Expression pattern of genes coding for steroidogenic enzymes involved in androgen production and promoter motif analysis of *11β-hydroxysteroid dehydrogenase* in catfish

A thesis submitted to University of Hyderabad for the award of the degree,

Doctor of Philosophy in

Animal Sciences

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April, 2015

Enrolment No.: 09LAPH01

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Hyderabad - 500 046, INDIA

DECLARATION

I, **Mr. A. Rajakumar** hereby declare that this thesis entitled **"Expression pattern of genes coding for steroidogenic enzymes involved in androgen production and promoter motif analysis of *11β-hydroxysteroid dehydrogenase* in catfish"** submitted by me under the guidance and supervision of **Prof. B. Senthilkumaran** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other University or Institute for the award of any degree or diploma.

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Signature of the Student

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CERTIFICATE

This is to certify that this thesis entitled **"Expression pattern of genes coding for steroidogenic enzymes involved in androgen production and promoter motif analysis of *11β-hydroxysteroid dehydrogenase* in catfish"** is a record of bonafide work done by **Mr. A. Rajakumar,** a research scholar for Ph.D. Animal Sciences program in the Department of Animal Biology, School of Life Sciences, University of Hyderabad under my guidance and supervision.

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

Signature of the Supervisor

Head
Department of Animal Biology

Dean
School of Life Sciences

Dedicated to

My GrandParents
&
Parents

Acknowledgements

- ✓ *I would like to acknowledge my heartfelt gratitude to my supervisor Prof. B. Senthilkumaran for his guidance, support, discussions and suggestions throughout the course of my work. I am very much thankful to him and Dr. C.C. Sudhakumari for all their encouragement and affection. I feel really privileged and fortunate to work under him.*
- ✓ *Special thanks to Prof. P. Reddana and Prof. Aparna Dutta-Gupta for their student friendly Ph. D. admission interview.*
- ✓ *I would like to thank my Doctoral Committee members, Prof. Aparna Dutta-Gupta and Dr. Bramanandam manavathi for their valuable suggestions.*
- ✓ *I thank the present Dean, Prof. P Reddanna and former Deans, Prof. R.P. Sharma, Prof. Aparna Dutta-Gupta, Prof. M. Ramanadham and Prof. A.S. Raghavendra for allowing me to use the school facilities.*
- ✓ *My sincere thanks to the present Head of Animal biology, Prof. B. Senthilkumaran and former Heads, Prof. Manjula Sritharan and Prof. S. Dayananda for providing good equipment facilities in the department.*
- ✓ *I owe my sincere thanks to Prof Aparna Dutta-Gupta for using her laboratory facilities and constant encouragement and support throughout my course.*
- ✓ *I would like to thank all the faculty members of School of Life Sciences for their help whenever needed.*
- ✓ *I thank all my teachers in the School GHST, VOC and My graduation, IGCAS and Pondicherry University.*
- ✓ *Special thanks to my teachers, Mr. Shanmugam (GHST), Mr. Balasubramanian (IGCAS), Dr. Suresh Yenugu (PU) and Prof. B. Senthilkumaran (UoH) for giving valuable basics and nuances on and off the subject at various times.*
- ✓ *I thank all my present and former collegues, Dr. G. Sreenivasulu, Dr. M.K, Rasheeda, Dr. K, Raghuveer, Dr. P. Sridevi, Dr. Swati Chakrabarty, Dr. S. Mamta, Ms. Y. Prathibha, Mr. R, Murugananthkumar,*

Ms. Laldinsangi, Mr. Yugantak Raj Gupta, Ms. S. Deepa, Dr. S. Durai, Dr. J. Varshini and Dr. Anshu Sharma for their help and keeping joyful environment in the laboratory.

- ✓ *I specially thank all my friends in Hyderabad and all the research scholars of SLS for their timely help.*
- ✓ *I also thank my chums, Mathavan, Shiva, Thiru, Dhana, Victor, Stephen, Janaki and Latchumikanthan for all the encouragement and joyful school days.*
- ✓ *I also thank my friends, Larance, Ganesh, Balaji, Chittu, Bobby, French, Sandeep, Jadeja, Avi, Joe, Shinde, Vijay, Ramachandran, Raj, Murugananth, Raghu, Maruthi, Venkatrao, Mohan, Thirupathi, Lakshun and other friends in GHST, VOC and IGCAS, PU, UoH for their help, all the fun and the lively days.*
- ✓ *I thank Mrs. Leena Bhashyam, Mrs. Monika Kannan and Mrs. Kalpana, for their help in Genomics and Proteomics facilities.*
- ✓ *Thanks to Mr. Rajendar, Mr. Mahesh and Mr. Pandu for maintaining the aquaculture facility.*
- ✓ *Thanks to all non-teaching staffs in the School, Department and Animal House for their help specially Mr. Ankineedu, Mrs. Jyoti, Mr. Murthy, Mr. Jagan, Mr. Gopi, Mr. Rangaswamy and Mr. Sreenu.*
- ✓ *I thank UoH for providing high speed internet connectivity and INFLIBNET.*
- ✓ *I thank Mr. Larry Page and Mr. Sergey Brin for providing best algorithms to search the scientific content with ease in seconds through Google and Google Scholar.*
- ✓ *I thank all the staffs in finance office and student service section for their help.*
- ✓ *I thank UoH (BBL) and CSIR (JRF &SRF) for giving me the financial assistance.*
- ✓ *I thank DST for providing Young scientist travel grant to attend JNS conference in Japan.*
- ✓ *I also thank DST, DBT, CSIR and UGC for funding our laboratory (Grants given to Prof. BS).*
- ✓ *DST-FIST, DST-PURSE, DBT-CREBB, UGC-SAP/CAS, UPE and University of Hyderabad funding to the Department and the School was also acknowledged.*
- ✓ *I specially thank my Grandparents, Parents, Sister, Brother and Uncle for their support.*

-Raj

Abbreviations

11β-h	11β-Hydroxylase
11β-hsd	11β-Hydroxysteroid dehydrogenase
17α,20β-DP	17α,20β-Dihydroxy-4-pregnen-3-one
17β-hsd	17β-Hydroxysteroid dehydrogenase
20β-hsd	20β-Hydroxysteroid dehydrogenase
Ad4BP/SF-1	Adrenal 4 binding protein/steroidogenic factor 1
cfGnRH	Catfish gonadotropin-releasing hormone
Cyp19a1a	Ovarian aromatase
Cyp19a1b	Brain aromatase
DAB	3,3′-Diaminobenzidine
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
Dmrt1	Doublesex and mab-3 related transcription factor 1
dph	Days post hatch
E_2	17β-Estradiol
EE_2	17α-Ethinylestradiol
EIA	Enzyme immune assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
IF	Immunofluorescence

IHC	Immunohistochemistry
IU	International Units
IVF	*In vitro* fertilization
L-15	Leibovitz -15
LH	Luteinizing hormone
MT	17α-Methyltestosterone
MS 222	Tricaine methanesulfonate
P450c17	Steroid 17α-hydroxylase/17,20 lyase
P450scc	Cholesterol side-chain cleavage enzyme (or)
Cyp11a1	Cytochrome P450, family 11, subfamily A, polypeptide 1
Poly(dI-dC)	Poly-deoxy-inosinic-deoxy-cytidylic acid
qPCR	Quantitative real-time PCR
RACE	Rapid Amplification of cDNA ends
Sox3	SRY related HMG-box3
Sry	Sex determining region Y
Star	Steroidogenic acute regulatory protein
T	Testosterone
TM-score	Template modeling score
Tph	Tryptophan hydroxylase
TR2/ nr2c1	Testicular receptor 2 /nuclear receptor subfamily 2, group C, member 1
TSI	Testicular somatic index
Wt1	Wilms' tumor suppressor 1

Contents

General Introduction

Reproductive success is vital for sustainability of a species and it is tightly regulated by sex-steroids, which exert variable effects based on the sex and stage of gonadal development and maturation. Sex-steroids regulate every aspect of reproduction in vertebrates including teleosts, which acts in coordination with several factors leading to the development and maturation of gametes with stringent regulation via hypothalamo-hypophyseal-gonadal (H-H-G) axis. Sex-steroids are produced mainly in the gonad and their production is regulated by several ways from gene expression changes to substrate limitation. As teleosts are evolutionarily diverse, there is a substantial variation in the steroidogenic pathway, but very little is known about their regulation both at mRNA and protein levels. Further, cellular and molecular processes underlying gonadal development, maturation and seasonal cycle, and the possible involvement of sex-steroids and transcription factors are worthwhile to study which will perhaps unravel various aspects of inter-relation between these processes. There are several factors like *doublesex and mab-3 related transcription factor 1* (*dmrt1*), *sry-related HMG-box 9* (*sox9*), *anti-mullerian hormone* (*amh*), *adrenal 4 binding protein/steroidogenic factor 1* (*Ad4BP/SF-1*), *wilms' tumor suppressor 1(wt1)* etc., are implicated in testicular differentiation and *ovarian aromatase* (*cyp19a1a*), *forkhead box protein L2* (*foxl2*), *wingless-type MMTV integration site family, member 4* (*wnt4*), *Ad4BP/SF-1* etc., were known to be involved in ovarian differentiation. Differential expression of certain steroidogenic enzyme genes during different stages of gonadal development, maturation and seasonal cycle results in the production of specific sex-steroids required for various physiological processes. The coordinated expression of many steroidogenic enzyme genes inculcate stringent control of sex-steroid biosynthesis needs to be explored in detail.

Sex determination and differentiation

Sex determination in lower vertebrates like fishes, exhibits broad variety of mechanisms based either on genotype and/or environmental factors (Fig. 1A & B). In vertebrates, sex differentiation occurs after sex determination, when a bipotential gonad develops into either testes or ovaries and the process is controlled by various factors/genes and hormones. The gonadal sex then regulates the phenotypic sex.

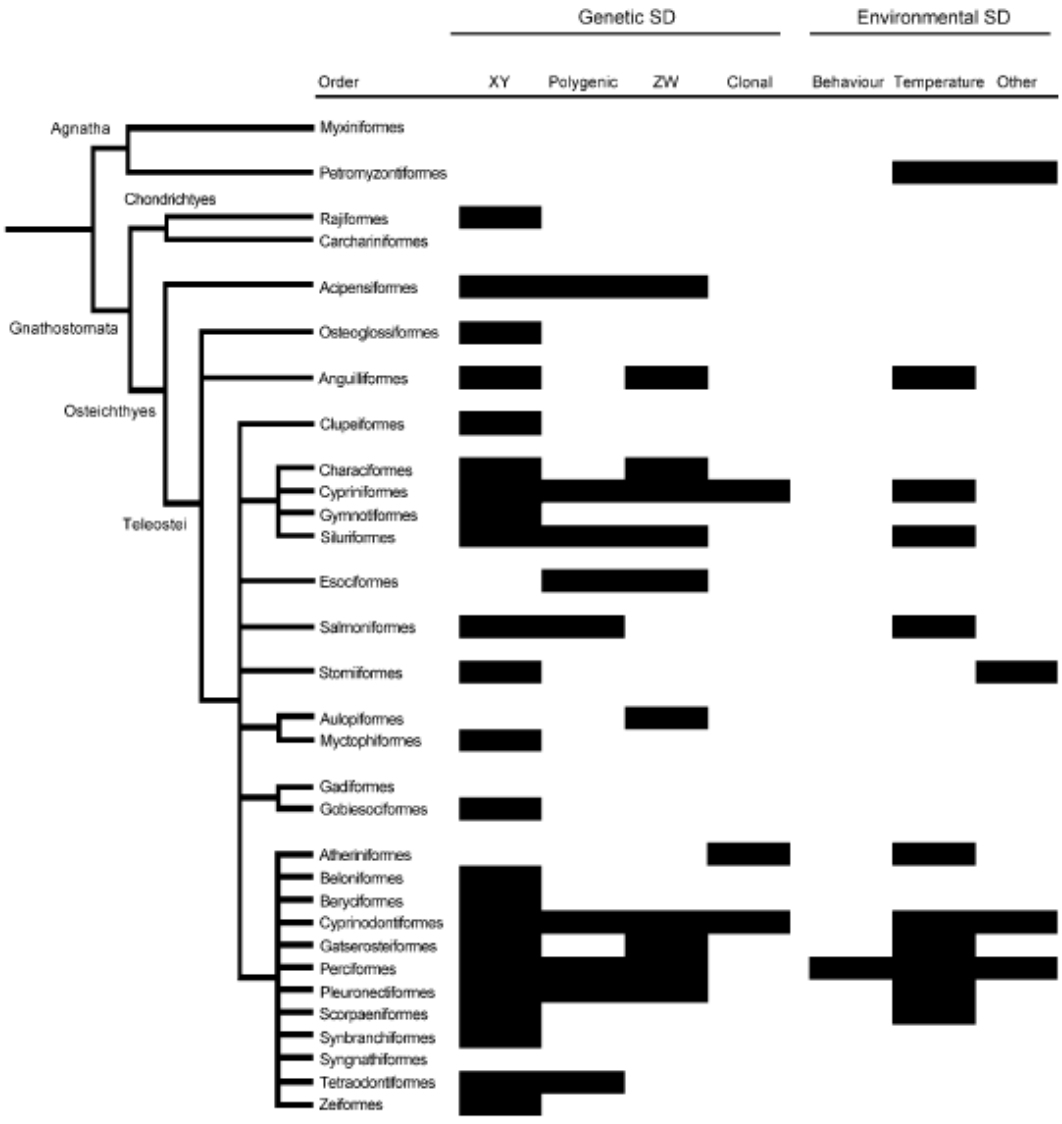


Fig. 1A. Summary of sex determination mechanisms utilized by fishes

Adapted from Devlin and Nagahama, 2002

In vertebrates, the phenotypic differences between both male and female occur as the result of differential expression of several genes during and after the development of testes or ovaries, whereas the sexual differentiation of the gonad is primarily triggered by a sex determining signal/gene (Takehana et al., 2014).

