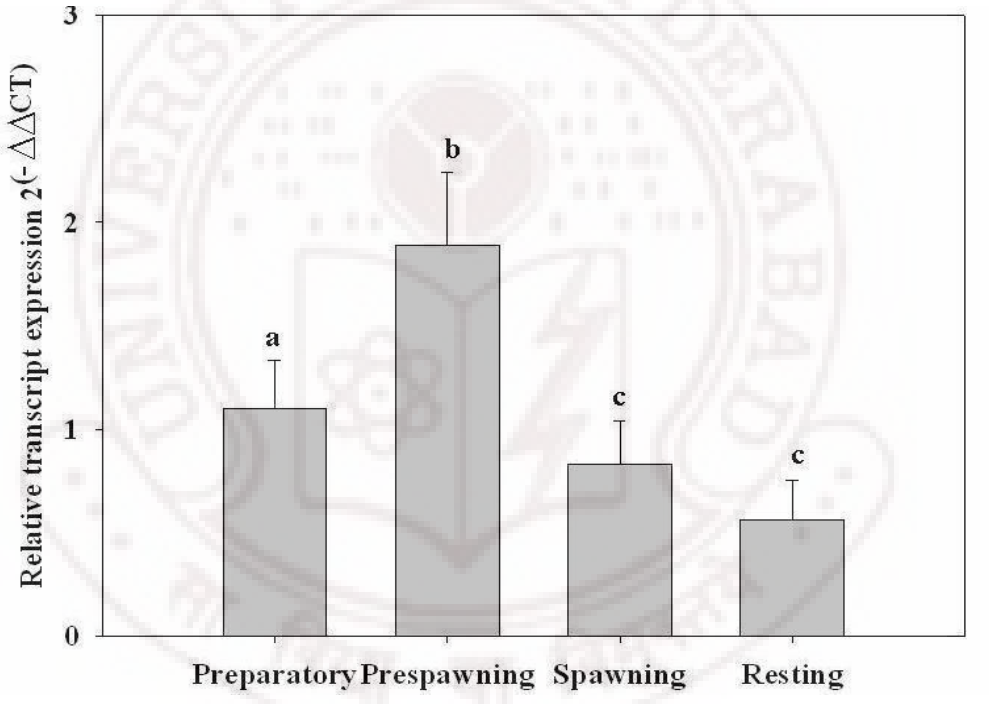



***Figure 5. Real-time quantitative RT-PCR analysis of 11β-H expression during different phases of testicular cycle in catfish. Data from real-time PCR were expressed as mean ± SEM. Significance between groups were tested by ANOVA followed by Tukey-Kramer's test using Graph pad prism 5 software. Means with different alphabets differ significantly (P<0.05).***

**Tissue distribution**

Tissue distribution study by semi-quantitative RT-PCR detected ubiquitous expression pattern of *11β-H*. However, the expression was much higher in gonad, gill, kidney and liver (Fig. 6).

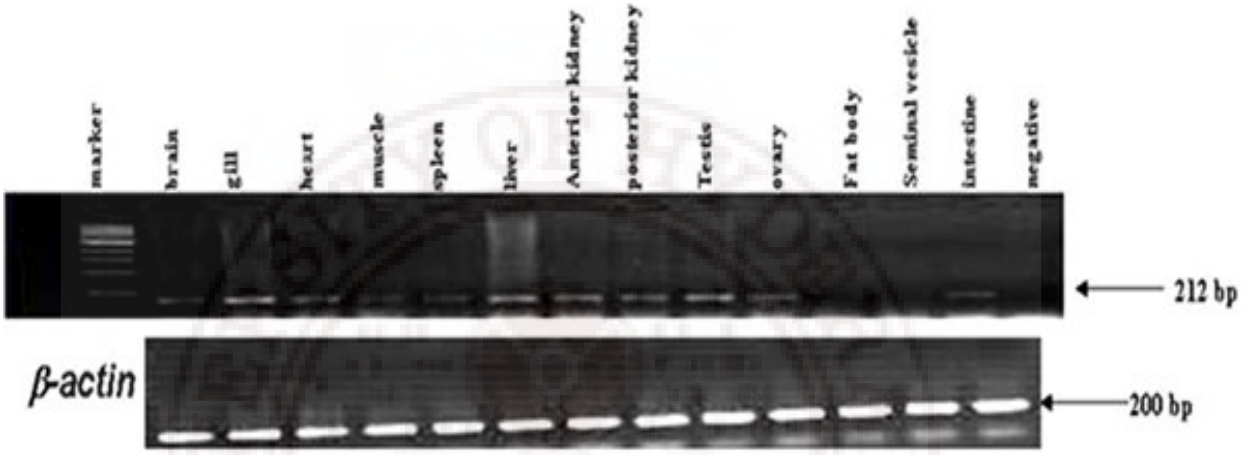



***Fig. 6. Representative gel showing spatial expression pattern of catfish 11β-H in different male tissues by semi-quantitative RT-PCR analysis. Negative control contains no cDNA template.***

**Changes in the expression of *11β-H* after hCG administration**

Significant increase in the expression of *11β-H* compared to control and saline treatment (data not shown in the gel picture) was observed from 4h after induction with sustained increase in the mRNA levels till 24h (Fig. 7A & B). The priming effect was negligible in the resting phase of testicular cycle. (Fig. 8A & B)