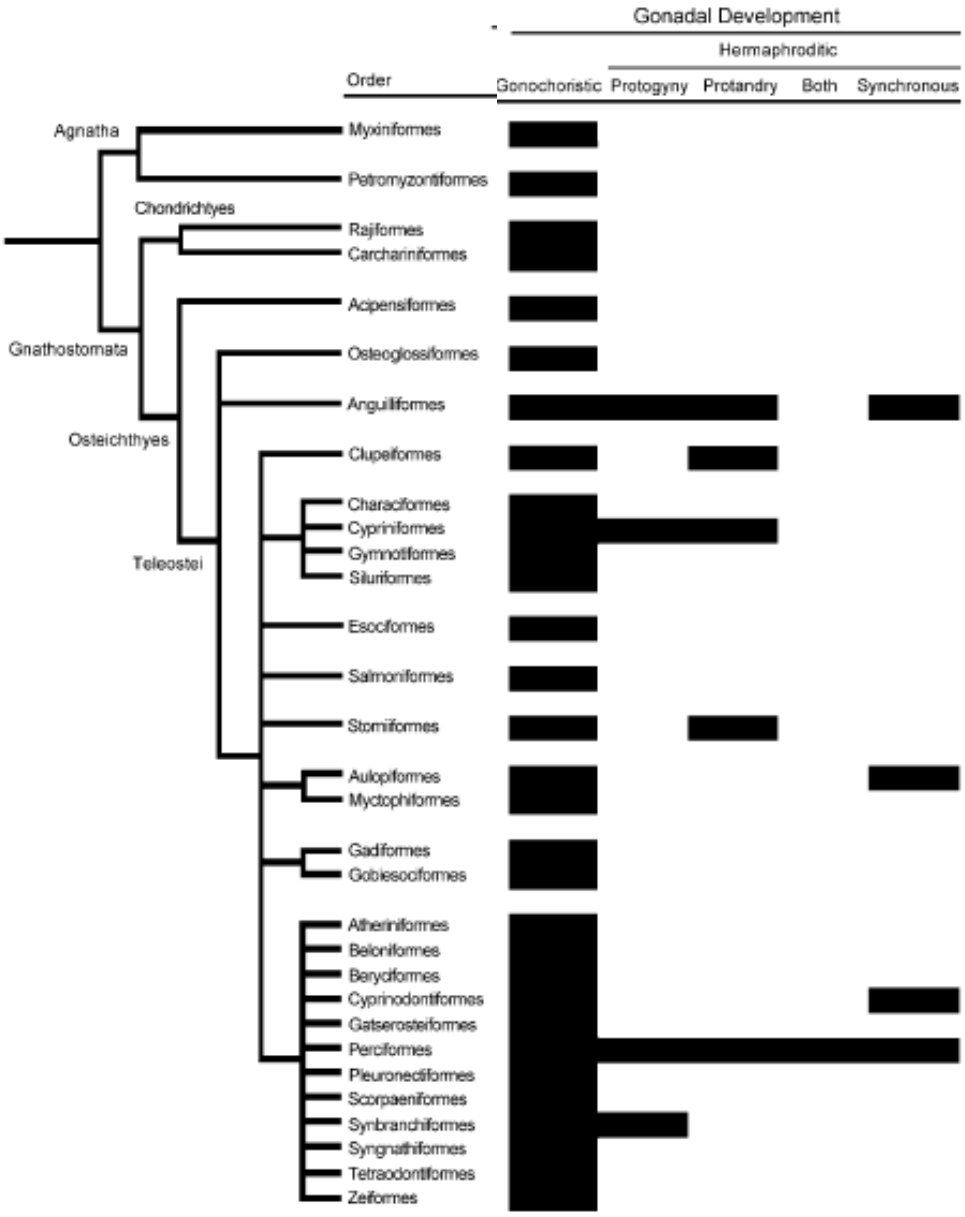


Fig. 1B. Summary of gonadal development and differentiation in fishes

Adapted from Devlin and Nagahama, 2002

The discovery of a gene that initiates testis determination in humans and other placental mammals (Sinclair et al., 1990) ignited the research on sex determination, and identification and evolution of sex determining genes. In mammals, gonadal development starts with formation of the bipotential gonad, which then differentiates into either testes or ovaries, which are dependent on the activation of either testis- or ovary-specific pathway (Fig. 2) while the opposite pathway is continuously repressed (Eggers et al., 2014).

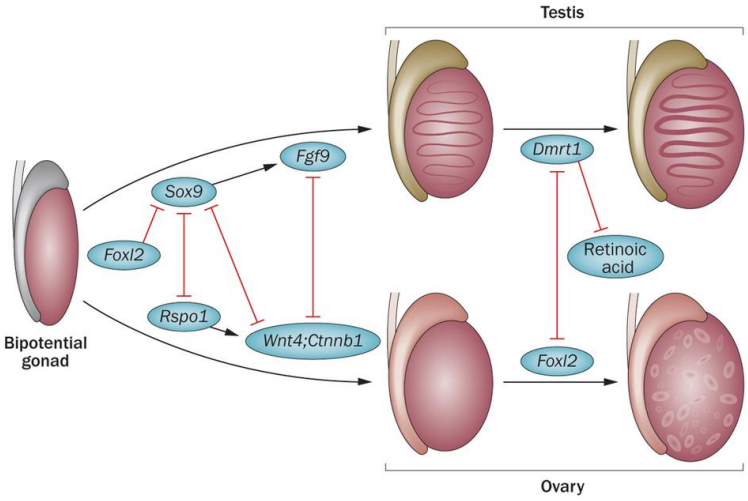


Fig. 2 Genes and pathways required for gonadal development and differentiation

Adapted from Eggers et al., 2014

A network of transcription factors (either for male or female or for both) tightly regulates the initiation and maintenance of these distinct pathways (Eggers et al., 2014). Sex determining region Y (Sry) is required and is sufficient to drive the testis-determining pathway (Koopman et al., 1991; Koopman, 1999) through the activation of *Sox9*, which is present, immediately downstream of *Sry*. All the factors involved in testicular differentiation and development from bipotential gonad is given below (Fig. 3). In contrast to the testicular pathway, no sex determining factor has been identified in the

ovarian pathway. However, several genes, such as *Foxl2*, *Rspo1*, *Ctnnb1*, and *Wnt4*, seem to work synergistically to ensure proper ovarian development (Eggers et al., 2014). In general, mammalian gonads are terminally developed into either testes or ovaries, while fish gonad often retains the ability to change the sex, making them semi-hermaphroditic. In teleosts, immature gonads can be directed to develop either into testes or ovaries, regardless of chromosomal background, after hormone treatments (Baroiller et al., 1999; Kobayashi et al., 2003).

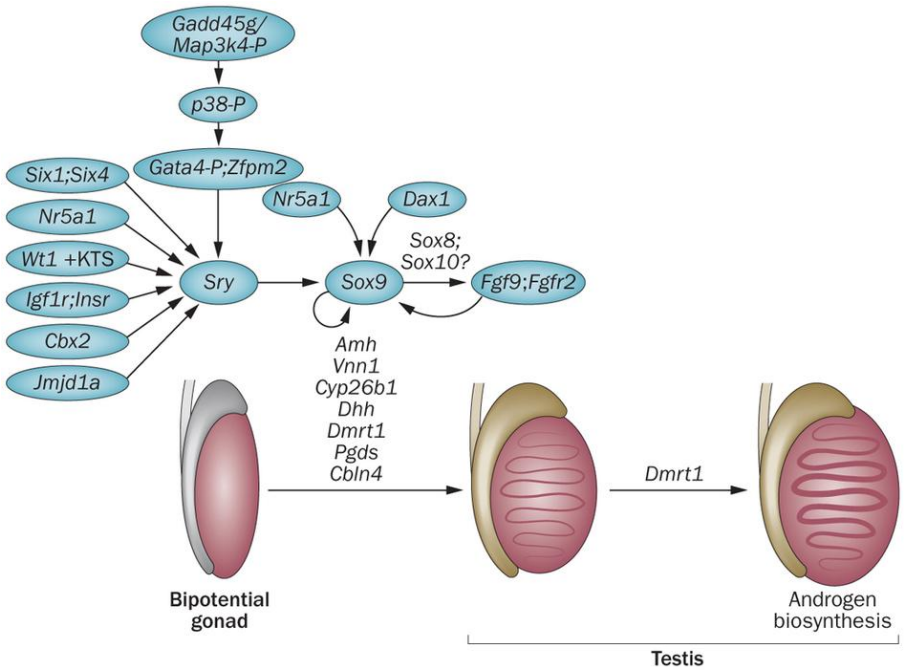
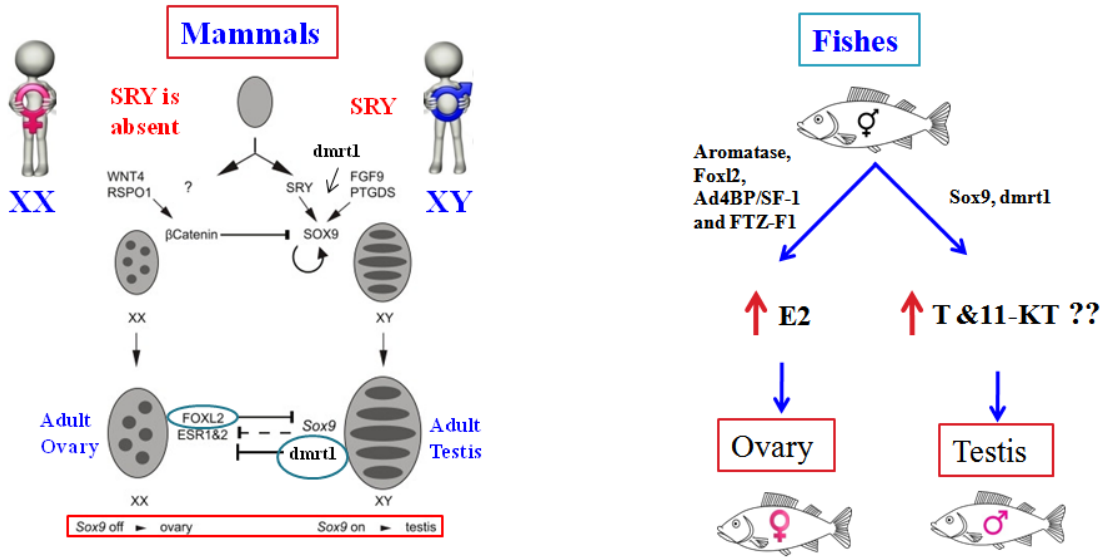


Fig. 3. Genes involved in the testicular development and differentiation

Adapted from Eggers et al., 2014

Contrary to the established fact that in mammals, the gonads developed (into testis or ovary) is final in adults, but recent studies have showed that, in females, the primary sex determining decision is not final. Loss of foxl2 in adult mouse granulosa cells can reprogram them into Sertoli cells (Uhlenhaut et al. 2009). Even in adult testis, sex is surprisingly labile. Loss of the *Dmrt1* in Sertoli cells activates *Foxl2* and reprograms

Sertoli cells into granulosa cells (Matson et al., 2011). These transdifferentiation resulted in male-to-female or female-to-male sex reversal in adult mice (Fig. 4). In the case of fishes, Foxl2 and Dmrt1 play antagonistic roles in sex differentiation via regulating *cyp19a1a* expression and estrogen production (Li et al., 2013). Understanding the evolution of new sex determination mechanisms requires the identification of novel, primary sex determining genes together with its molecular pathways.



Uhlenhaut et. al., 2009, **Cell** 139, 1130-1142
Matson et al., 2011, **Nature** 476, 101-104

Fig. 4. Gonadal development and differentiation in mammals and fishes

Sex chromosomes harbour a sex determining gene/signal that triggers sexual development of the organism. Conversely, different sex chromosome systems have known to be evolved in teleosts (Fig. 1A) and other vertebrates (Fig. 5). Though sex determining gene and possible determination mechanism were known in few fishes, but sexual plasticity exists, which makes it better model to study these processes and mechanisms involved in transdifferentiation. In fishes only very few species have a sex determining gene (Eg: dmy, gsdfy, amhr2), analogous to *sry* (Takehana et al., 2014).

Dmy is expressed in male somatic gonadal cells at the time of initial sex determination, and provides the first example of a sex determining gene in lower vertebrates (Matsuda et al., 2002). Sox9 is the direct target of Sry, which interacts with *Sox9* and up regulates its transcription in developing pre-Sertoli cells during male development (Kent et al. 1996). Two different forms of *Sox9* genes have been identified in teleosts (Klüver et al., 2005; Raghuveer and Senthilkumaran, 2010) and they were found to have arisen during the teleost-specific whole genome duplication events (Berthelot et al., 2014).

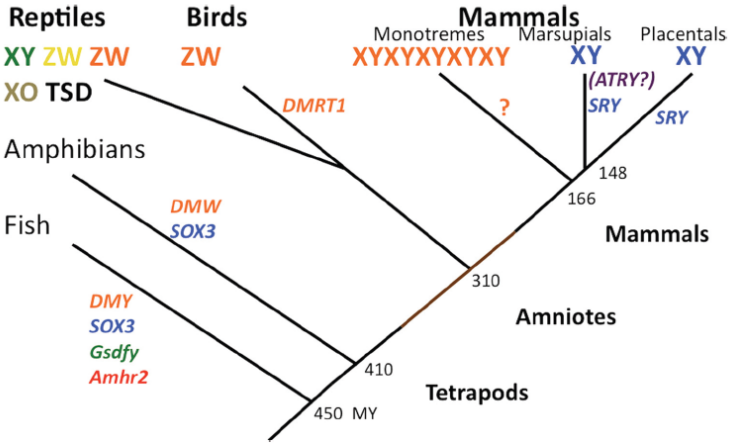


Fig. 5. Evolution of sex determining genes in vertebrates

Adapted from Graves, 2013

A recent study (Takehana et al., 2014) in *Oryzias dancena* (the Indian medaka) has unraveled the importance of Sox3 in sex determination. The locus on the Y chromosome of *O. dancena* contains a cis-regulatory element that upregulates the neighbouring Sox3 in the developing gonad. Further, sex-reversed phenotypes in $Sox3^{Y}$ transgenic fish and $Sox3^{Y}$ loss-of-function mutants all revealed the importance of Sox3 in sex determination (Takehana et al., 2014). In addition, Sox3 initiates testicular differentiation by upregulating the expression of Gsdf, which is highly conserved in fish and thus a novel sex determination mechanism was suggested to have evolved (Takehana et al., 2014).

Sex differentiation appears to be relatively conserved in several vertebrate species across the phyla except for Sry. There is no Sry in other non-mammalian vertebrates, or even in prototherian mammals like the platypus (Wallis et al., 2007). *Dmrt1*, *sox9*, *amh*, *Ad4BP/SF-1*, *wt1* etc., were shown to play different roles in testicular differentiation while, *cyp19a1a*, *foxl2*, *wnt4*, *Ad4BP/SF-1* etc., were involved in ovarian differentiation (Raghuveer et al., 2011). However in teleosts, changes in the expression pattern of these factors vary based on the sex-, stage- and timing during gonadal development, maturation and seasonal cycle cohesively, which are yet to be explored in detail. Dmrt1 belongs to DM domain gene family of putative transcription factor which is conserved across invertebrates to vertebrates. It is mostly expressed in testis of various vertebrate species implicating its role in testicular differentiation (Raymond et al., 1998). In the Nile tilapia, Dmrt1 suppresses the female pathway by repressing *cyp19a1a* transcription and estrogen production in the gonads (Wang et al., 2010). Ad4BP/SF-1 is a member of the NRA51 superfamily (Morohashi and Omura, 1996) and is a transcriptional activator of steroidogenic enzyme genes based on *in vitro* cell culture transcription assays and its *in vivo* expression profiles. It plays an important role in the transcriptional regulation of steroidogenic enzymes genes like *cytochrome P450 side-chain cleavage enzyme (P450scc/cyp11a1), cytochrome P450 17α-hydroxylase/c17-20 lyase (P450c17), 3β-hydroxysteroid dehydrogenase (3β-hsd)* and *cyp19a1a* (Hu et al., 2001; Parker et al., 2002). In zebrafish, *Ff1b* and *Ff1d* are functional homologues of *Ad4BP/SF-1*, which show higher levels of expression in the Leydig and Sertoli cells of testes (Chai and Chan, 2000). Fetal and adult testes express high levels of *Ad4BP/SF-1* in the testosterone (T) synthesizing Leydig cells and the non-steroidogenic, germline-supporting Sertoli cells. A

recent study (Buaas et al., 2012) revealed that Ad4BP/SF-1 is necessary for the steroidogenic gene program in fetal and adult Leydig cells, ovarian theca and stromal cells and in fetal adrenocortical cells. Moreover, it also controls cell morphology, signaling and the expression of non-steroidogenic markers in these cells.

In addition to the involvement of transcription factors, sex-steroids/sex-steroid analogues administered at the time of sex determination can strongly influence the course of sex differentiation in fishes. Thus, the role of sex-steroids and expression of steroidogenic enzyme genes are critical in assignment of gonadal determination as well as subsequent differentiation.

Steroidogenesis and Gonadal Differentiation

Steroidogenesis is a multi-step complex cascade of biosynthetic pathway which involves a number of enzymatic conversions and precursor intermediates which results in the formation of the sex-steroids (Leusch and MacLatchy, 2003). The processes occur in Leydig cells of testis and thecal and granulosa cells of the ovary which are dependent on the delivery of the substrate, cholesterol from the cytoplasm into the inner mitochondrial membrane with the help of a membrane transporter (Clark et al., 1994), steroid acute regulatory protein (StAR). Cyp11a1 hydroxylates carbons at 20^{th} and 22^{nd} positions, and removes a six-carbon residue side chain (C22-C27) of cholesterol and giving rise to pregnenolone, which is then the sole precursor for the synthesis of all the steroid hormones through a multi-step enzymatic process (Young et al., 2005). Movements of cholesterol and its subsequent conversion into pregnenolone are the presumed rate-limiting step of the steroidogenic pathway (Sugawara et al., 1997; Stocco, 2000). Schematic diagram of steroidogenesis pathway in the gonad of teleosts is given in fig. 6A

& B. T and 11-ketotestosterone (11-KT) are the potent androgens in males while, estradiol-17β (E_2) is an important estrogen in females (Borg, 1994). The hormonal balance between estrogens and androgens are crucial for sex differentiation in teleosts. Hence enzymes and transcription factors, which are involved either directly or through sex-steroid biosynthesis, are critical. In-depth analysis of these will provide vital cues in understanding tissue- and stage-specific expression and regulation of sex-steroids. In teleosts, sex-steroids influence the development of germ cells and other cell-types during the process of gonadal sex differentiation (Devlin and Nagahama, 2002). E_2 is considered to be responsible for inducing and maintaining ovarian development and growth. In general, in fishes, T is not directly involved in the mechanisms of differentiation, but participates as precursor for 11-KT and E_2 production (Nakamura et al., 1998; Baroiller et al., 1999). If steroids are critical for directing initial sex differentiation in fishes, rather than being a consequence of it, then the early expression of steroidogenic enzyme genes and sex specific differences in steroid production should be apparent prior to morphological differentiation of the gonad. Steroidogenic enzymes such as Cyp11a1, P450c17, 3β-hsd, Cyp19a1a, 17β-hydroxysteroid dehydrogenase (17β-hsd), 11β-hydroxylase (11β-h) and 11β-hydroxysteroid dehydrogenase (11β-hsd) were expressed much prior to sex differentiation in the Nile tilpia (Kobayashi et al., 1998; Tao et al., 2013) and catfish (Raghuveer et al., 2011; Chapter 1; 2; 3; 4). However, sex specific expression of steroidogenic enzyme and transcription factor genes together with its promoter level regulation were not clearly understood. In addition coordinated expression of several genes involved in steroidogenesis is critical for the production of sex-steroids. Further, in fish, both pituitary gonadotropins (GtH), follicle stimulating hormone (FSH)

and luteinizing hormone (LH) stimulate gonadal sex-steroid production directly by activating Leydig cells while, Sertoli cell function is predominantly regulated by FSH (Schulz et al., 2010).

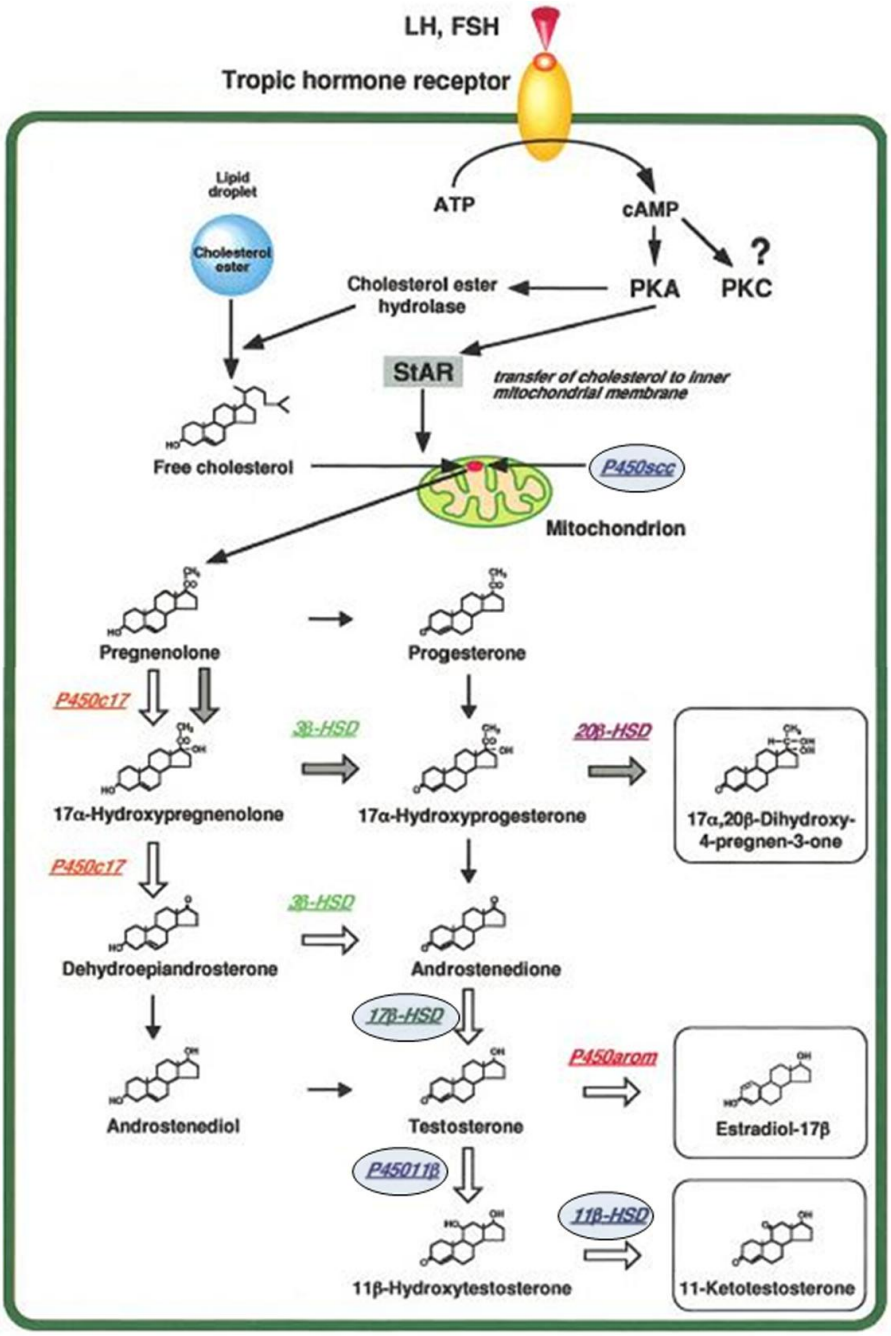


Fig. 6A. Steroidogenesis pathway in the gonad of teleosts

Adapted from Young et al., 2005

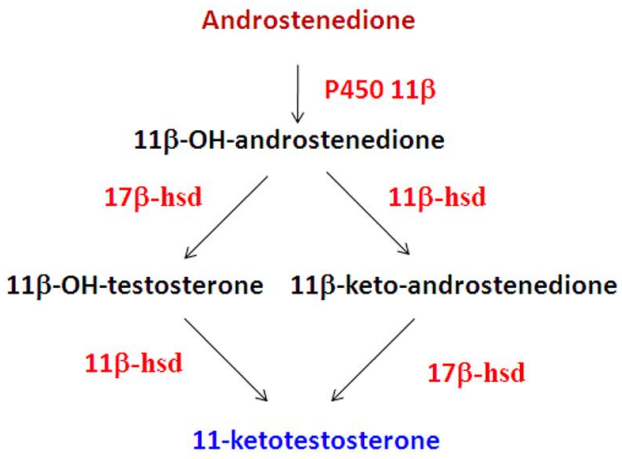


Fig. 6B. Other possible conversion of androstenedione into 11-KT in teleosts

In general, natural influence of sex-steroids more importantly estrogen has a pivotal role in ovarian differentiation while, such a phenomenon of sex-steroids regulating testicular differentiation is not evident. For example, Cyp19a1a is a key enzyme involved in the conversion of androgens to estrogens and plays a crucial role in ovarian differentiation and recrudescence (Simpson et al., 1994; Nagahama, 2005; Rasheeda et al., 2010).

Reproductive success of a species mainly depends on synchronized mechanisms including gonadal differentiation, development and gametogenesis with respect to favorable environmental season for the sustainability of young ones. In fish, sexual maturation is important as it signals the end of the growth phase of gonadal development. In most teleosts, a subsequent wave of germ-cell differentiation and growth (recrudescence) occurs for future spawning phases. The initiation of puberty in teleosts is characterized by the onset of spermatogenesis in males (Schulz and Miura, 2002) and vitellogenesis in females (Patino and Sullivan, 2002). In males, androgen production remains high throughout sexual maturation, even during the period of 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) synthesis in few teleosts (Mayer et al., 1990,

1992; Schulz et al., 1994; Chapter 2; 3), while in others, androgens were declined at sexual maturation where, 17α,20β-DP level increases (Miura et al., 1991; Liu et al., 2000; Scott et al., 2010). FSH receptors are found at all stages of spermatogenesis, but LH receptors are only detected on Leydig cells during the time of spermiation (Miwa et al., 1994). *In vitro* treatment of testis with GtHs at different stages also revealed that LH had a greater capability than FSH to stimulate 17α,20β-DP from late stage testis (Planas and Swanson, 1995). In sex changing fishes, the existence of synchronous hermaphrodites (both male and female germ tissues present) reveals that changing levels of sex-steroids might have limited role in the sex differentiation. Further, other local, paracrine influences, difference in sex-steroid perception and levels of glucocorticoids might also play significant roles. Hence it is important to study, other factors involved in these processes together with steroidogenesis.

Why fishes?

Teleost are excellent models to study sex determination, differentiation, maturation and steroidogenesis from evolutionary point of view, as they have several more gene homologues than the mammals and can exhibit different reproductive mechanism such as gonochorism, protandry, protogyny, true hermaphroditism, gynogenesis and androgenesis. Unlike mammals, in fishes cystic (semi-cystic in few fishes) spermatogenesis was seen, in which, group of Sertoli cells surrounding and nursing synchronously developing germ cell clones. Basic testicular architecture together with different cell types of spermatogenesis in comparison with mammalian testis is given below (Fig. 7).

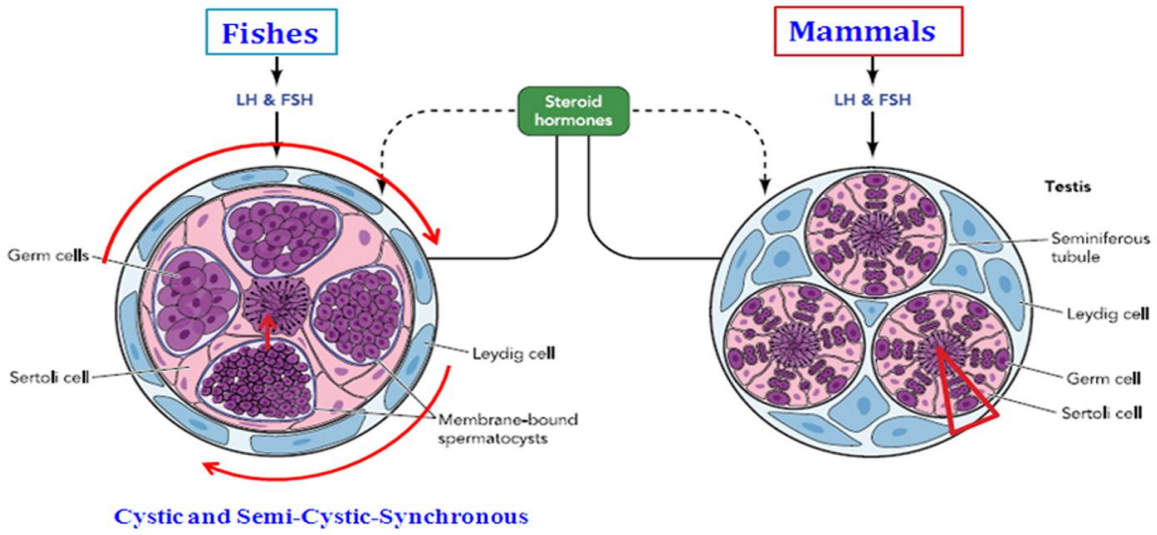


Fig. 7. Testicular organization and spermatogenesis in fishes in comparison with mammals

Adapted from Maruska and Fernald, 2011

In a given stage, germ cells in different stages of spermatogenesis (of different size) exist in testis (Schulz et al., 2010). Sertoli cells in cystic type might be more efficient in supporting germ cell development. Further, Sertoli cells retain their capacity to proliferate in adult fish (Schulz et al., 2005) unlike mammals. GtHs elicit a surge in the secretion of 11-KT by Leydig cells which stimulate Sertoli cells to produce several mediators such as activin B, insulin-like growth factor 1 (igf-1) and amh. An overview of endocrine mechanisms of teleostean spermatogenesis is given in fig. 8.