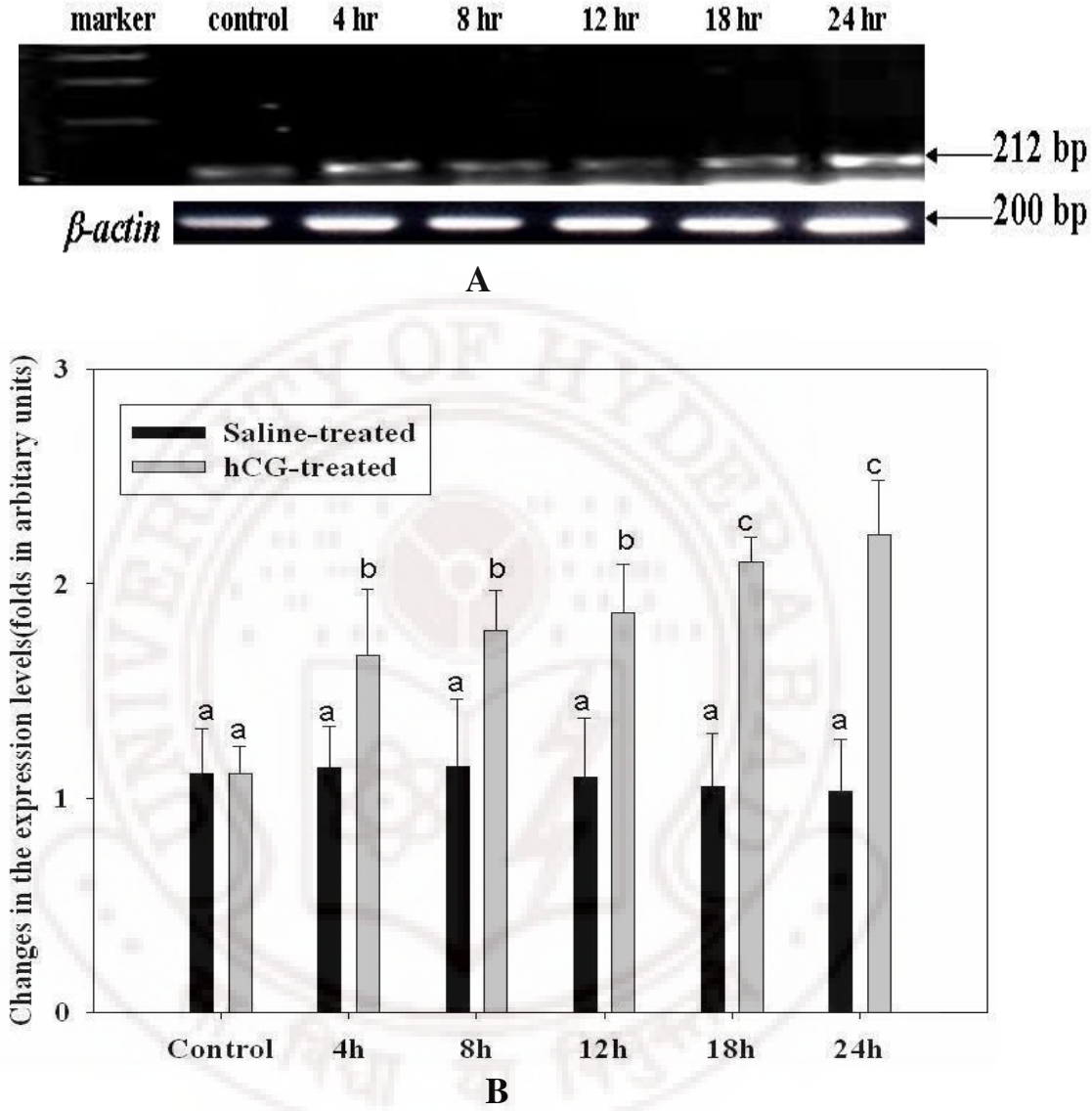
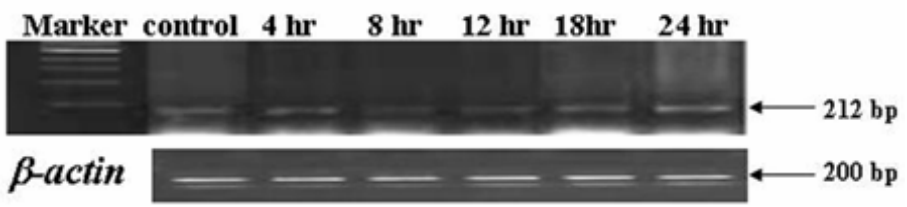
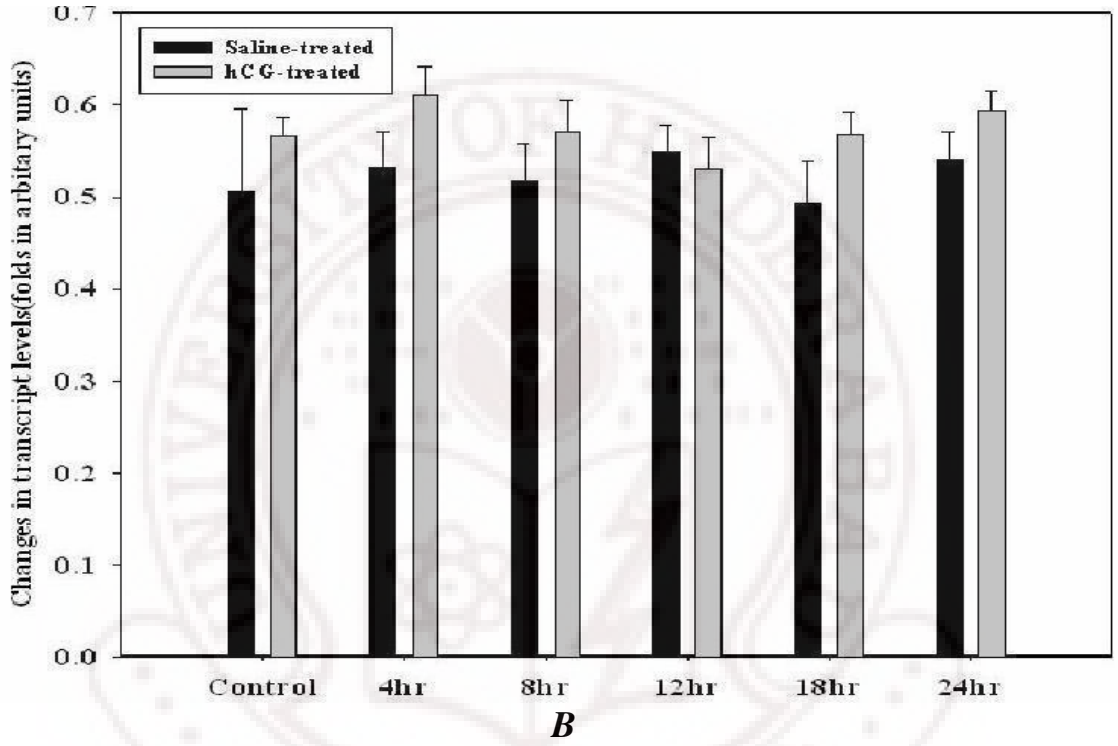



***Fig. 7. Representative gel showing the semi-quantitative RT-PCR analysis of 11β-H (A) expression (B) graphical representation of the changes in the expression pattern compared to control after hCG- induction in the prespawning phase. X-axis represents hours after treatment. Statistical analysis was done by one way ANOVA followed by Dunnett's test using Graph pad prism 5 software. Values are mean ± SEM, n=5, common alphabets indicate means that are not significantly different. Means with different alphabets differ significantly (P<0.05).***




**A**




***B***

***Fig. 8. Representative gel showing the semi-quantitative RT-PCR analysis of 11β-H (A) expression (B) graphical representation of the changes in the expression pattern compared to control after hCG- induction in the in the resting phase. X-axis represents hours after treatment. Statistical analysis was done by one way ANOVA followed by Dunnett's test using Graph pad prism 5 software. Values are mean ± SEM, n=5, (P<0.05).***

**Ontogeny of *11β-H***

Real-time RT-PCR analysis detected expression of *11β-H* from 55 dph with levels showing a constant increase corresponding to the onset of spermatogenesis in male larvae. We monitored expression of *11β-H* till 260 dph at different periods. The expression pattern

of *11β-H* in adult testis was already shown in tissue distribution analysis. No amplification of *11β-H* transcript was detected in female larva during 45, 55, 75 and 90 dph however from 150 dph there was faint expression in the ovarian samples (Fig. 9).

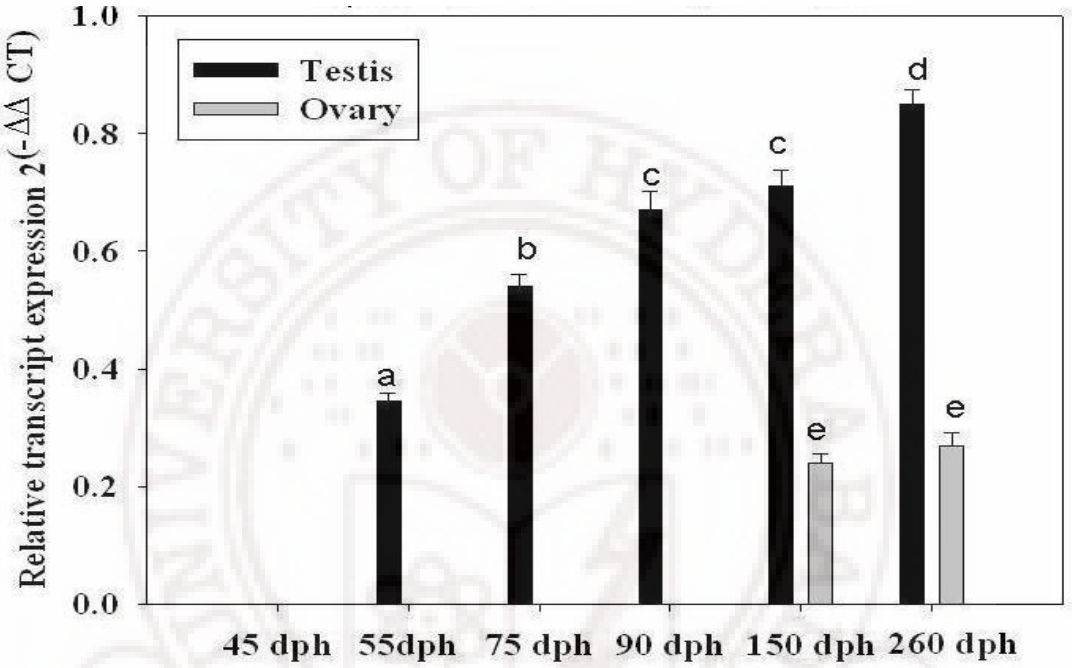



***Figure 9. Real-time quantitative RT-PCR analysis of temporal expression pattern of catfish 11β-H in larvae at 45, 55, 75, 90, 150 and 260 days post hatch (dph). X-axis represents larval age from the time catfish hatched. Data from real-time PCR were expressed as mean ± SEM. Significance between groups were tested by ANOVA followed by Tukey-Kramer's test using Graph pad prism 5 software. Means with different alphabets differ significantly (P<0.05). No detection was seen in 45 dph.***

**Discussion**

In the present study, we tried to investigate the role of *11β-H* in testis formation and spermatogenesis using *Clarias gariepinus* as experimental model. To accomplish this, a partial cDNA encoding *11β-H* was isolated from catfish testicular tissue. Catfish *11β-H* (partial cDNA sequence) showed 67-76% homology to *11β-H* cDNA sequences reported from other teleosts and 40-48% with *cyp11a1* (P450 cholesterol side chain cleavage

enzyme), *11β-H* and *cyp11b2* cDNA sequences from higher vertebrates. The close relationship of teleostean *11β-H* with *cyp11b2* is due to the evolution of *cyp11b2* from *11β-H* during tetraploidization event, when the ray finned fishes got diversified from the lobe-finned fishes. This is evident from the highly conserved characteristic regions of cytochrome P450 found in both the isoforms (Nonaka *et al*., 1995) except for the presence of two amino acids G288 and A320 in *cyp11b2*, which are essential for aldosterone synthase activity (Curnow *et al*., 1997). No report exists in teleosts that could confirm the presence of aldosterone in fish except for trace amounts in plasma and interrenal cells (Blair-West *et al*., 1977) in few fish species. Further report from the Japanese eel demonstrated the absence of aldosterone synthase activity by *11β-H* (Jiang *et al*., 1998), indicating, cortisol might serve as both mineralocorticoid and glucocorticoid hormone (Greenwood *et al*., 2003). All these findings suggest the teleost *11β-H* is involved mainly in hydroxylation at $C_{11}$ position of androgens (testosterone, androstenedione) and corticosteroids (cortisol).