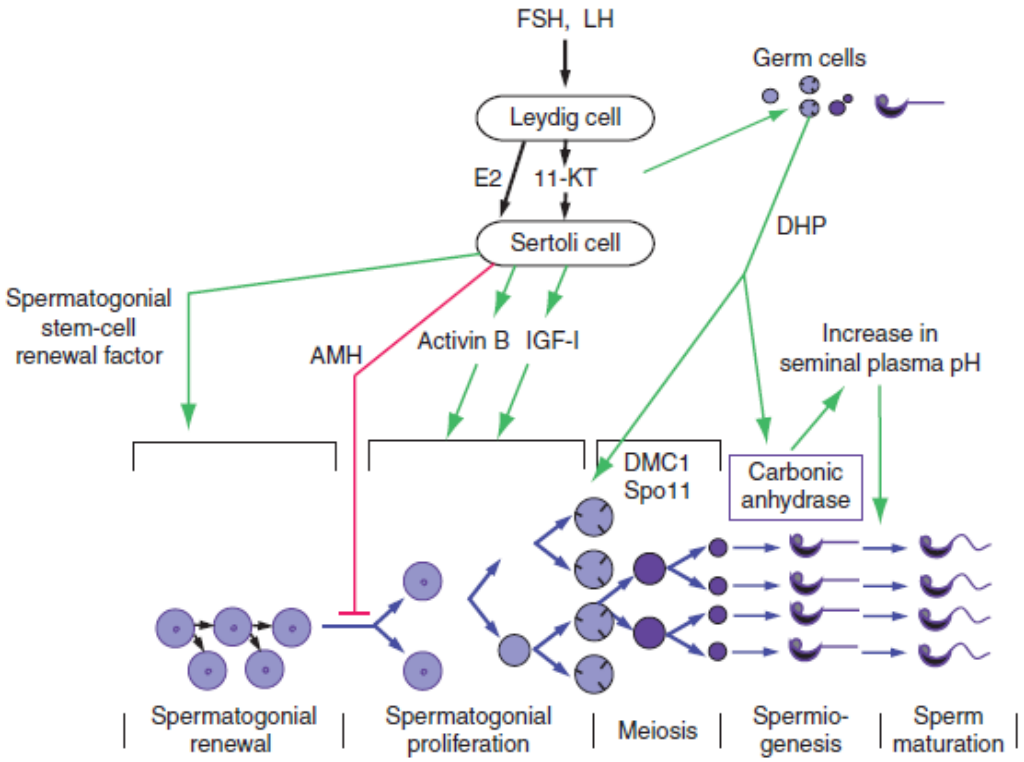


Fig. 8. Endocrine mechanisms regulating spermatogenesis in teleosts

Adapted from Yaron and Levavi-Sivan, 2011

Vastly different reproductive mechanisms in teleosts are explained based on the specific genome duplication events that occurred in the course of evolution (Fig.9A & B). Fishes are the most diversified group of vertebrates and comprise 33,000 known species (as per Fishbase ver. [02/2015]), which is more than the combined total of all other vertebrate species: mammals, amphibians, reptiles and birds. Increasing database of genomic information on characterized fish genes strongly suggests that many multi-gene families studied so far in fishes contains more functional members than in mammals (Wittbrodt et al., 1998). Though many fishes are diploid organisms like human, there are greater number of genes in fishes which possibly caused by additional ancient genome duplications that had happened in the lineage leading to modern ray-finned fishes (Eg: teleosts) but not along the lineage leading to tetrapods (Eg: mammals) (Fig.9A & B).

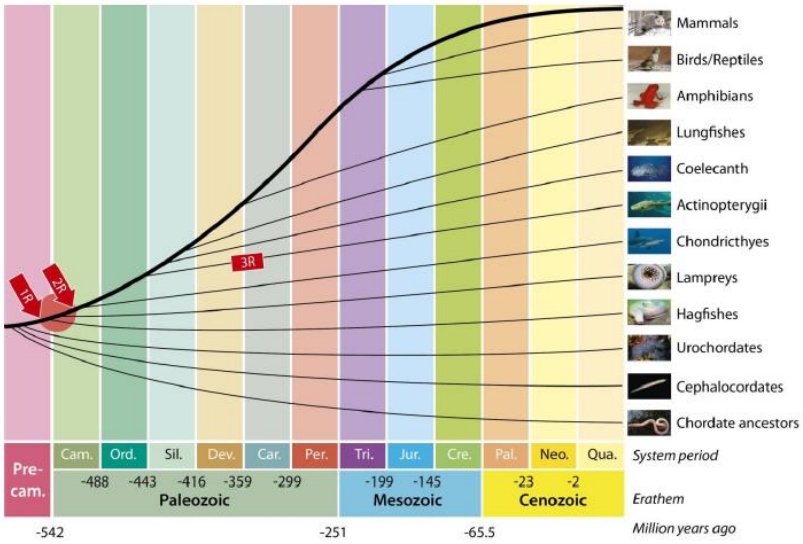


Fig. 9A. Evolution of vertebrates in context of the proposed whole genome duplications

Adapted from Larhammar et al., 2009.

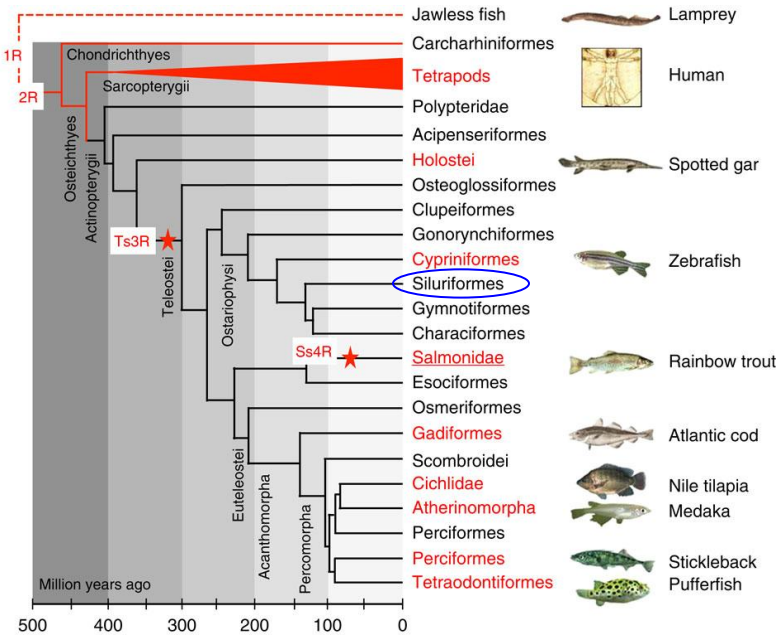


Fig. 9B. Evolution of fishes (Actinopterygii) in context of the proposed whole genome duplications **Adapted from Berthelot et al., 2014**

1, 2 and 3R are the suggested whole genome duplication events. 3R (Ts3R and Ss4R) is considered as a fish-specific genome duplication, thought to have provided genetic raw materials for the physiological, morphological, and behavioral diversification of the

fishes (Meyer and Van de Peer, 2005; Opazo et al., 2013). Approximately 30% of zebrafish genes are duplicates (Amores et al., 1998; Postlethwait et al., 1998). The enormous species diversity together with the presence of numerous gene duplicates makes fishes an excellent model system for studying gene evolution and function.

In addition to their species diversity and the presence of gene duplicates, fishes are more sensitive to sex-steroids and hence sex reversal studies are possible. Functional female-to-male sex reversal can be induced by exposure of juvenile (Baroiller and Guiguen, 2001). Androgen treatment is most effective in inducing masculinization and the most commonly employed androgen in sex-reversal studies is 17α-methyltestosterone (MT), while E_2 and 17α-ethinylestradiol (EE_2) is the most regularly used estrogens to induce feminization. The manipulation of sex differentiation in fish with exogenous steroids has been extensively reviewed (Devlin and Nagahama, 2002). Fishes are also becoming increasingly important indicators of environmental health, with respect to water body contamination by xeno- /sex-steroid mimics and habitat restriction.

Why catfishes?

Though studies are available on sexual development and basic steroidogenesis of daily breeder like zebrafish and seasonal breeder like rainbow trout, expression changes together with the involvement of steroidogeneic enzyme genes and transcription factor regulation were never analyzed cohesively in detail. Further, teleosts with seasonal reproductive cycle (Eg. Catfish) are ideal models for studying gonadal regulation, as gonadal maturation and regression occurs seasonally. Hence, to understand these processes in detail, the present study was conducted using catfish, *Clarias batrachus*, an annual breeder which might provide basic understanding and additional molecular details

for comparative analysis. Furthermore, unlike other fishes, Siluriformes (catfishes) have well developed seminal vesicles which support milt production. Catfish takes around a year to mature and undergo seasonal reproduction. Scientific classification and different phases of catfish reproductive cycle are given in Fig. 10.

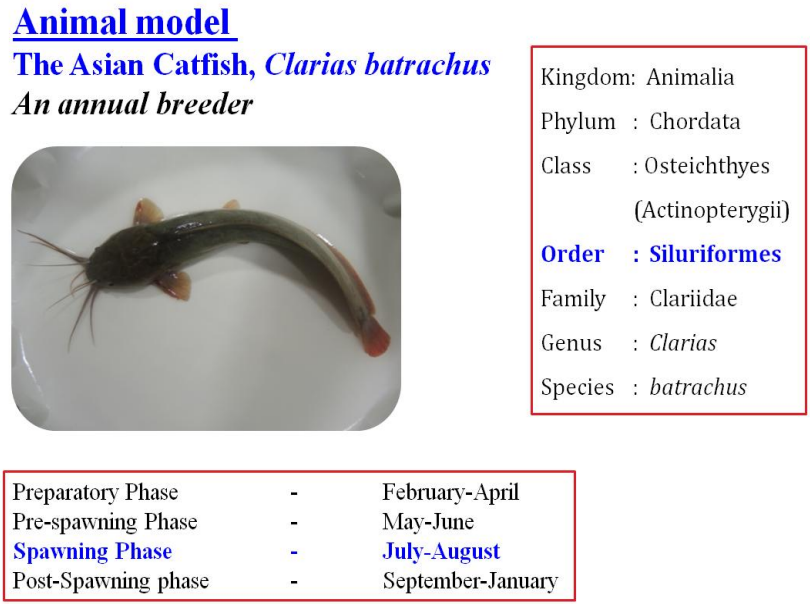


Fig. 10. Scientific classification and different phases of seasonal reproductive cycle of catfish

Catfishes respond efficiently to human chorionic gonadotropin (hCG) induction and it can produce thousand of eggs with hatchability rate of ~80%. hCG can bind to LH receptor and activate both adenylate cyclase and phospholipase c mediated pathways (Bogerd et al., 2001; Vischer et al., 2003; Choi and Smitz, 2014) and hence it is widely used in many hormonal induction studies including catfish. Morphological sex distinction is possible in catfishes by careful observation of genital papillae in juveniles and adults. During breeding season female can be differentiated easily by the increase in the abdomen size due to the increase in size of the ovary. *In vitro* fertilization (IVF) can be easily carried out during breeding season in catfish. Female fishes were stripped to

release eggs and male fishes were sacrificed for milt. As fishes have external fertilization, embryonic development can be monitored easily under microscope. Different stages of catfish embryonic development are given in the fig. 11.

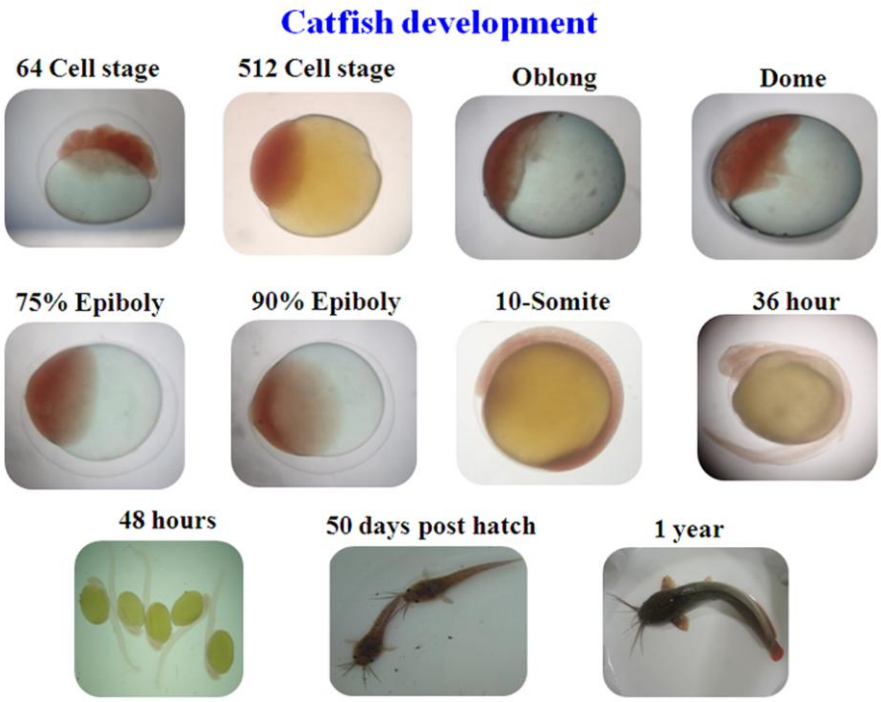


Fig. 11. Embryonic development in catfish

Juvenile catfish used for the present study were obtained by IVF. Juveniles caught from the wild might have encountered xeno-estro/-androgenic environments which might give variable results when sex differentiation and endocrinological studies were undertaken. Hence, IVF was carried out to obtain juvenile catfishes and were used for ontogenic and endocrine disruption studies in the present doctoral thesis.

In the backdrop of this existing information, the present thesis work is an effort to understand cohesively the implication of steroidogenic gene expression with the regulation by transcription factors during gonadal development, maturation and seasonal reproductive cycle in male teleosts. Relative to the advantage of a seasonal

breeder like catfish in understanding gonadal development, maturation and quiescence and further reactivation in breeding season, *C. batrachus* have been utilized for the current studies. The results in the seasonal breeder can be used for comparative analysis with daily breeders which might provide interesting insights. In addition, regulation of steroidogenic enzyme genes at the promoter level and their transcription factor control might provide multi -step, -process control at multiple levels, which might help in tissue- stage- and sex- specific expression. We also extended our study to understand the effect of sex-steroid analogues/pesticide on the expression of important genes during gonadal development in an idea to understand the changes in altered endocrine physiology. Each chapter has been carefully planned based on the specific objectives to understand the importance of steroidogenic enzyme genes involved in androgen production with reference to sexual development and maturation. The results of these studies were compiled into six chapters in this doctoral thesis.

Note: Abbreviations mentioned in page nos. vii and viii are followed in the thesis chapters

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Chapter 1

Expression analysis of *cyp11a1* during gonadal development, recrudescence and after hCG induction and sex-steroid analog treatment in catfish, *Clarias batrachus*

Abstract

Gonadal steroidogenesis is regulated primarily by the delivery of substrate, cholesterol into the inner mitochondrial membrane and its subsequent conversion to pregnenolone by Cyp11a1. In this study, full length cDNA of *cyp11a1* (2581 bp) was cloned from catfish testis to investigate the importance of Cyp11a1 by analyzing the expression of *cyp11a1* during gonadal development, seasonal reproductive cycle, after hCG induction and sex-steroid analog treatment. Phylogenetic analysis revealed that the Cyp11a1 is more conserved across teleosts. Tissue distribution analysis revealed that the *cyp11a1* expression was higher in the testis followed by the brain, head kidney, muscle and ovary compared to other tissues analyzed. High expression of *cyp11a1* in the head kidney and muscle revealed that Cyp11a1 could potentially regulate the extra-gonadal and/or circulating steroid levels in teleosts. Developing and mature testes showed higher expression of *cyp11a1* than the ovary of corresponding age group. Further, *cyp11a1* expression was higher during pre-spawning and spawning phases of testicular cycle and was found to be upregulated by hCG, *in vivo* and *in vitro*, which indicates the possible regulation by GtH. Exposure of MT (1 μg/l) and EE_2 (1 μg/l) for 21 days during catfish testicular development showed lower *cyp11a1* expression levels in the testis and brain indicating a certain feedback intervention. These results suggest possible role for Cyp11a1 in the testicular development and recrudescence.

1. Introduction

Cyp11a1 involved in the biosynthesis of pregnenolone, a sole precursor for all important steroids like E_2, T and 17α,20β-DP and corticosteroids. This enzyme is located on the

matrix side of the inner mitochondrial membrane and is a part of electron transport system (Chung et al., 1997). Movement of cholesterol and its further conversion to pregnenolone are the presumed rate-limiting step of the steroidogenic pathway (Stocco, 2000) and thus Cyp11a1 occupies a key regulatory part in the biosynthesis of steroids. One of the important factors which regulate the activity of Cyp11a1 is the availability of its substrate, cholesterol. Cholesterol is stored in the lipid droplet away from the mitochondria and hence it has to be transported to the inner mitochondrial membrane for steroidogenesis. This step is very slow and requires the facilitation of the star protein (Stocco and Clark, 1996). In general, sex-steroids act as inducers of gonadal differentiation and development in teleosts and hence regulation of steroidogenic enzyme genes like *cyp11a1* is very critical for steroidogenesis vis-à-vis gonadal development. Consistent with this, many steroidogenic enzyme genes were expressed prior to the gonadal differentiation in teleosts (Kobayashi et al., 1998; Nakamura et al., 1998; Nakamoto et al., 2010; Raghuveer et al., 2011). Further, in many species of teleosts, sex reversal can be induced by treatment with exogenous sex-steroids during critical period of gonadal differentiation (Nakamura et al., 1998; Sudhakumari and Senthilkumaran, 2013). Temporally, *cyp11a1* in zebrafish is expressed as two waves, first during embryogenesis and the second around the period of gonadal differentiation (Hsu et al., 2002). Expression of *cyp11a1* was detected from interstitial/Leydig cells of the testis and telencephalon and hypothalamus of the brain of zebrafish (Hsu et al., 2002). *Cyp11a1* is also expressed in the cytoplasm of oocytes, as a maternal transcript, and in yolk syncytial layer during early embryogenesis (Hsu et al., 2002; Hu et al., 2004). Cyp11a1 present in yolk syncytial layer facilitates pregnenolone synthesis, which is essential for microtubule

stabilization (Hsu et al., 2006). The transcription factor, Ad4BP/SF-1 binds to the *cyp11a1* promoter and plays an important role in the tissue-specific and hormonally regulated expression (Hu et al., 2004). Though few studies are available on cloning, characterization and localization of *cyp11a1* in few teleosts (Hsu et al., 2002; Hu et al., 2004; Kazeto et al., 2006), and ovarian cycle in channel catfish (Kumar et al., 2000), sex specific differential expression together with developmental and testicular cycle variations along with artificial GtH induction were never explored in an annually reproducing teleost. Such an attempt will provide important clues to understand recrudescence of testicular function to time reproductive events annually by initiating steroidogenesis. On this perspective, the present study was conducted using catfish, *C. batrachus* an annually breeding freshwater teleost, wherein, full length cDNA encoding Cyp11a1 was cloned and expression was analyzed during ontogeny, seasonal testicular cycle and after hCG induction and sex-steroid analog treatment.

2. Materials and methods

2.1. Animals, sampling and sex-steroid analogs exposure

The reproductive cycle of *C. batrachus* comprises of preparatory (February-April), pre-spawning (May-June), spawning (July-August) and resting/Post-spawning (September-January) phases (Joy et al., 2000). *C. batrachus* hatchlings at different age groups were obtained by IVF during breeding season (July-August) and reared in fresh water tanks under ambient photothermal conditions in the aquaculture facility at University of Hyderabad. Catfish juveniles were fed live tubeworms and commercially available feed pellets *ad libitum*. Sampling for ontogenic studies was done at different time points 0, 10,

20, 30, 40, 50, 100, 150, 200 and 250 days post hatch (dph) and adult. For 0 dph whole trunk was used for total RNA isolation while for 10, 20, 30 and 40 dph mesonephric gonadal complex was used. During gonadal development, the sex of the gonads was determined by histological examination of gonads which showed that the gonadal differentiation into testis and ovary occurs around 45-50 dph. Testes appear like a slender thread while ovaries appear as a transparent pouch devoid of sperm and oocytes, respectively. From 50 dph onwards, gonadal sex can be differentiated by morphological examination and hence, the testis and ovary were dissected out for total RNA isolation. Adult *C. batrachus* reared for two year in the outdoor tanks before it was sacrificed for experiments on the seasonal cycle and hCG (Pubergen, Uni-Sankyo Pvt., Ltd., India) induction *in vivo* and *in vitro*. For tissue distribution analysis, different tissues from both male and female catfishes (testis, ovary, brain, liver, head kidney, gills, intestine, muscle, heart and spleen) were collected during pre-spawning phase of catfish reproductive cycle. The preparatory phase testis contains predominantly spermatogonia and spermatocytes and spermatids. Pre-spawning phase testis contains more number of spermatids and lesser number of sperms, while the number of spermatogonia and spermatocytes were decreased than the testis of preparatory phase. Testis of spawning phase contains spermatids and sperms in high number, while spermatogonia and spermatocytes were negligible. Post-spawning/resting phase testis shows lots of empty lumens with relatively less number of spermatogonia and almost no sperm. For sex-steroid analog studies, juvenile catfish of 50 dph were divided into three groups of 50 larva each and maintained in aquarium tanks containing filtered water with or without treatment compounds of 1 µg/l of MT (97% purity; Wako, Wako Pure Chemical Industries Ltd., Chuo-Ku, Osaka, Japan) or 1 µg/l of

EE_2 (98% purity; Sigma, St. Louis, MO, USA). Filtered water containing treatment compounds were replenished daily. After 21 days of exposure, catfishes were sacrificed by anesthetizing with 100 mg/L of MS 222 (Sigma) in ice-cold water. All experiments and sacrifice procedures followed guidelines of institutional animal ethics committee. Both EE_2 and MT used in the study were first dissolved in absolute ethanol, air-dried and reconstituted in milliQ water. The dosages were chosen based on pilot studies which were comparable to other related studies done in our laboratory, as well as elsewhere (Orn et al., 2003; Pawlowski et al., 2004; Raghuveer et al., 2005).

2.2. Molecular cloning of full length *cyp11a1* cDNA from catfish gonads

In brief, total RNA was isolated from catfish gonads using Sigma TRI reagent, after their quality and quantity assessment using a NanoDrop spectrophotometer (ND-2000, NanoDrop Technologies and Wilmington, DE, USA), cDNA synthesis was carried out using 5 µg total RNA, oligo dT and SuperScript® III according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The quality of cDNA was checked by amplifying the internal control gene, *β-actin*. A set of degenerate primers (DF and DR/Table 1) for *cyp11a1* was designed based on the available nucleotide sequences of *cyp11a1* of vertebrates from the NCBI GenBank database. PCR amplification was performed at 94 °C (1 min) and 35 cycles of 94 °C (30 s), 53 °C (30 s), 72 °C (1 min) and 72 °C (10 min) using degenerate primers (Table 1). The partial cDNA fragment obtained was cloned into pGEM®-T easy vector (Promega, Madison,WI, USA) and sequenced bi-directionally. Rapid Amplification of cDNA ends (RACE) strategy was used to obtain the full length cDNA of *cyp11a1* by using the gene specific primers which were designed based on the sequence data of the partial cDNA. The 5′ and 3′RACE cDNA templates

were prepared by using SMARTer™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Touchdown PCR reactions were performed to obtain 5′ and 3′ ends by following manufacturer's universal thermal cycling conditions (Clontech). The obtained cDNA fragments were cloned into pGEM®-T easy vector (Promega) and sequenced bi-directionally using dye terminator cycle sequencing method in an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). High fidelity advantage® 2 polymerase (Clontech) was used for partial and full length cDNA amplifications in a 2720 thermal cycler (Applied Biosystems). After assembling the overlapping DNA fragments 2581 bp full length cDNA with open reading frame (ORF) of 1563 bp was obtained. ORF primers (Table 1) were designed and the cDNA cloned was reconfirmed by comparing with other *cyp11a1* of other teleosts using Lasergene software 7.1.0 (DNASTAR, Madison, WI, USA), NCBI-BLAST and ClustalW2 under default parameters.

2.3. Phylogenetic analysis

The phylogenetic tree of Cyp11a1 protein was constructed by ClustalW (http://clustalw.ddbj.nig.ac.jp/) using the neighbor-joining method with Gonnet protein weight matrix and a bootstrap analysis with 1000 replicates was used to assess the strength of the nodes in the tree. The deduced amino acid sequence of catfish *cyp11a1* together with other teleosts was used for phylogenetic tree construction. The phylogenetic tree was displayed using the TreeView software package version 1.6.6. The following are the GenBank accession numbers of Cyp11a1 sequences used for constructing the phylogenetic tree: *Danio rerio* (AF527755), *Gobiocypris rarus* (JN858106), *Fundulus heteroclitus* (AB471800), *Ictalurus punctatus* (NM_001200312), *Anguilla japonica*

(AY654741), *Oryzias latipes* (NM_001163086), *Odontesthes bonariensis* (GQ381266), *Oncorhynchus mykiss* (S57305), *Tautogolabrus adspersus* (GU596480) and *C. batrachus* (KF739411, this study).

2.4. Quantitative real-time PCR (qPCR)

Total RNA extraction was carried out using TRI-reagent® (Sigma) as per the manufacturer's protocol. Reverse transcription was performed using 1 µg of total RNA, random hexamers and verso® reverse transcriptase (Thermo Scientific Inc., Waltham, MA, USA). All qPCR primers were designed for the amplicon length of ~220 bp and all reactions were performed in triplicate for three different samples using gene specific primers (Table 1) and power SYBR® Green PCR master mix (Applied Biosystems) in a 7500 fast thermal cycler (Applied Biosystems) according to the manufacturer's universal thermal cycling conditions. Melting-curve analysis was performed to check the specificity of PCR amplification and all assays were performed with no template controls which yielded no amplification. Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification and *cyp11a1* expression was normalized against the expression of *18S rRNA* (GenBank accession no.: KM018296) to generate a ΔCt value (Ct of target gene-Ct of reference gene). The change in the gene expression was calculated by using $2^{-\Delta Ct}$ method. For sex-steroid analog studies, relative expression was calculated by using the comparative Ct ($2^{-\Delta\Delta Ct}$) method by taking the control as a calibrator.

Table 1. List of primers used for cDNA cloning and qPCR analysis

Sl.no.	Primer Name	Primer Sequence 5′ to 3′	Usage

1.	**cyp11a1 DF**	**ATGTTYAARWCYACCTSDCCYATG**	**RT-PCR**
2.	**cyp11a1 DR**	**AGGAAGAKYTGCATYTCNGTYTC**	**RT-PCR**
3.	**cyp11a1 5P**	**CTACACCTCCAGCCATCAGCTCAGTC**	**RACE**
4.	**cyp11a1 5N**	**ACATGGAAGGGGGGATGTACAGC**	**RACE**
5.	**cyp11a1 3P**	**GTATGCCATGGGCAGGGACTATCG**	**RACE**
6.	**cyp11a1 3N**	**ACGTCAATGCCTTGGGCGCAGA**	**RACE**
7.	**cyp11a1 ORFF**	**ATGTGCATGATGGTGGTGAG**	**ORF cloning**
8.	**cyp11a1 ORFR**	**TTACTGTTGTAAAGGCTTTAT**	**ORF cloning**
9.	**cyp11a1 RTF**	**TCAACCAAGCGGACCACTGT**	**qPCR**
10.	**cyp11a1 RTR**	**TCAGGATGCCGTGCCAACTC**	**qPCR**
11.	**18S rRNA F**	**GCTACCACATCCAAGGAAGGCAGC**	**qPCR**
12.	**18S rRNA R**	**CGGCTGCTGGCACCAGACTTG**	**qPCR**

IUPAC Nucleic acid codes: R- A or G; Y- C or T; S- G or C; W- A or T; K- G or T; M- A or C; B- C or G or T; D- A or G or T; H- A or C or T; V- A or C or G; N- any base

2.5. hCG induction, *in vivo* and *in vitro*

To understand the neuroendocrine control and seasonal effect of GtHs on the expression of steroidogenic enzyme genes, hCG (Pubergen) inductions were carried out both *in vivo* and *in vitro* during preparatory phase of the reproductive cycle. During the mid-preparatory phase (March), male catfishes weighing about 170-200 g, were collected and used of hCG induction experiments. For each time point (0, 6, 12, 18 and 24 h) 3 male (in total 30 fishes with saline controls) were maintained in the glass tank and acclimatized for a fortnight. Later, all catfishes were intraperitoneally injected a single dose (1000 IU/Kg body weight) of hCG. Fishes were sacrificed at end of the time points, by immersing them in ice-cold water with MS 222, to collect testis. The age matched control fishes were injected with fish physiological saline (0.6% NaCl W/V) and sacrificed for every time point similar to hCG groups. The testis samples were snap-frozen in liquid

nitrogen and stored at -80 °C until total RNA preparation. This procedure was repeated twice with different batches of fishes. Total RNA preparation, reverse transcription and qPCR analysis were performed using the methods described earlier, to analyze the changes in the expression pattern of *cyp11a1* in testis. For *in vitro* studies, testes from catfish during preparatory phase (March) were dissected out under sterile conditions and kept in ice-cold Leibovitz (L-15) culture medium (Sigma) and testicular slices of ~100 µm thickness were prepared using a McIlwain tissue chopper (Vibratome, Ted Pella Inc., Redding, California, USA). Testicular slices were then transferred carefully into tissue culture plates containing 2 ml of L-15 medium supplemented with 10 mM HEPES, 10% FBS and antibiotic (penicillin, 100 IU/ml, streptomycin, 0.1 mg/ml). Testicular slices were cultured up to 24 h at 22 °C in the presence of 100 IU/ml of hCG. At each time interval 0, 6, 12 and 24 h, slices were collected, washed with ice-cold phosphate buffer (pH 7.4) before proceeding with total RNA isolation. Fish physiological saline was added for control, and samples were collected corresponding to each time point similar to the hCG group. This *in vitro* procedure was repeated again with different batches of catfishes. Total RNA extraction, reverse transcription and qPCR analysis were performed as described earlier, to analyze the changes in the expression pattern of *cyp11a1* after hCG induction. The change in the gene expression was calculated by using $2^{-\Delta Ct}$ method.

2.6. Statistical analysis

All data were presented as mean of different samples with Standard Error of the Mean (SEM). All data passed homogeneity and normality tests which were compared by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls' (SNK) post-hoc test and all statistical analyses were performed using SigmaPlot 11.0 software (Systat

Software Inc., Chicago, IL, USA). A probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning of *cyp11a1* from catfish gonads

A partial cDNA of 704 bp of *cyp11a1* obtained was cloned from catfish testis. Based on the sequence data, gene specific RACE primers were designed and used for 5′ and 3′ RACE. The cDNA fragments obtained through 5′ (1062 bp) and 3′RACE (976 bp) were cloned, sequenced and confirmed by comparing with *cyp11a1* of other teleosts using Lasergene software 7.1.0 (DNASTAR), NCBI-BLAST and ClustalW. After assembling of all the cDNA fragments corresponding to *cyp11a1*, yielded 2581 bp of full length cDNA with ORF of 1563 bp, which were submitted to GenBank (KF739411). The *cyp11a1* ORF of 1563 bp were cloned from catfish testis, which was sequenced bi-directionally and confirmed by comparing with *cyp11a1* cDNA of other vertebrates. The 3′ untranslated region (UTR) of *cyp11a1* was 769 bp with a variant polyadenylation signal, TTTAAA and poly-A^+ tail.