The pattern of seasonal change in *11β-H* expression correlated well with catfish testicular cycle with relatively high transcripts levels in preparatory and prespawning phases followed by a decline in transcript levels as testis progresses to mature and spermiate. These results corroborates well with seasonal expression profile of *11β-H* obtained from the rainbow trout and sea bass (Liu *et al*., 2000; Kusakabe *et al*., 2002; Vinas and Piferrer, 2008). However in salmonids, plasma 11-KT levels and expression of steroidogenic enzyme genes leading to 11-KT production were low during early spermatogenesis and peaked at spermiation (Borg, 1994; Maugars and Schmitz, 2008). Irrespective of the observed discrepancy in the expression pattern of *11β-H*, in the circannual breeding fishes,

all fishes displayed an indispensable role of *11β–HSD2* in initiating spermatogenesis validated explicitly by Miura *et al.* (1991a) in the Japanese eel and also in our experimental model (Chapter 2).

Spatial expression pattern revealed abundant expression in testis, liver, kidney and gills and faint expression in brain, heart, and ovary. In agreement with our findings sea bass and zebrafish also displayed low expression in brain, rectum, oesophagus, and gills in addition to prominent expression in testis and head kidney which are sites actively involved in androgen and corticosteroid synthesis (Socorro *et al*., 2007; Wang and Orban, 2007). However no expression was detected in ovary of eel, rainbow trout and medaka (Jiang *et al*., 1996; Liu *et al*., 2000; Kusakabe *et al*., 2002; Yokota *et al*., 2005). On the other hand, in protogynous honeycomb grouper (Alam *et al*., 2005) and in protandrous anemone fish (Miura *et al*., 2008) *11β-H* was immunolocalized in thecal cells surrounding the follicles. Further, zebrafish ovary also reported relatively less expression of *11β-H* than testicular tissue (Wang and Orban, 2007).

Ontogeny study by real-time PCR, detected *11β-H* transcripts from 55 dph in male larvae after the critical period of sex differentiation (Raghuveer and Senthilkumaran, 2009) suggesting that 11-KT might not be required for testis formation in catfish. Nevertheless, expression of *11β-H* preceded the initiation of spermatogenesis which was found to be around 70 dph in *Clarias gariepinus* (Cavaco *et al*., 1997) indicating its role in testis growth and maintenance. Concomitant with our results, studies from zebrafish and the Nile tilapia (Wang and Orban, 2007; Ijiri *et al*., 2008) demonstrated that *11β-H* was not the primary inducer of testis differentiation but *dmrt1* and *amh,* factors originating from Sertoli cells were expressed before testis differentiation. In congruous to the findings in Nile

tilapia, in catfish also *dmrt1* was expressed during testicular differentiation (Raghuveer and Senthilkumaran, 2009). Contrary reports also exist from fish belonging to varying reproductive strata: gonochoristic, protogynous, protandry, bidirectional sex changing fish, that demonstrate pivotal role of 11-KT during testicular differentiation and early detection of *11β-H* expression (Liu *et al*., 2000; Blazquez *et al*., 2001; Alam *et al.,* 2006; Papadaki *et al*., 2005; Socorro *et al*., 2007). Furthermore in Nile tilapia there are equivocal reports where Baroiller *et al*. (1998) and D'cotta *et al*. (2001) have shown expression of *11β-H* during testicular differentiation but Ijiri *et al*. (2008) have reported its expression only after gonadal differentiation. So discrepancy prevails among teleosts on the role of endogenous 11-KT as inducer of testicular differentiation but there are no two opinions in the capacity of exogenous steroids in efficiently masculizing the female population to phenotypic/functional male on treatment with 11-oxo androgens (Baron *et al*., 2005; 2008) which is made feasible by down regulating *cyp19a1* expression but a parallel up-regulation of *11β-H* is not observed elucidating that testis development is only feasible if the male hormones can override the levels of estradiol-17β (Viziaano *et al*., 2007). The above ontogeny results and seasonal expression profiles indicates that *11β-H* is indeed involved in initiating the spermatogenic cycle and testis growth as it is one of the important enzymes involved in the synthesis of 11-KT. We further studied the sensitivity of this gene to gonadotropins that facilitate gonadal development in few fishes (Schulz and Miura, 2002). This was demonstrated by administering hCG intraperitoneally to catfish at two crucial phases, testicular recrudescence and quiescence. The choice of these two distinct phases (prespawning and resting) will make clear whether hCG, that induced expression of *11β-H* (Jiang *et al*., 1996), *11β-HSD2* (Miura *et al*., 1991b), and plasma 11-KT levels (Khan *et al*.,

1987) in salmonids could show similar stimulatory effect in catfish. Present study revealed hCG could efficiently enhance the transcript levels of *11β-H* only in the prespawning phase with no change in the resting phase. Earlier we showed (Chapter 2) similar observations with respect to the terminal steroidogenic enzyme gene, *11β-HSD2* involved in 11-KT synthesis. Although gonadotropins modulated the transcription of *11β-H* and *11β-HSD2* in prespawning phase testis, consisting primary spermatogonia (type B), spermatocytes and spermatids, its effect in stimulating the quiescent phase testis was inconsequential. Thus, fluctuating levels of gonadotropins (FSH→LH) are essential in advancing the testicular cycle and spermatogenesis (Penaranda *et al*., 2009) but it alone cannot trigger spermatogenesis in a otherwise inert testis prompting to speculate that other factors chiefly the age of fish (Chiba *et al*., 1997), the testicular phase (Miura *et al*., 1997) and photoperiod/season (Sakai *et al*., 1989) might also exert a significant role in testis growth and development.

In conclusion, a partial 11β-hydroxylase cDNA sequence that is homologous to *11β-H* was cloned from the testis of catfish. *11β-H* transcripts were found to be high during preparatory and prespawning phases of reproductive cycle, marking the onset of spermatogenesis. During hCG induced *in vivo* study, only the testis of prespawning phase responded positively while the testis of quiescent phase could not be activated. Ontogeny study revealed that *11β-H* is expressed after gonadogenesis indicating that it might not be the primary switch required for testis formation. Spatiotemporal expression pattern tend to suggest a role of *11β-H* in modulating 11-KT levels which in turn may entrain the testicular cycle. Further studies are required to propose an important role for *11β-H*.

**References**

Alam M.A., Bhandari R.K., Kobayashi Y., Nakamura S., Soyano K., Nakamura M., 2006. Changes in androgen-producing cell size and circulating 11-ketotestosterone level during female-male sex change of honeycomb grouper *Epinephelus merra*. Mol. Reprod. Dev., 73:206-214.

Baker M.E., 2004. Evolutionary analysis of 11β-hydroxysteroid dehydrogenase-type 1,-type 2, -type 3 and 17β-hydroxysteroid dehydrogenase-type 2 in fish. FEBS Letters, 574: 167–170.

Baroiller J.F., Guiguen Y., Iseki K., Fostier A., 1998. Physiological role of androgens on gonadal sex differentiation in two teleost fish, *Oncorhynchus mykiss* and *Oreochromis niloticus*. J. Exp. Zool., 281:506–507.

Baroiller J.F., Guiguen Y., 2001. Endocrine and environmental aspects of sex differentiation in gonochoristic fish. Exs., 91: 177–201.

Baron D., Houlgatte R., Fostier A., Guiguen Y., 2005. Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol. Reprod., 73: 959–966.

Baron D., Houlgatte R., Fostier A., Guiguen Y., 2008. Expression profiling of candidate genes during ovary-to-testis trans-differentiation in rainbow trout masculinized by androgens. Gen. Comp. Endocrinol., 156:369–378.