3.2. Phylogenetic and sequence analyses

Multiple alignment of deduced amino acid sequence of catfish Cyp11a1 in comparison with other teleost counterparts revealed considerable homology with *I. punctatus* (92%) and *O. mykiss* (76%) than the rest analyzed. The deduced Cyp11a1 amino acid sequence in comparison with other teleosts shows high homology in steroid binding and heme binding domains (Fig. 7). The phylogenetic analysis of Cyp11a1 from different teleosts

revealed that Cyp11a1 of *C. batrachus* showed more homology with Cyp11a1 of *I. punctatus* and formed a separate clade (Fig. 1).

3.3. Tissue distribution of *cyp11a1* in different tissues of adult catfish

Expression of *cyp11a1* was higher in the testis followed by the brain, head kidney, muscle, ovary, intestine and spleen. The expression *cyp11a1* was negligible in other tissues analyzed (Fig. 2).

3.4. Expression of *cyp11a1* during gonadal ontogeny

qPCR analysis of *cyp11a1* at different stages of catfish gonadal development revealed that both male and female gonads express *cyp11a1* differentially ($P < 0.05$) throughout gonadal development (Fig. 3). The expression of *cyp11a1* was higher in males than in the females during gonadal development and at maturity (Fig. 3).

3.5. Expression of *cyp11a1* during different phases of testicular cycle

qPCR analysis revealed that the expression of *cyp11a1* was significantly higher ($P < 0.05$) during pre-spawning and spawning phases of testicular cycle than the rest of the phases analyzed (Fig. 4).

3.6. Expression of *cyp11a1* in the testis after hCG induction, *in vivo* and *in vitro*

The hCG induction of catfish revealed significantly enhanced *cyp11a1* expression in testis during the entire duration of the study when compared to 0 h. The *cyp11a1* expression increased significantly ($P < 0.05$) with maximum expression at 24 h after hCG induction *in vivo* (Fig. 5A), while the expression of *cyp11a1* was maximum at 12 h (Fig. 5B) *in vitro*.

3.7. Expression of *cyp11a1* in the gonads and brains of MT and EE_2 treated catfish

MT and EE_2 treatments (50-71 dph) decreased the expression of *cyp11a1* significantly ($P < 0.05$) in the testis to 62 and 17% (Fig. 6A) and in the ovary to 33 and 78% (Fig. 6B), respectively. In the male brain, *cyp11a1* expression decreased to 7 and 31% (Fig. 6C) and in the female brain to 24 and 26%, respectively (Fig. 6D).

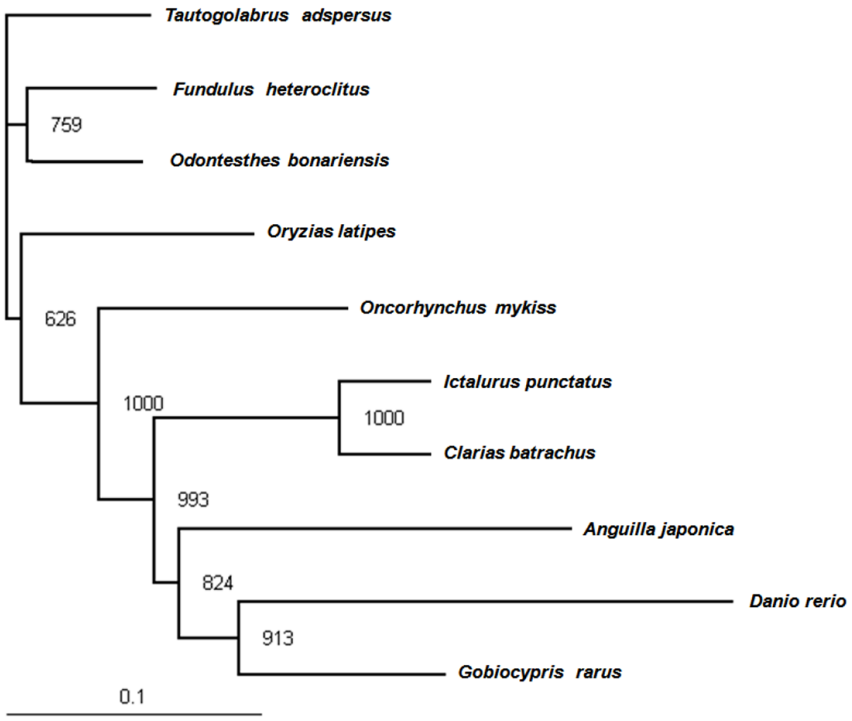

Fig. 1. Phylogenetic analysis of Cyp11a1 of catfish. The phylogenetic analysis was performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/) and was displayed using the TreeView software package version 1.6.6. GenBank accession numbers of sequences used for constructing the phylogenetic tree were given in the section 2.3.

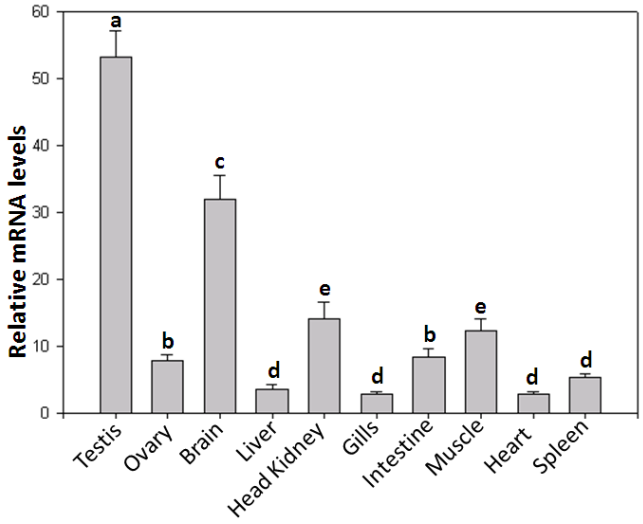

Fig. 2. Relative mRNA levels of *cyp11a1* in different tissues of adult catfish. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

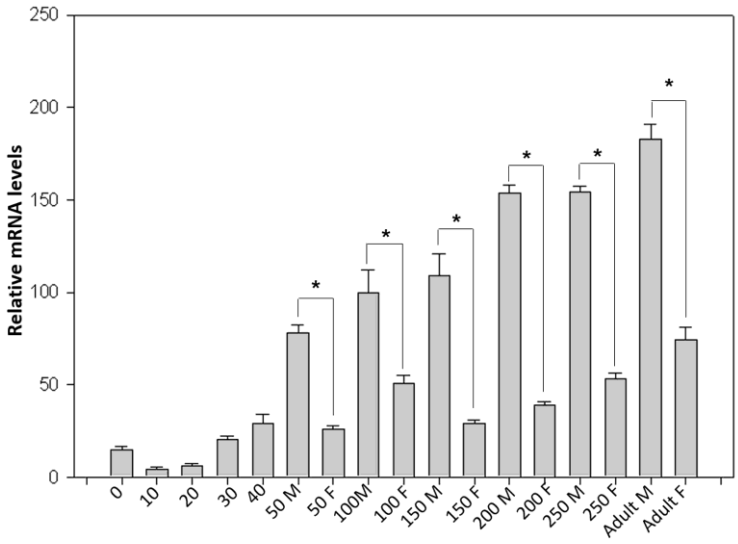

Fig. 3. Relative mRNA levels of *cyp11a1* in different stages of gonadal development. M-Male and F-Female. All data were expressed as mean ± SEM. *****indicates means with significantly higher *cyp11a1* mRNA levels compared with the corresponding females of the same age group (*, $P < 0.05$; ANOVA followed by SNK post-hoc test).

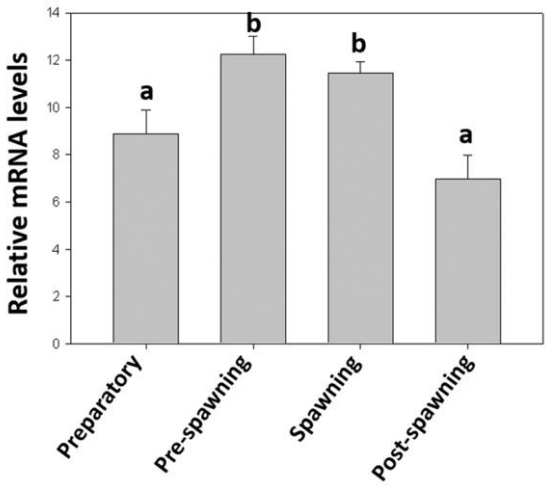

Fig. 4. Relative mRNA levels of *cyp11a1* in the testis of catfish during different phases of reproductive cycle. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

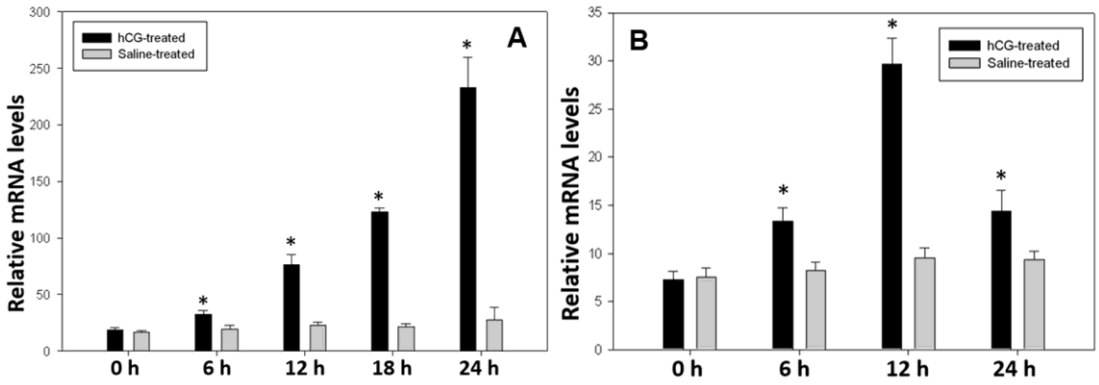

Fig. 5. Relative mRNA levels of *cyp11a1* in the testis of catfish at different time intervals after hCG induction. (A) *in vivo* and (B) *in vitro*. All data were expressed as mean ± SEM. *****indicates means with significantly higher *cyp11a1* mRNA levels compared with the 0 h (*, $P < 0.05$, ANOVA followed by SNK post-hoc test).

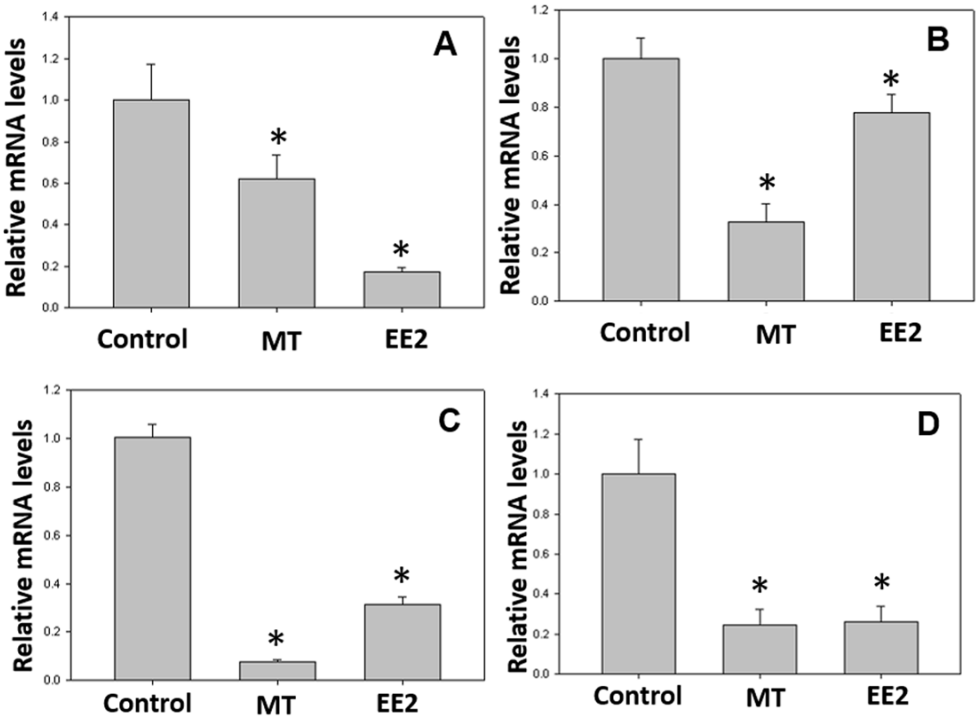

Fig. 6. Relative mRNA levels of *cyp11a1* in the gonad and brains of catfish after MT and EE_2 treatments. (A) Testis, (B) Ovary, (C) Male brain and (D) Female brain. All data were expressed as mean ± SEM. *indicates means with significantly lower *cyp11a1* mRNA levels compared to control (*, $P < 0.05$, ANOVA followed by SNK post-hoc test).

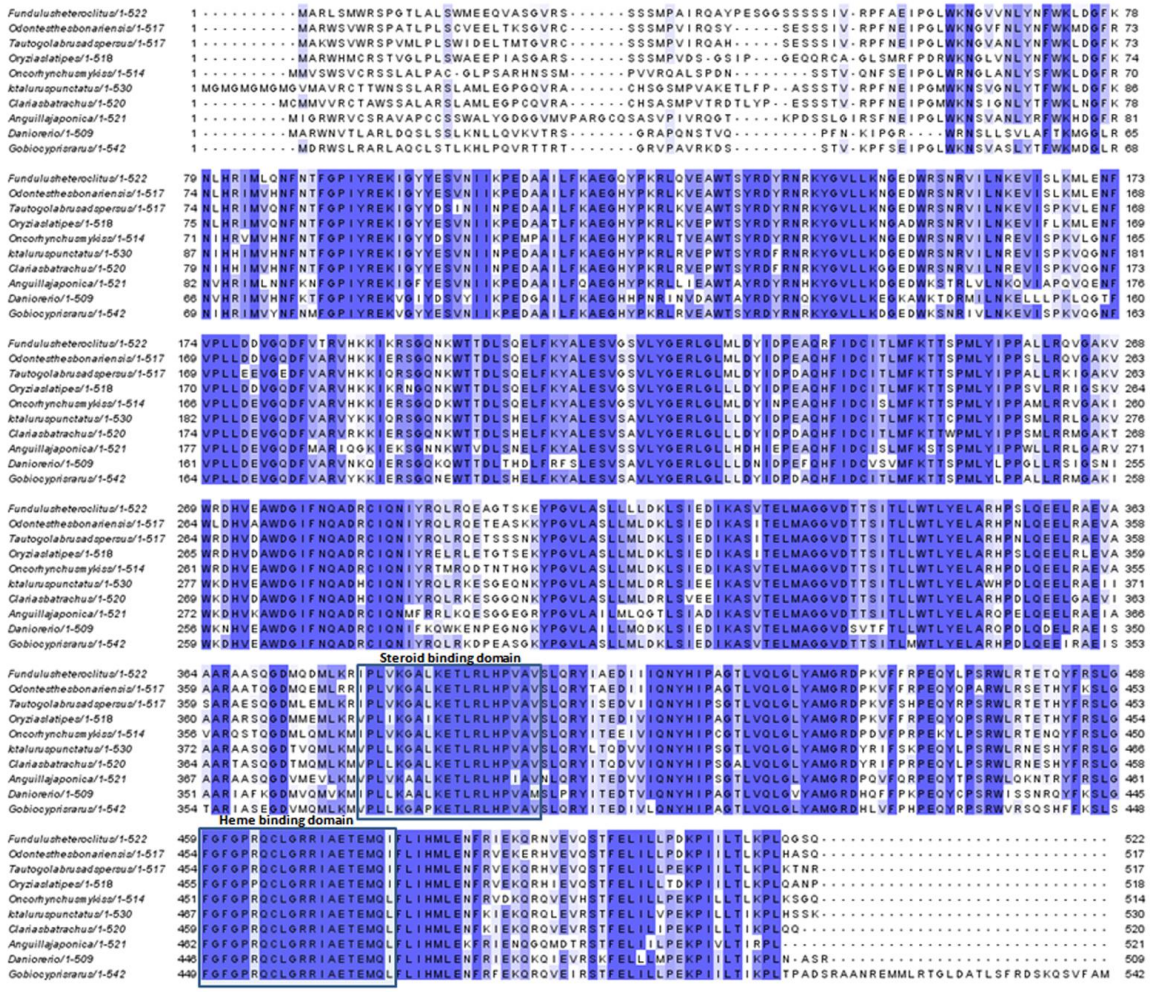

Fig. 7. Multiple alignment of the aminoacid sequences of Cyp11a1 from catfish and other teleosts. The multiple alignment was created using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and high homologous regions are shaded using Jalview 2.8 and the conserved domains are shown as rectangles. GenBank accession numbers for the sequences used for multiple alignment are given in the section 2.3.