Blair-West J.R., Coghlan J.P., Denton D.A., Gibson A.P., Oddie C.J., Sawyer W.H., Scoggins B.A., 1977. Plasma renin activity and blood corticosteroids in the Australian lungfish *Neoceratodus forsteri*. J. Endocrinol., 74: 137–142.

Blázquez M., Felip A., Zanuy S., Carrillo M., Piferrer F., 2001. Critical period of androgen-inducible sex differentiation in a teleost fish, the European sea bass. J. Fish Biol., 58:342–358.

Borg B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol., 109C: 219–245.

Cardwell G.R., Liley N.R., 1991. Hormonal control of sex and color change in the stoplight parrotfish, *Sparisoma viride*. Gen. Comp. Endocrinol., 81:7-20.

Cavaco J.E.B., Lambert J.G.D., Schulz R.W., Goos H.J.Th., 1997. Pubertal development of male African catfish, *Clarias gariepinus*. *In vitro* steroidogenesis by testis and interrenal tissue and plasma levels of sexual steroids. Fish Physiol. Biochem., 16: 129-138.

Cavaco J.E.B., Bogerd J., Goos H.J.Th., Schulz. R.W., 2001. Testosterone inhibits 11-ketotestosterone-induced spermatogenesis in African catfish (*Clarias gariepinus*). Biol. Reprod., 65:1807 - 1812.

Chiba H., Miura T., Nakamura M., Yamauchi K., 1997. Differentiation and development of Leydig cell, and induction of spermatogenesis during testicular differentiation in the Japanese eel, *Anguilla japonica*. Fish Physiol.Biochem., 16:187-195

Curnow K.M., Mulatero P., Emeric Blanchouin N., Aupetit Faisant B., Corvol P., Pascoe L., 1997. The amino acid substitutions Ser288Gly and Val320Ala convert the cortisol producing enzyme, CYP11B1, into an aldosterone producing enzyme. Nat. Struct. Biol., 4:32-35.

D'Cotta H., Fostier A., Guiguen Y., Govoroun M., Baroiller J.F., 2001. Search for genes involved in the temperature-induced gonadal sex differentiation in the tilapia, *Oreochromis niloticus*. J. Exp. Zool., 290:574-585.

Devlin R.H., Nagahama Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture, 208:191-364.

Govoroun M., McMeel O.M., D'Cotta H., Ricordel M.J., Smith T., Fostier A., Guiguen Y., 2001. Steroid enzyme gene expression during natural and androgen–induced gonadal differentiation in the rainbow trout *Oncorhynchus mykiss*. J. Exp. Zool., 290:558-566.

Greenwood A.K., Butler P.C., White R.B., De Marco U., Pearce D., Fernald R.D., 2003. Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns, and transcriptional activities. Endocrinology, 144: 4226-4236.

Ijiri S., Kaneko H., Kobayashi T., Wang D.S., Sakai F., Paul-Prasanth B., Nakamura M., Nagahama Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol. Reprod., 78:333-341.

Jiang J.Q., Kobayashi T., Ge W., Kobayashi H, Tanaka M., Okamoto M., Nonaka Y., Nagahama Y., 1996. Fish testicular 1lβ-hydroxylase: cDNA cloning and mRNA expression during spermatogenesis. FEBS Letters, 397:250-252.

Jiang J.Q., Young G., Kobayashi T., NagahamaY., 1998. Eel (*Anguilla japonica*) testis 11β-hydroxylase gene is expressed in interrenal tissue and its product lacks aldosterone synthesizing activity. Mol. Cell. Endocrinol., 146: 207-211.

Kusakabe M., Kobayashi T., Todo T., Lokman P.M., Nagahama Y., Young G., 2002. Molecular cloning and expression during spermatogenesis of a cDNA encoding testicular 11 β -hydroxylase (P45011β) in rainbow trout (*Oncorhynchus mykiss*). Mol. Reprod. Dev., 62:456-469.

Khan I.A., Lopez E., Leloup-Hâtey J., 1987. Induction of spermatogenesis and spermiation by a single injection of human chorionic gonadotropin in intact and hypophysectomized immature European eel (*Anguilla anguilla* L.). Gen. Comp. Endocrinol., 68:91-103.

Kobayashi M., Aida K., Stacey N.E., 1991. Induction of testis development by implantation of 11-ketotestosterone in female goldfish. Zool. Sci., 8: 389-393.

Liu S.J., Govoroun M., D'Cotta H., Ricordel M.J., Lareyre J.J., McMell O.M. Smith T., Nagahama Y., Guiguen Y., 2000. Expression of cytochrome P450 (11beta) (11beta-hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. J. Steroid Biochem. Mol. Biol., 75:291-298.

Liu J-F., Guiguen Y., Liu S-J., 2009.Aromatase (P450arom) and 11β-hydroxylase (P45011β) genes are differentially expressed during the sex change process of the protogynous rice field eel, *Monopterus albus*. Fish Physiol. Biochem., 35:511-518.

Maugars G., Schmitz M., 2008. Gene expression profiling during spermatogenesis in early maturing male Atlantic salmon parr testes. Gen. Comp. Endocrinol., 159:178-187.

Miura T., Yamauchi K., Nagahama Y., Takahashi H., 1991a. Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool. Sci., 8:63-73.

Miura T., Yamauchi K., Takahashi H., Nagahama Y., 1991b. Involvement of steroid hormones in gonadotropin-induced testicular maturation in male Japanese eel (*Anguilla japonica*). Biomed. Res., 12:241-248.

Miura T., Kawamura S., Miura C., Yamauchi K., 1997. Impaired spermatogenesis in the Japanese eel, *Anguilla japonica*: possibility of the existence of factors that regulate entry of germ cells into meiosis. Dev. Growth Differ., 39:685-691.

Miura S., Horiguchi R., Nakamura M., 2008. Immunohistochemical Evidence for 11β-hydroxylase (P45011β) and androgen production in the gonad during sex differentiation and in adults in the protandrous anemone fish *Amphiprion clarkii*. Zool. Sci., 25: 212-219.

Nonaka Y., Takemori H., Halder S.K., Sun T.J., Ohta M., Hatano O., Takakusu A., Okamoto M., 1995. Frog cytochrome P450 (11β, aldo), a single enzyme involved in the final steps of glucocorticoid and mineralocorticoid biosynthesis. Eur. J. Biochem., 229:249–256.

Ozaki Y., Higuchi M., Miura C., Yamaguchi S., Tozawa Y., Miura T., 2006. Role of 11β-hydroxysteroid dehydrogenase in fish spermatogenesis. Endocrinology, 147:5139-5146.

Penaranda, D.S., Perez, L., Gallego, V., Jover, M., Tveiten, H., Baloche, S., Dufour, S., Asturiano, J. F., 2009. Molecular and physiological study of the artificial maturation process in European eel males: From brain to testis. Gen. Comp. Endocrinol., doi:10.1016/j.ygcen.2009.08.006.

Pandian T.J., Sheela S.G., 1995. Hormonal induction of sex reversal in fish. Aquaculture, 138:1–22.

Papadaki M., Piferrer F., Zanuy S., Maingot E., Divanach P., Mylonas C.C., 2005. Growth, sex differentiation and gonad and plasma levels of sex steroids in male- and female-dominant populations of *Dicentrarchus labrax* obtained through repeated size grading. J. Fish Biol., 66: 938-956.

Raghuveer K., Garhwal R., Wang D.S., Bogerd J., Kirubagaran R., Rasheeda M.K., Sreenivasulu G., Bhattacharya N., Tharangini S., Nagahama Y., Senthilkumaran B., 2005. Effect of methyl testosterone and ethynyl estradiol–induced sex differentiation on catfish,

*Clarias gariepinus*: expression profiles of *dmrt1*, cytochrome P450 aromatase and 3β-hydroxysteroid dehydrogenase. Fish Physiol. Biochem., 31: 143-147.

Raghuveer K., Senthilkumaran B., 2009. Identification of multiple dmrt1s in catfish: localization, dimorphic expression pattern, changes during testicular cycle and after methyltestosterone treatment. J. Mol. Endocrinol., 42:437-448.

Sakai N., Ueda H., Suzuki N., Nagahama Y., 1989. Steroid production by amago salmon (*Oncorhynchus rhodurus*) testes at different development stages. Gen Comp Endocrinol., 75:231-240.

Shankar D.S., Kulkarni R.S., 2000. Effects of cortisol on testis of freshwater fish *Notopterus notopterus* (Pallas). Indian J. Exp. Biol., 38:1227-1230.

Schulz R.W., Miura T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem., 26:43-56.

Schulz R.W., Liemburg M., Garcia-López Á., Dijk W., Bogerd J., 2008. Androgens modulate testicular androgen production in African catfish (*Clarias gariepinus*) depending on the stage of maturity and type of androgen. Gen. Comp. Endocrinol., 156:154-163.

Socorro S., Martins R.S., Deloffre L., Mylonas C.C., Canario A.V.M., 2007. A cDNA for European sea bass (*Dicentrachus labrax*) 11β-hydroxylase: gene expression during the thermosensitive period and gonadogenesis. Gen. Comp. Endocrinol., 150:164-173.

Viñas J., Piferrer F., 2008. Stage-specific gene expression during fish spermatogenesis as determined by laser-capture microdissection and quantitative-PCR in sea bass (*Dicentrarchus labrax*) gonads. Biol. Reprod., 79:738-747.

Vizziano D., Randuineau G., Baron D., Cauty C., Guiguen Y., 2007. Characterization of early molecular sex differentiation in rainbow trout, *Oncorhynchus mykiss*. Dev. Dyn., 236: 2198-2206.

Wang G-M., Ge R-S., Latif S.A., Morris D.J., Hardy M.P., 2002. Expression of 11β-hydroxylase in rat Leydig cells. Endocrinology, 143:621-626.

Wang X.G., Orban L., 2007. Anti-Mullerian hormone and 11β-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. Dev. Dyn., 236:1329–1338.

Yokota H., Abe T., Nakai M., Murakami H., Eto C., Yakabe Y., 2005. Effects of 4-tert-pentylphenol on the gene expression of P450 11beta-hydroxylase in the gonad of medaka (*Oryzias latipes*). Aquat. Toxicol., 71: 121–132.

# *Chapter 4*

## Thiourea-induced alterations in the expression of some steroidogenic enzymes in air-breathing catfish *Clarias gariepinus*

## Chapter 4: Thiourea-induced effect on gonadal recrudescence


### Abstract

Previous study from our laboratory on thiourea-induced thyroid hormone depletion in mature male demonstrated that thyroid hormones play a significant role in testicular function of catfish. In the present study, we aimed to analyze the changes in the expression pattern of several steroidogenic enzyme genes using semi quantitative RT-PCR after thyroid hormone depletion by thiourea in adult male and female catfish. There was a marked decrease in the *11-βH* expression in the testis while no changes were observed in kidney. A marked decrease in *11β-HSD2* transcript level in testis, liver and kidney were observed in the thiourea-treated males. The observed results corroborate our earlier findings on testicular regression after thyroid hormone depletion. In females, expression of *cyp19a1* increased in the experimental group when compared to control. No significant changes were observed in the transcript levels of 3β-hydroxysteroid dehydrogenase, cytochrome p450c17α enzyme, and 20β-hydroxysteroid dehydrogenase in both males and females. Thus, thyroid hormones might regulate expression of terminal steroidogenic enzyme genes and thereby reproduction in catfish.


### Introduction

Thyroid hormone plays a crucial role in embryogenesis, sex steroid metabolism and reproduction in mammalian and human system (Jannini *et al*., 1995; Krassas *et al*., 2004). In teleosts its role in metamorphosis, embryogenesis and vitellogenesis is well documented (Cyr and Eales, 1988; Weber *et al*., 1992; Liu and Chan, 2002; Reine and Leatherland, 2003). Previous reports demonstrated histological and biochemical changes in testis on

thyroid hormone depletion illustrating significant role of thyroxine in fish reproduction (Misra and Panday, 1985; Matta *et al*., 2002). Studies from our laboratory on thiourea-induced thyroid (hormone) depletion indicated alteration in hypothalamo-hypophyseal-testicular (HHT) axis by depleting cfGnRH immunoreactivity (ir) neurons in preoptic area *vis à vis* on LH ir cells in the pituitary (Swapna *et al*., 2006). In the same study, we also suggested that HHT axis alteration is one of the causative factors for the reduction in tissue and serum levels of testosterone and 11-ketotestosterone (11-KT). Thus, it is plausible that thyroxine exerts a direct or indirect effect on HHT axis by modulating sex steroid levels. To understand this at molecular level, we intended to analyze the effect of thyroid hormone depletion on the expression pattern of some steroidogenic enzyme genes involved in production of sex steroids.

**Materials and Methods**

Adult (recrudescence) air-breathing male and female catfishes (200-250g) were purchased from local fish markets of Hyderabad, India. They were acclimatized for 3 weeks by maintaining in aquarium tanks filled with filtered tap water under natural photoperiod and ambient temperature (26 ± 2°C) and fed with minced goat liver *ad libitum* during acclimation and experimentation. Thyroid hormone depletion was induced by adding thiourea (SRL, Mumbai, India) to a final concentration of 0.03% in well-aerated aquarium as per the methodology described in detail by Swapna *et al.* (2006). For a period of 21 days, group I, control fishes (n=10), were maintained in filtered water whereas group II, male fishes (n=5) and group III, female fishes (n=5) were maintained in filtered water

containing thiourea. Water in control and treated tanks were replenished daily along with thiourea for the treated group alone. After 21 days of treatment, fish from both the groups were weighed and sacrificed. Serum was obtained by centrifugation at 1500g, lyophilized and stored briefly in -80°C ultra low freezer for the estimation of 3, 3, 5'- triiodothyronine ($T_3$) levels. $T_3$ levels in control and thiourea-treated fish were measured as per the method described by Swapna *et al*. (2006). Testes, liver and kidney tissues from male catfish and ovarian tissue from female catfishes were collected for isolation of total RNA using Tri-reagent. Total RNA from respective tissues were reverse transcribed to cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA) and oligod$T_{18}$ primers following the manufacturer's instructions. To clone partial cDNA fragments a set of degenerate primers for each steroidogenic enzyme genes were designed from conserved region by aligning the existing sequences (GenBank database) of teleost 11β-hydroxylase (*11β-H*), 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2)*, 3β-hydroxysteroid dehydrogenase (*3β-HSD*), cytochrome p450c17α enzyme (*cyp17*), 20β-hydroxysteroid dehydrogenase (*20β-HSD)* and ovarian aromatase (*cyp19a1)* using Lasergene software, (release 3.05; DNASTAR, Madison, WI, USA). *11β-H, 11β-HSD2, 3β-HSD, cyp17* and *20β-HSD* partial cDNA fragments were amplified from testis first stand cDNA templates. Similarly, *3β-HSD*, *cyp19a1*, *cyp17 20β-HSD*, partial cDNA fragments were amplified from ovarian first stand cDNA templates. Following are the degenerate primers for *11β-H*, Fw: 5' CCT NGG SCC CAT WTA CAG GSA G 3', Rv: 5' GTC GTG TCC ACY SCY CCB GCC AT3', *11β-HSD2*, Fw1:5'GCG GTS YTC ATC ACM GGY TGT GA 3', Rv1:5' CCA AAG

AAR TTS ACY TCC ATR CA3', Rv2:5'GCT GCY TTS GAG GYY CCA TA3', *cyp19a1*, Fw: 5' TGG WYK GGN ATH GGB ACD GC3', Rv1:5'GGV CCD GTB RVG CTT TRG 3'. The sequences of degenerate primers for *3β-HSD*, *CYP17*, *20β-HSD* were reported by Raghuveer *et al*. (2005) and Sreenivasulu *et al*. (2005). PCR was performed at 94°C (7 min), 55-60°C (1.5min), 72°C (1.5min), for 30 cycles and 72°C final extension for 10 min using Gene AMP PCR system 9700 (Applied Biosystems, USA) thermal cycler. The efficiency of the transcription was checked by performing a PCR for *β-actin* a constitutively expressed gene. All the partial cDNA fragments were cloned in pGEMT-easy vector (Promega, Madison, WI, USA) and the sequence was determined. The identity of amplified partial cDNA was analyzed by NCBI-BLAST. Gene specific primers were designed for *11β-H*, *11β-HSD2*, *cyp19a1*, *3β-HSD*, *CYP17* and *20β-HSD* from the sequences obtained from partial cDNA fragments. Semi-quantitative RT-PCR was opted to observe the relative expression pattern of the above mentioned steroidogenic enzyme genes in liver, kidney, testis and ovary by following the method of Kwon *et al*., (2001). The PCR conditions [94°C (1 min), 50-60°C (1 min), 72°C (1 min), for 30 cycles and final extension at 72°C for 10 min] were standardized for all the templates and their expression patterns studied. PCR product of each gene obtained from specific primers were cloned in pGEM-T Easy vector (Promega) and the amplicon size and identity confirmed by sequencing.