4. Discussion

In this study, we report on the molecular cloning of catfish *cyp11a1* and its expression during early gonadal development, different phases of seasonal reproductive cycle, after

hCG induction and sex-steroid analog treatment in juveniles during early gonadal development.

The deduced Cyp11a1 amino acid sequence in comparison with other teleosts showed high homology in steroid and heme binding domains (Fig. 7). In zebrafish, a duplicated copy of *cyp11a* gene, *cyp11a2*, was found which shares 85% identity with *cyp11a1* (Goldstone et al., 2010; Parajes et al., 2013). However, in catfish, *C. batrachus*, only one *cyp11a1* was found, even after varying the reaction conditions and/or primers in RT-PCR and RACE. The deduced aminoacid of the cloned catfish *cyp11a1* showed considerable homology with Cyp11a1 of channel catfish (92%) and rainbow trout (76%) than the rest analyzed. The phylogenetic analysis showed that catfish Cyp11a1 formed separate clade with the Cyp11a1 of channel catfish.

Expression of *cyp11a1* was higher in the testis, brain, head kidney, muscle and ovary signifying its main role in steroidogenic tissues and additional role in non-steroidal tissues as well. Cyp11a1 could also potentially regulate the extra-gonadal and local steroid levels in the teleosts. *Cyp11a1* expression was detected much earlier starting from 0 dph till adulthood. Developing and mature testes showed higher *cyp11a1* expression than the ovary of corresponding age group. Interestingly, *cyp11a1* expression is sexually dimorphic during mouse embryogenesis and is apparent at embryonic day 12.5 in the testis, while, it is not expressed in the developing ovary (Hatano et al., 1994; Ikeda et al., 1994). Similar to the present study, ubiquitous and sex specific expression was found in *O. bonariensis* (Blasco et al., 2010). Further, *cyp11a1* expression was found to be higher during pre-spawning and spawning phases of testicular cycle, which shows the possible importance of sex-steroid production during these stages and further role in steroid

regulated gametogenesis. The increase in *cyp11a1* expression during testicular cycle might have indirectly increased the levels of steroid hormones by sustained substrate availability for sex-steroid production. In comparison to the present study, *cyp11a1* expression was higher during late stages of spermatogenesis in rainbow trout (Kusakabe et al., 2006).

To understand the GtH regulation of *cyp11a1* expression, hCG induction was carried out during preparatory phase of catfish reproductive cycle. The expression of *cyp11a1* was increased by hCG both at *in vivo* and *in vitro* treatments to maximum levels at 24 and 12 h respectively. Changes in the expression of *cyp11a1* after hCG induction showed GtH modulation of steroid levels indirectly. Further, dynamic changes in the levels of steroids during gametogenesis are critical for germ cell proliferation, differentiation and maturation.

MT and EE_2 are commonly used sex-steroid analogs for sex reversal studies, which were used during critical period of gonadal development (Piferrer, 2001; Orn et al., 2003; Pawlowski et al., 2004). In the present study, MT and EE_2 treatment down regulated *cyp11a1* expression in the gonads and brains of catfish. MT treatment during very early development skewed the population towards male (Raghuveer and Senthilkumaran, 2009). However, no skewing of sex was found in the present study, which might be due to the low dose of MT as well as the different developmental stages (50 dph) of catfish. Decrease in the expression of *cyp11a1* caused by EE_2 in the gonads and brains of catfish during gonadal development shows possible multiple targets for EE_2. Further EE_2 acts more similar to E_2 and modulated the expression of steroidogenic enzyme genes along with their enzyme activity (Govoroun et al., 2001; Filby et al., 2007). Indeed, it may be

assumed that the MT and EE_2 treatments might increase *cyp11a1* expression, yet on the contrary, the expression was decreased in both gonads and brains which might reveal either feedback intervention or steroidal treatment effect. Further studies on this line are necessary to substantiate the detailed mechanism involved in GtH induced *cyp11a1* regulation and also at the level of promoter motifs during gonadal development and maturation both in the gonads and brains of teleosts, which might provide interesting insights in this process.

5. Conclusions

The present study substantiates the importance of *cyp11a1* in gonadal development and maturation in catfish. The present work also demonstrated the GtH dependency of *cyp11a1* using hCG, *in vivo* and *in vitro.* Decreased expression of *cyp11a1* in juvenile catfish treated with sex-steroid analogs indicates differential effects based on the stage of gonadal development. Sexual dimorphic and differential expression of *cyp11a1* during gonadal development in addition to recrudescence in catfish can further corroborate the importance of Cyp11a1 in catfish reproduction in initiating steroidogenesis.

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Chapter 2

Molecular cloning and expression analysis of *17β-hydroxysteroid dehydrogenase 1* and *12* during gonadal development, recrudescence and after *in vivo* hCG Induction

Abstract

17β-hsds are important steroidogenic enzymes as they are involved in both T and E_2 biosynthesis. Full length cDNAs of *17β-hsd 1* (1791 bp) and *12* (1073 bp) were cloned from catfish gonad which encode protein of 295 and 317 amino acid residues, respectively. To understand the importance of these enzymes in teleost reproduction, mRNA expression was analyzed during gonadal development, seasonal reproductive cycle and after hCG induction. Phylogenetic analysis revealed that the 17β-hsd 1 and 12 share high homology with their respective 17β-hsd forms from other teleosts and both the forms belong to short chain dehydrogenase/reductase family. Tissue distribution analysis showed that the *17β-hsd 1* expression was higher in the ovary and gills, while *17β-hsd 12* was higher in the testis, ovary, brain, intestine and head kidney compared to other tissues analyzed. Developing and mature ovary showed higher expression of *17β-hsd 1*, while *17β-hsd 12* was higher in the testis than the ovary of corresponding stages. Further, *17β-hsd 1* and *12* transcripts together with E_2 and T levels were found to be modulated during different phases of the seasonal reproductive cycle. Expression of *17β-hsd 1* and *12* was upregulated after hCG induction, suggesting possible regulation by GtH. Our findings suggest that 17β-hsd 1 and 12 might play important role in regulating gonadal development and gametogenesis through modulation of sex-steroid levels.

1. Introduction

17β-hsds are multifunctional enzymes which catalyze the interconversion between 17β-hydroxy- (active) and 17-keto- (inactive) steroids and thereby regulate the specific levels of substrates for sex-steroid biosynthesis (Adamski and Jakob, 2001; Moeller and

Adamski, 2009). Most 17β-hsds belong to short chain dehydrogenase/reductase (SDR) and aldo-ketoreductase (AKR) protein superfamilies (Oppermann et al., 1999; Penning, 2003) which has been isolated and characterized in many mammals and few teleosts. 17β-hsds share SDR conserved motifs, which consist of the cofactor binding domain (TGxxxGxG), the tetrad of active site (N-S-Y-K), the structural stabilization domain NNAG, and the PGxxxT domain (Oppermann et al., 2003; Penning, 2003; Lukacik et al., 2006; Marchais-Oberwinkler et al., 2011). Although most 17β-hsds share high homology in sequence and structure, they differ in substrate specificity, catalytic coenzyme preference, reaction direction and tissue distribution (Poirier, 2003; Moeller and Adamski, 2009; Prehn et al., 2009).

17β-hsd 1 is a key enzyme catalyzing the final step of the synthesis of E_2. In most vertebrates, the most prominent site of expression is the ovary, but in zebrafish it can also be found at low levels in other tissues like skin, muscle and eye (Mindnich et al., 2004a). 17β-hsd 1 is predominantly involved in interconversion of estrone (E_1) to E_2 in rat, mice and teleosts (Zhou et al., 2005). Interestingly in the Nile tilapia, 17β-hsd 1 can also catalyze the conversion between androstenedione and T but less efficiently (Zhou et al., 2005). 17β-hsd 12 is the most recent addition to this enzyme family and subsequent phylogenetic analysis revealed its close relationship to 17β-hsd 3 and was suggested that 17β-hsd 12 would be an ancestor of 17β-hsd 3 (Mindnich et al., 2004a). Zebrafish contains two paralogous copies of 17β-hsd 12 (A and B) and both are expressed widely in several tissues of both the developing and adult fish (Mindnich et al., 2004a; 2004b). 17β-hsd 12 has been shown to be involved in the biosynthesis of E_2 and the elongation of

the essential very long fatty acids (Rantakari et al., 2010). 17β-hsd 12 was first characterized as a 3-ketoacyl-CoA reductase which is specifically involved in long and very long-chain fatty acid biosynthesis (Moon and Horton, 2003) in mammals.

17β-hsds are required for the production of E_2 and T and thus indirectly involved in the process of sex differentiation and gametogenesis in fish (Guiguen, 2000; Mindnich et al., 2004b; Nagahama, 2005). It is well known that sex-steroids can play important roles in the process of sex differentiation and gametogenesis of non-mammalian vertebrates like teleosts (Nagahama, 2000). Hence, the expression and regulation of steroidogenic enzyme genes are critical for steroidogenesis as well as gonadal differentiation and development. Further, the analysis of 17β-hsds, in an annually reproducing teleost was never attempted. Such an attempt might provide important clues to understand gonadal development together with recrudescence. To this end, to understand the possible involvement of steroids and the expression of steroidogenic enzyme genes on gonadal development together with recrudescence, the present study was conducted in *C. batrachus*. Full length cDNA encoding 17β-hsd 1 and 12 were cloned from catfish gonads and the expression was analyzed during ontogeny, seasonal cycle and after hCG induction. In addition, the levels of T in serum and testicular tissue and E_2 in serum during different stages of reproductive cycle were also measured.

2. Materials and methods

2.1. Animals

IVF, sampling for ontogeny, tissue distribution and seasonal reproductive cycle were done as described in the chapter 1. Prior to sampling, blood was collected from different

stages of catfishes by caudal puncture and the collected blood was centrifuged at 2500 g and 20 °C for 20 min for serum separation.

2.2. Molecular cloning of full length *17β-hsd* cDNAs from catfish gonads

Total RNA isolation and cDNA synthesis were done as described in the chapter 1. In brief, a set of degenerate primers were designed individually for *17β-hsd 1* and *12* based on the available nucleotide sequences of both *17β-hsd 1* and *12* of teleosts from the NCBI GenBank database. PCR amplification was performed at 94 °C (1 min) and 35 cycles of 94 °C (30 s), 52 °C (30 s), 72 °C (1 min) and 72 °C (10 min) using degenerate primers (Table 1) of either *17β-hsd 1* (DF1 and DR1) or *17β-hsd 12* (DF12 and DR12). The partial cDNA fragments obtained were cloned into pGEM®-T easy vector (Promega) and sequenced bi-directionally. Based on the sequence data, RACE primers were designed and used for full length cDNA amplification by using SMARTer RACE kit by following manufacturer's instructions (Clontech). In the 3′RACE of *17β-hsd 12* two fragments were amplified which upon analysis, showed that both are *17β-hsd 12* but a variant form. All the overlapping DNA fragments were assembled and the full length cDNA of *17β-hsd 1* (1791 bp with ORF of 888 bp) and *17β-hsd 12* normal type (1073 bp with ORF of 954 bp) and variant (921 bp with ORF of 690 bp) were obtained. For ORF cloning and further analysis only *17β-hsd 12* normal type was used. ORF primers were designed for *17β-hsd 1* and *12* and the cDNAs were cloned into pGEM®-T easy vector (Promega) and sequenced bi-directionally. The analysis of sequence homology of the cloned cDNA sequences to *17β-hsd 1* and *12* of other vertebrates were carried out by using NCBI-BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the assembled

sequences were translated using EditSeq of Lasergene 7.1.0 (DNASTAR). The analysis of homology of the deduced amino acid sequence of catfish *17β-hsd 1* and *12* with *17β-hsd 1* and *12* of other teleosts were carried out by using ClustalW multiple alignment tool using default parameters.

2.3. Phylogenetic analysis

Multiple alignment of deduced amino acid sequences were performed based on 17β-hsd 1 and 12 sequences of different teleosts and mammals using ClustalW (DDBJ) as described earlier in the chapter 1. The following are the GenBank accession numbers of deduced amino acid sequences used for constructing the phylogenetic tree: 17β-hsd 1 [*D. rerio* (NM_205584), *O. niloticus* (NM_001279795), and *O. latipes* (XM_004071297), *A. japonica* (AY498620), *Mus musculus* (NM_010475), *Rattus norvegicus* (NM_012851), *Bos taurus* (NM_001102365), *Homo sapiens* (NM_000413) and *C. batrachus* (KM034751)] and 17β-hsd 12 [*D. rerio* 12a (NM_200881), *D. rerio* 12b (NM_199613), *Salmo salar* 12b (BT045689), *M. musculus* (NM_019657), *R. norvegicus* (NM_032066), *B. taurus* (NM_001101307), *H. sapiens* (NP_057226) and *C. batrachus* (JN848590)].

2.4. qPCR

Total RNA isolation, reverse transcription and qPCR were done as described in the chapter 1. qPCR primers were designed for the amplicon length of ~250 bp and all reactions were performed in triplicate for three different samples using gene specific primers (Table 1). Change in the gene expression was calculated by using $2^{-\Delta Ct}$ method.