## Results

### Thiourea induced thyroid hormone depletion during testicular recrudescence

The $T_3$ level in the serum of thiourea treated male fish and control were 0.29 ± 0.02 and 0.69 ± 0.01 ng/ml, respectively. On the other hand, $T_3$ levels in the serum of thiourea treated female fish and control were 0.21 ± 0.02 and 0.57 ± 0.03 ng/ml, respectively. The RT-PCR performed using specific primers amplified *11β-H, 11β-HSD2* and *cyp19a1* products of 212, 296, and 262 bp that showed homology to previously reported medaka, the Nile tilapia and channel catfish, respectively. The expression of *11β-H* declined in the thiourea treated males both in the liver and testis after 21 days of treatment. There was no significant change in *11β-H* in the head kidney. The expression of *11β-HSD2* decreased in testis, liver and kidney following thyroid hormone depletion by thiourea. In adult female fishes, thiourea treatment resulted in significant increase in *cyp19a1* transcript levels when compared to the control group. There was no significant change in the transcript levels of, *3β-HSD, CYP17*, *20β-HSD* in both males and females.

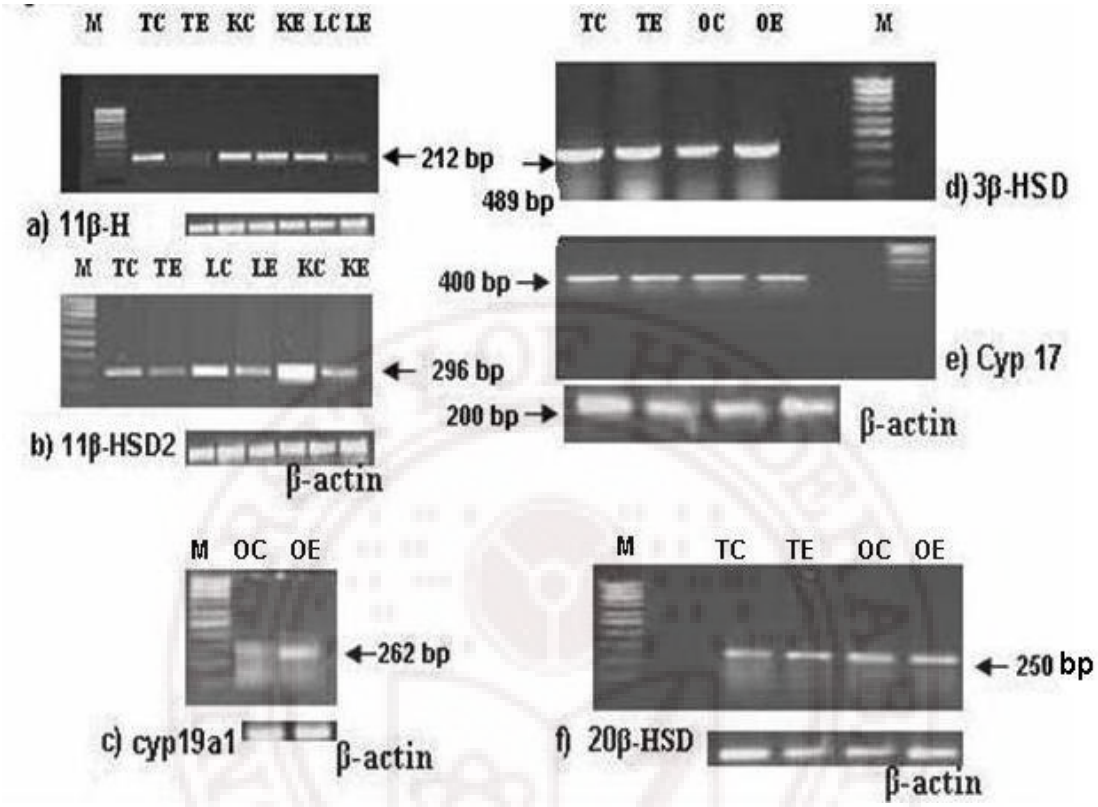



**Fig.1:** ***RT-PCR analysis (representative gel) of the expression pattern of a) 11β-hydroxylase, b) 11β-hydroxysteroid dehydrogenase type 2, d) 3β-hydroxysteroid dehydrogenase, e) cytochrome p450c17α enzyme, and f) 20β-hydroxysteroid dehydrogenase in adult thiourea treated male. c) cyp19a1 in adult thiourea treated female. TC, control testis; TE, thiourea treated testis; KC, male control kidney; KE, male treated kidney; LC, male control liver; LE, male treated liver; OC, ovary control; OE, ovary treated.***

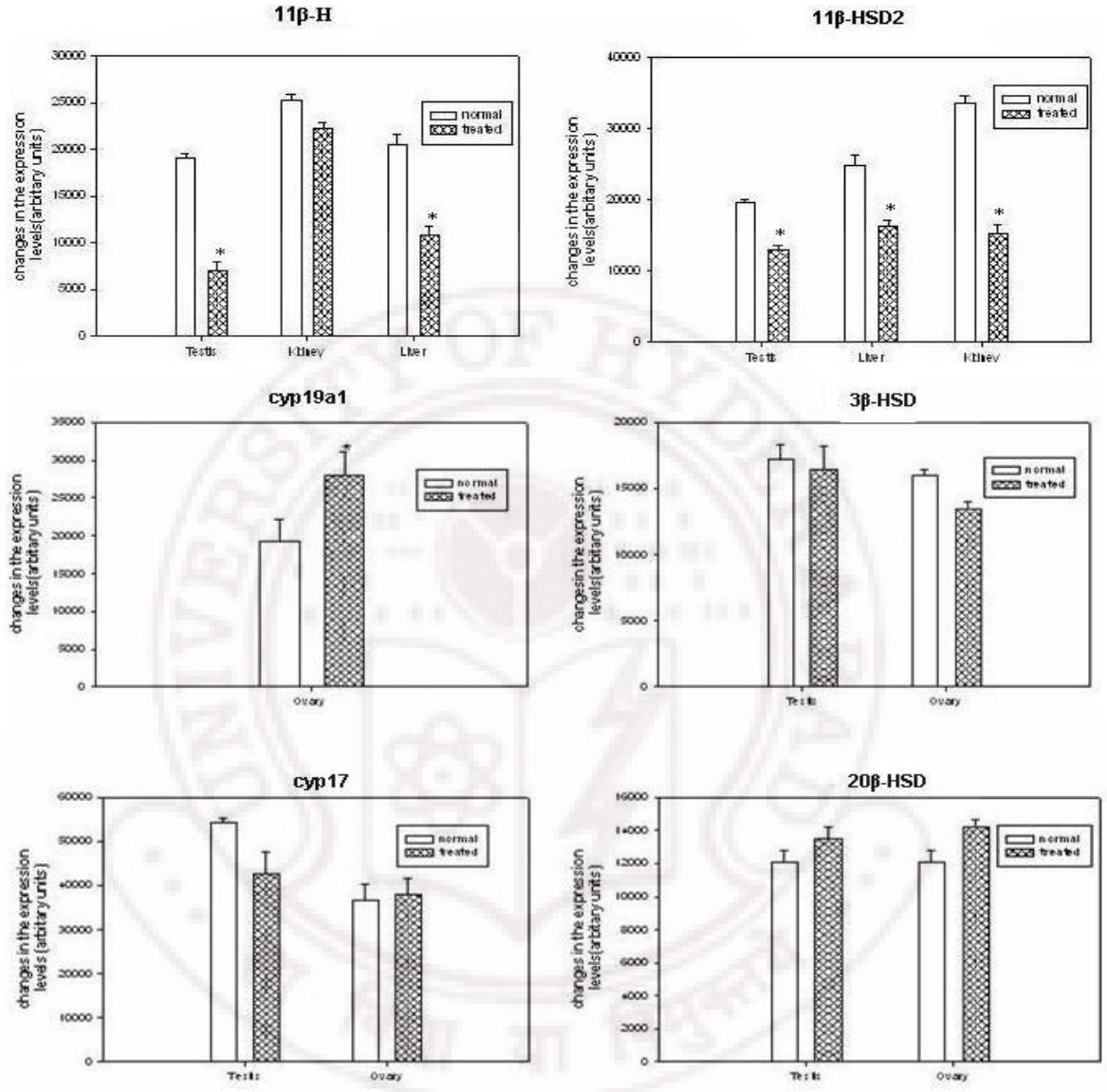



**Fig. 2:** ***Graphical representation of the changes in the expression pattern of 11β-hydroxylase, 11β-hydroxysteroid dehydrogenase type 2, 3β-hydroxysteroid dehydrogenase, cytochrome p450c17αenzyme, and 20β-hydroxysteroid dehydrogenase in adult thiourea treated male. cyp19a1, in adult thiourea treated female. Values are mean ± SEM, n=5. Statistical analysis was done by one way ANOVA followed by Students Newman- Keul's test using Sigma stat 3.5 software. Level of significance was set at P<0.05. * Significant over control.***

## Discussion

The significant decrease in $T_3$ level in the serum after thiourea treatment confirms the effective depletion of thyroid hormone by thiourea. Physiologically relevant androgen for the initiation of spermatogenesis in catfish is 11-KT and any change in its level alters testicular function (Cavaco *et al*., 1998). Previous report by Swapna *et al*. (2006) indicated that thiourea-induced thyroid hormone depletion leading to testicular regression (by reducing 11-KT) might be due to the alteration in transcript levels of *11β-H* and *11β-HSD2* in testis. In accordance to this, present study demonstrated a decline in the expression of aforementioned steroidogenic enzyme genes. Further, reduction in the transcript level of *11β-HSD2* in liver, the major site for 11-KT production (Cavaco *et al*., 1997) and in kidney by thyroid hormone depletion hampered spermatogenesis leading to testicular regression which may be attributed to the decrease in physiological concentration of 11-KT produced by these tissues. Histological results by Supriya *et al*. (2005) showed that there was not much impact of thyroid hormone depletion on the ovarian structure and function in adults though a considerable increase in the *cyp19a1* transcript levels was evident in the treated fish in the present investigation. The results obtained thus hint that the depletion of thyroid hormone either directly or indirectly modulates the steroidogenic enzyme expression in the gonad and peripheral tissues leading to drop in androgen level and testicular regression In females, it may have a variable role which require further studies (see Swapna and Senthilkumaran, 2007) at molecular level though physiological studies show modulation of ovarian function.

## References

Cavaco J.E.B., Vischer H.F., Lambert J.G.D., Goos H.J.Th., Schulz R.W., 1997. Mismatch between patterns of circulating and testicular androgens in adult African catfish, *Clarias gariepinus*. Fish Physiol. Biochem., 17, 155-162.

Cavaco J.E.B., Vilrokx C., Trudeau V.L., Schulz R.W., Goos H.J.Th, 1998 .Sex Steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). American J. Physiol. Reprod., 275: 1793-1802.

Cyr D.G., Eales J.G., 1988. Influence of thyroidal status on ovarian function in rainbow trout, *Salmo gairdneri*. J. Exp. Zool. 248:81-87.

Jannini E.A., Ulisse S., D'Armiento M., 1995. Thyroid hormone and male gonadal function. Endocr. Rev., 16: 443-459.

Krassas G.E. Pontikides N. 2004. Male reproductive function in relation with thyroid alterations. Clin. Endocrinol. Metab. 18:183-195

Kwon J.Y., Mc. Andrew B.J., Penman D.J., 2001. Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in gigantic male and female Nile Tilapia *Oreochromis niloticus*. Mol. Reprod. Dev., 59: 359-370.

Matta S.L.P., Vilela D.A.R., Godinho H.P., França L.R., 2002. The goitrogen 6-*n*-propyl-2-thiouracil (ptu) given during testis development increases sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model. Endocrinology, 143: 970-978.

Misra M., Panday K., 1985. Effect of thiourea on the testicular steroidogenesis and secondary sexual characters of a tropical freshwater fish, *Mystus vittatus* (Bloch). Annals Endocrinol., 46: 421-425.

Liu Y.W., Chan W.K., 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. Differentiation, 70: 36-45.

Raghuveer K., Rahul G., Wang D.S., Bogerd J., Kirubagaran R., Rasheeda M.K., Sreenivasulu G., Nupur B., Tharangini S., Nagahama Y., Senthilkumaran B., 2005. Effect of methyl testosterone and ethynyl estradiol-Induced sex differentiation on catfish, *Clarias gariepinus*: Expression profiles of *Dmrt1*, cytochrome P450 aromatases and 3β-hydroxysteroid dehydrogenase. Fish Physiol. Biochem., 31:143-147.

Reine J.C., Leatherland J.F., 2003. Trafficking of l-triiodothyronine between ovarian fluid and oocytes of rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem Physiol., 136B: 26-74.

Sreenivasulu G., Swapna I**.,** Rasheeda M.K., Ijiri S., Adachi S., Thangaraj K., Senthilkumaran B., 2005. Expression of 20β-hydroxysteroid dehydrogenase and P450 17α-hydroxylase/c17-20lyase during hCG-induced *in vitro* oocyte maturation in snake head murrel *Channa striatus*. Fish Physiol. Biochem., 31:227– 230.

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

Swapna I., Senthilkumaran B., 2007. Thyroid hormones modulate the hypothalamo-hypophyseal-gonadal axis in teleosts: Molecular insights. Fish Physiol. Biochem., 33:335-345.

Supriya A., Raghuveer K., Swapna I., Rasheeda M.K., Kobayashi T., Nagahama Y., Dutta-Gupta A., Majumdar K.C., Senthilkumaran B., 2005. Thyroid hormone modulation of ovarian recrudescence of air-breathing catfish, *Clarias gariepinus*. Fish Physiol. Biochem., 31:267-270.

Weber G.M., Okimoto D.K., Richman N.H., Garu E.G., 1992. Patterns of thyroxine and triiodothyronine in serum and follicle-bound oocytes of the tilapia, *Oreochromis mossambicus*, during oogenesis. Gen. Comp. Endocrinol., 85:392-404.

# *Consolidated summary*

Present study concentrated on studying the expression pattern of three important terminal enzymes (cytochrome P450 ovarian aromatase-*cyp19a1*, cytochrome P450 11β-hydroxylase-*11β-H* and 11β-hydroxysteroid dehydrogenase-*11β-HSD2)* during gonad differentiation, by real-time PCR which might be controlling the estrogen and androgen ratio during gonad formation and thereby assisting in initiating and establishing the differentiation of the bipotent gonads to develop either as male or female. Further present study also aimed to investigate the role of these steroidogenic enzymes during puberty and reproductive cycle in catfish by analyzing their expression and activity at various stages of gamete development and maturation. Additionally attempts were made to understand the interplay of steroidogenic enzyme genes and their modulators such as gonadotropins and thyroxine during various stages of gametogenesis. These aspects have been studied as four major chapters and the result and important findings are summarized below.

**Chapter 1: Molecular cloning, expression and enzyme activity of ovarian aromatase (*cyp19a1*) during ovarian development and oogenesis in air-breathing catfish *Clarias gariepinus* and *in vivo* hCG-modulation of *cyp19a1* during female reproductive cycle**

To investigate the specific role of cytochrome P450 ovarian aromatase *(cyp19a1*) during ovarian development and annual reproduction in air-breathing catfish *C. gariepinus* we initially cloned full length cDNA of *cyp19a1* from the catfish ovarian tissue containing 1551 bp of open reading frame (ORF), which displayed 79% homology with channel catfish *cyp19a1*. Characterization of the encoded protein in non–steroidogenic COS-7 cells illustrated that *cyp19a1* ORF could efficiently catalyze the aromatization reaction by producing estradiol-17β ($E_2$) from testosterone. Tissue distribution pattern revealed the

predominance of ovarian form in the ovary with trace amount being detected in other tissues including brain. The seasonal expression profile of *cyp19a1* measured using relative real-time PCR in connection with the different ovarian follicular stages revealed, high expression in the prespawning phase when compared to spawning, preparatory and regressed phases which corroborated with the measured serum $E_2$ levels. The enzymatic activity assessed by means of a sensitive radiometric assay in ovarian tissues collected during different phases recorded results consistent with the expression levels. Ontogeny results displayed sexual dimorphism, with relatively early expression of ovarian form in ovary than the testis clearly indicating its role in female sex differentiation. Further to understand the role of gonadotropins in modulating the *cyp19a1* transcripts and enzyme activity, we performed an *in vivo* study by injecting human chorionic gonadotropin (hCG) in adult catfish at three phases and observed phase-dependent stimulatory effect of hCG in the preparatory and prespawning phases. However, in spawning phase induction of transcripts was not sustained suggesting that the stage of oocyte during ovarian cycle was crucial and gonadotropins could not override the induction of *cyp19a1* transcripts once the follicles have undergone or undergoing meiotic maturation. These results demonstrates specific role of *cyp19a1* during ovarian differentiation by displaying dimorphic expression during the period of gonadal differentiation in catfish, and a distinct expression pattern and activity during ovarian cycle, indicating its involvement in maintaining the periodicity of the ovarian cycle by modulating the levels of $E_2$.

**Chapter 2: Cloning, expression and enzyme activity analysis of testicular 11β-hydroxysteroid dehydrogenase during seasonal cycle and after hCG induction in air-breathing catfish *Clarias gariepinus***

A full length cDNA encoding 11β-hydroxysteroid dehydrogenase type 2 (*11β-HSD2*) was cloned from testis of air-breathing catfish, *C. gariepinus* which showed high sequence homology to zebrafish and eel. *11β-HSD2* ORF was then transfected to COS-7 cells. The transfected cells converted 11β-hydroxytestosterone (11-OHT) to 11-ketotestosterone (11-KT) at a considerable rate than mock transfected cells. Tissue distribution analysis by RT-PCR revealed prominent expression in testis, anterior kidney and liver. Expression of *11β-HSD2* in the testes was assayed by real-time PCR during four testicular phases (preparatory, prespawning, spawning and resting phase) and was found to peak during the prespawning phase and gradually decline during the spawning and resting phases. With $NAD^+$, testicular microsomes oxidized 11-OHT with apparent $K_m$ of 56 ± 4 nM and $V_{max}$ of 55 ± 6 pmol/h/mg-protein, respectively. Seasonal *11β-HSD2* dehydrogenase activity in testicular tissues revealed highest production of 11-KT during the prespawning phase. Serum 11-KT levels corroborated well with the levels of transcripts and activity of 11β-HSD2. *In vivo* hCG administration enhanced *11β-HSD2* expression in the testis, especially during the prespawning phase, at 4, 8, 12 and 24h after induction. It also augmented 11-KT production by testicular microsomes at 8 and 24h. Ontogeny study indicated that this enzyme is expressed after the fate of gonad is determined. However, levels of *11β-HSD2* transcripts were significant during testicular differentiation. Thus the spatiotemporal expression results supported with dehydrogenase activity and circulating 11-KT. Based on these observations, present study clearly indicated a major role for *11β-HSD2* during testicular differentiation and seasonal testicular cycle in catfish.

**Chapter 3: Cloning and expression analysis of testicular 11β-hydroxylase during seasonal cycle and after hCG induction in air-breathing catfish *Clarias gariepinus***

Cytochrome P450 11β-hydroxylase (*11β-H)* gene is involved in production of 11β-OHT the precursor for the synthesis of 11-KT, a potent androgen for several male fishes. In the present study, a partial *11β-H* cDNA sequence (768bp) was cloned from air-breathing catfish *C. gariepinus* which shared high homology with zebrafish (76%) and rainbow trout (72%). Tissue distribution analysis revealed expression of *11β-H* in most of the tissues with predominance in testis, anterior kidney, liver and gills. Ontogeny study by real-time PCR from 45 days post hatch (dph) till 260 dph in male and female catfish larvae, detected the expression of *11β-H* from 55 dph suggesting that 11-KT might not be required during testis formation but subsequent increase in transcript levels clearly indicated a crucial role of *11β-H* in advancing testicular growth and development. Bleak expression was detected in female catfish larvae from 150 dph. Expression of *11β-H* correlated well with testicular recrudescence, displaying maximum expression in the prespawning phase. Administration of hCG elevated *11β-H* mRNA levels from 4h in the prespawning testis while priming was ineffective in the resting phase. These results tend to suggest that onset of spermatogenesis during testicular recrudescence is marked by corresponding increase in the mRNA levels of steroidogenic enzymes *11β-H* and *11β-HSD2* (Chapter 2).

**Chapter 4: Thiourea-induced alterations in the expression of some steroidogenic enzymes in air-breathing catfish *Clarias gariepinus***

Previous study from our laboratory on thiourea-induced thyroid hormone depletion in mature male demonstrated that thyroid hormones play a significant role in testicular function of catfish. In the present study, we aimed to analyze the changes in the expression pattern of several steroidogenic enzyme genes using semi quantitative RT-PCR after

thyroid hormone depletion by thiourea in adult male and female catfish. There was a marked decrease in the *11-βH* expression in the testis while no changes were observed in kidney. A marked decrease in *11β-HSD2* transcript level in testis, liver and kidney were observed in the thiourea-treated males. The observed results corroborate our earlier findings on testicular regression after thyroid hormone depletion. In females, expression of *cyp19a1* increased in the experimental group when compared to control. No significant changes were observed in the transcript levels of 3β-hydroxysteroid dehydrogenase, cytochrome p450c17α enzyme, and 20β-hydroxysteroid dehydrogenase in both males and females. Thus, thyroid hormones might regulate expression of terminal steroidogenic enzyme genes and thereby reproduction in catfish.

In conclusion, present study demonstrated crucial role of *cyp19a1* in catfish ovarian development and oogenesis, with early expression during ovarian differentiation and marked correlation in the seasonal expression, enzyme activity and plasma estradiol-17β levels during different follicular stages. On the other hand, *11β-H* and *11β-HSD2* genes were not detected at the time of testis formation but their levels increased with the onset of spermatogenesis. Further seasonal variations in 11*β-H* and *11β-HSD2* transcripts vis-à-vis levels of 11-KT during various phases of reproductive cycle attribute specific role for these enzymes in the entrainment of testicular cycle. Thyroid hormone depletion using thiourea and gonadotropin (hCG) exerted phase-dependent effects during gonadal recrudescence by modulating the expression of these steroidogenic enzyme genes.

# *Research Publications*

## From Ph. D thesis work:

1. **Rasheeda, M. K.**, Sreenivasulu, G., Swapna, I., Raghuveer, K., Wang, D. S., Thangaraj, K., Dutta-Gupta, A., Senthilkumaran, B. (2005). Thiourea-induced alterations in the expression of some steroidogenic enzymes in the air-breathing catfish, *Clarias gariepinus*. **Fish Physiol. Biochem. 31:275-279**.

2. **Rasheeda M. K.,** Kagawa, H., Kirubagaran, R., Dutts-Gupta, A., Senthilkumaran**,** B. (2010). Cloning, expression and enzyme activity analysis of testicular 11β-hydroxysteroid dehydrogenase during seasonal cycle and after hCG induction in air-breathing catfish *Clarias gariepinus* **(Revised manuscript submitted to J. Steroid Biochem. Mol. Biol.)**.

3. **Rasheeda M. K.,** Sridevi P., Senthilkumaran**,** B. (2010). Cytochrome P450 aromatases: Impact on gonad development, recrudescence and effect of hCG in catfish **(Manuscript under revision, Gen. Comp. Endocrinol.)**.

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

1. Swapna, I., Rajasekhar, M., Supriya, A., Raghuveer, K., Sreenivasulu G., **Rasheeda, M. K.**, Majumdar, K. C., Kagawa, H., Tanaka, H., Dutta-Gupta A., Senthilkumaran, B. (2006). Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus.* **Comp. Biochem. Physiol. 144A:1-10**.

2. Sreenivasulu, G., Swapna, I., **Rasheeda, M. K.**, Ijiri, S., Adachi, S., Thangaraj, K., Senthilkumaran, B. (2005). Expression of 20β-hydroxysteroid dehydrogenase and P450 17α-hydroxylase/c17-20lyase during hCG-induced *in vitro* oocyte maturation in snake head murrel *Channa striatus*. **Fish Physiol. Biochem. 31:227– 230**.

3. Swapna, I., Sreenivasulu, G., **Rasheeda, M. K.,** Thangaraj, K., Kirubagaran, R., Okuza, K., Kagawa, H. Senthilkumaran. B. (2005). Seabream GnRH: Partial cDNA cloning, localization and stage dependent expression in the brain and ovary of snake head murrel, *Channa striatus*. **Fish Physiol. Biochem. 31:157-161**.

4. Supriya, A., Raghuveer, K., Swapna, I., **Rasheeda, M. K.,** Kobayashi, T., Nagahama, Y., Dutta-Gupta, A., Majumdar, K.C., Senthilkumaran, B. (2005). Thyroid hormone modulation of ovarian recrudescence of air-breathing catfish, *Clarias gariepinus*. **Fish Physiol. Biochem. 31:267-270**.

5. Raghuveer, K., Rahul, G., Wang, D.S., Bogerd, J., Kirubagaran, R., **Rasheeda, M. K**., Sreenivasulu, G., Nupur, B., Tharangini, S., Nagahama, Y., Senthilkumaran, B. (2005). Effect of methyl testosterone and ethynyl estradiol-induced sex differentiation on catfish, *Clarias gariepinus*: Expression profiles of *Dmrt1*, cytochrome P450 aromatases and 3β-hydroxysteroid dehydrogenase. **Fish Physiol. Biochem. 31:143-147**.