Table 1. List of primers used for cDNA cloning and qPCR analysis

S.no.	Primer Name	Primer Sequence 5′ to 3′	Usage
1.	17β-hsd1 DF1	TSATYACCGGYTGCTCCTCKGGMAT	RT-PCR
2.	17β-hsd1 DR1	GTGTKSACYGSSCCRCACT	RT-PCR
3.	17β-hsd1 5P	ACACCAGGATGTCCACTCGGCCCTCG	RACE
4.	17β-hsd1 5N	AGGTCCCTCACACTCTCCAGCAAGCGC	RACE
5.	17β-hsd1 3P	GTCACAGGCAGTATGGGAGGGCTACAAGG	RACE
6.	17β-hsd1 3N	AGGGTGTCTGTGAGAGTCTGGCCAT	RACE
7.	17β-hsd1 ORFF	ATGGAGTGGAAGGTAGTTCTC	ORF cloning
8.	17β-hsd1 ORFR	TTATTTCTGTGCTCTTATTTC	ORF cloning
9.	17β-hsd1 RTF	GACATCCTGGTGTGTAATGCAGG	qPCR
10.	17β-hsd1 RTR	CTGCCTGTGACCAGGATCCGT	qPCR
11.	17β-hsd12DF12	GTTGTBCANGGDRSYCANKMYGG	RT-PCR
12.	17β-hsd12DR12	GAGAAGAADKYYACAAAVGYYTT	RT-PCR
13.	17β-hsd12 5P	CGATGGCAAAGCCACGCCGTGCAAGC	RACE
14.	17β-hsd12 5N	TCCGTAAGTAGCCCCCGTCACAAC	RACE
15.	17β-hsd12 3P	GATGACTCGGCTTGTGCTGCCTAAGATGG	RACE
16.	17β-hsd12 3N	AGCGGCATGTACCCTGTTCCACTC	RACE
17.	17β-hsd12 ORFF	ATGGAGGCGGCAGATATTTTG	ORF cloning
18.	17β-hsd12 ORFR	TTAGCCCTGCTTTTGCTTCT	ORF cloning
19.	17β-hsd12 RTF	AGCCATCGAGAGCAAGTACCATGT	qPCR
20.	17β-hsd12 RTR	AAGCCGAGTCATCTGACAAACCGA	qPCR
21.	18S rRNA F	GCTACCACATCCAAGGAAGGCAGC	qPCR
22.	18S rRNA R	CGGCTGCTGGCACCAGACTTG	qPCR

IUPAC Nucleic acid codes: R- A or G; Y- C or T; S- G or C; W- A or T; K- G or T; M- A or C; B- C or G or T; D- A or G or T; H- A or C or T; V- A or C or G; N- any base

2.5. Enzyme immune assay (EIA)

Serum and tissue levels of T were measured by using EIA kit (Shrivatsav et al., 2012) and for serum levels of E_2, Cayman's EIA kit was used by following respective manufacturer's protocol. Serum from both male and female catfishes and testicular tissue collected from catfishes during different stages of recrudescence were stored at -80 °C

briefly until assayed. Samples were analyzed following the manufacturer's instructions in a microplate reader (Bio-Rad model 680; Bio-Rad Laboratories, Hercules, CA). The dilutions of series of serum samples and/or tissue extracts from catfish with differing hormone concentrations were linear with standards of T and E_2. Intra- and inter-assay variations were within the limits specified in the respective manufacturer's protocol. The cross reactivity of T antisera to other naturally occurring C27-, C21-, C19- and C18-steroids is less than 0.1%. The cross- reactivity of the E_2 antisera for E1 is 12%, estradiol-17-glucoronide (10%) and estriol (0.3%).

2.6. hCG induction

During the mid-preparatory phase (March), adult catfishes (male and female) weighing about 170-200 g, were intraperitoneally injected a single dose (1000 IU/Kg body weight) of hCG and sacrificed in different time intervals as described in the chapter 1. Total RNA isolation, reverse transcription and qPCR analysis were performed as described earlier in the chapter 1.

2.7. Statistical analysis

All data were presented as mean of different samples with SEM. All data were compared by Mann-Whitney Rank Sum Test or Kruskal-Wallis one-way ANOVA by ranks followed by SNK post hoc test. All statistical analyses were performed using SigmaPlot 11.0 software (Systat Software Inc.). A probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning of *17β-hsd 1* and *12* from catfish gonads

A partial cDNA of 521 bp encoding for 17β-hsd 1 was cloned from catfish ovary and a different partial cDNA of 479 bp encoding for 17β-hsd 12 was cloned from catfish testis. Based on the sequence data, gene specific RACE primers were designed and used for 5′ and 3′RACE. The cDNA fragments obtained through 5′ (801 bp) and 3′RACE (728 bp) for *17β-hsd 1* and 5′ (346 bp) and 3′RACE (372 and 252 bp) for *17β-hsd 12* were cloned and verified by comparing with *17β-hsd 1* and *12* of other teleosts using Lasergene software 7.1.0 (DNASTAR), NCBI-BLAST and ClustalW. After assembling all the cDNA fragments corresponding to *17β-hsd 1* and *12*, full length cDNA of 1791 bp (*17β-hsd 1*), 1073 bp (*17β-hsd 12* regular type), and 921 bp (*17β-hsd 12* variant) with ORFs of 888 bp (*17β-hsd 1*), 954 bp (*17β-hsd 12* regular type), and 690 bp (*17β-hsd 12* variant) were obtained. The cDNA sequences were submitted to GenBank, *17β-hsd 1* (KM034751), *17β-hsd 12* (JN848590) and *17β-hsd 12* variant (KM034752). The *17β-hsd 1* and *12* (normal type) ORF were cloned from catfish testis, sequenced bi-directionally and confirmed with the assembled full length *17β-hsd 1* and *12* cDNA of other vertebrates. *17β-hsd 12* normal type was used for all the expression and phylogenetic analyses.

3.2. Sequence and phylogenetic analyses

The multiple alignment of deduced 17β-hsd 1 (Fig. 1) and 12 (Fig. 2) amino acid sequence in comparison with other species revealed considerable homology with 17β-hsd 1 and 12 of other teleosts specifically at coenzyme binding motif, active sites, catalytic

center and other important motifs specific for SDR superfamily (Figs. 1 and 2). Phylogenetic analysis revealed that catfish 17β-hsd 1 and 12 in comparison with other vertebrate counterparts showed considerable homology with 17β-hsd 1 and 12 of other teleosts by forming separate clades (Fig. 3). 17β-hsd 1 of catfish was more similar to *D. rerio* (75.6%) while, 17β-hsd 12 showed high homology with *D. rerio* 17β-hsd 12b (84.9%) and 12a (65.6%).

3.3. Tissue distribution of *17β-hsd 1* and *12* in different tissue of adult catfish

Expression of *17β-hsd 1* was high in the ovary followed by gills, head kidney, muscle, heart and intestine (Fig. 4A) while *17β-hsd 12* was expressed ubiquitously with higher levels in the testis, ovary, brain, intestine and head kidney (Fig. 4B).

3.4. Expression of *17β-hsd 1* and *12* during gonadal ontogeny

qPCR analysis of *17β-hsd 1* and *12* at different stages of catfish gonadal development showed that both male and female gonads express *17β-hsd 1* and *12* differentially throughout gonadal development till maturity. The expression of *17β-hsd 1* was higher in females compared to males of corresponding age group (Fig. 5A). In contrast, *17β-hsd 12* was higher in males than the females during gonadal development and at maturity (Fig. 5B).

3.5. Expression of *17β-hsd 1* and *12* during different phases of the seasonal cycle

The qPCR analysis showed that the expression of *17β-hsd 1* was significantly higher ($P < 0.05$) during the pre-spawning phase of catfish ovarian cycle (Fig. 6A) compared to other

phases. The expression of *17β-hsd 12* was higher in the preparatory and pre-spawning phases than in the other phases of catfish testicular cycle (Fig. 6B).

3.6. Changes in expression of *17β-hsd 1* and *12* in the gonads after hCG induction

hCG induction during the mid-preparatory phase (March) of catfish reproductive cycle revealed that *17β-hsd 1* expression enhanced significantly ($P < 0.05$) in the ovary and *17β-hsd 12* expression in the testis. The *17β-hsd 1* expression increased significantly ($P < 0.05$) with maximum expression at 18 h after hCG induction in the ovary (Fig. 7A), while the expression of *17β-hsd 12* was maximum at 24 h (Fig. 7B) after hCG induction in testis.

3.7. Levels of T and E_2 during different phases of recrudescence

The levels of T was found to be higher in the serum (Fig. 8A) of catfishes of pre-spawning (2.22 ± 0.39 ng/ml) and spawning (2.02 ± 0.20 ng/ml) than the rest of the phases analyzed. In testicular tissue high levels of T (Fig. 8B) was found during pre-spawning phase (30.75 ± 1.67 pg/mg tissue) followed by spawning phase (21.15 ± 1.90 pg/mg tissue), while the T levels dropped to 14.42 ± 1.04 pg/mg tissue in the post spawning phase. The levels of serum E_2 was found to be higher (Fig. 9) in the pre-spawning phase (2.10 ± 0.15 ng/ml) followed by preparatory (1.12 ± 0.17 ng/ml), spawning (1.07 ± 0.24 ng/ml) and post spawning (0.50 ± 0.15 ng/ml) phases of catfish.

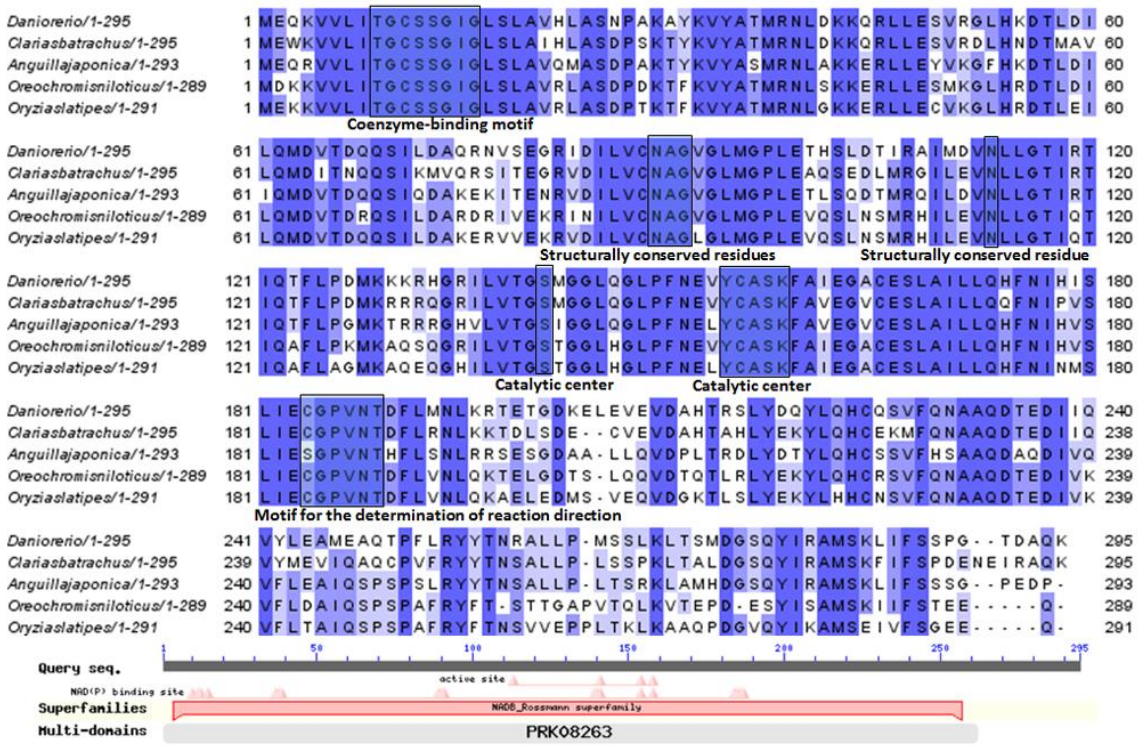

Fig. 1. Multiple alignment of the aminoacid sequences of 17β-hsd 1 from catfish and other teleosts. The multiple alignment was created using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and high homologous regions are shaded using Jalview 2.8 and important domains are shown as rectangles. The GenBank accession numbers of sequences used are: *D. rerio* (NM_205584), *O. niloticus* (NM_001279795), and *O. latipes* (XM_004071297), *A. japonica* (AY498620) and *C. batrachus* (KM034751). Protein-BLAST of catfish 17β-hsd 1 showed coenzyme binding motif, active sites, catalytic center and other important motifs specific for SDR superfamily.

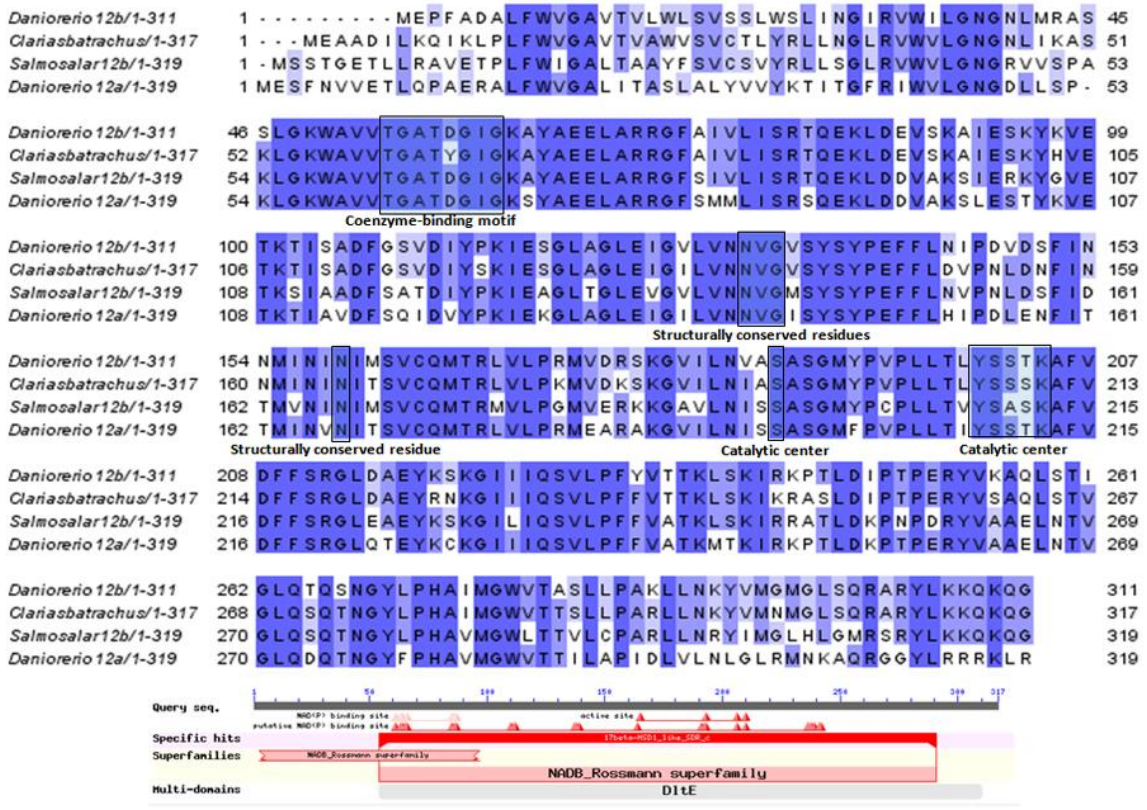

Fig. 2. Multiple alignment of the aminoacid sequences of 17β-hsd 12 from catfish and other teleosts.

The multiple alignment was created using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and high homologous regions are shaded using Jalview 2.8 and important domains are shown as rectangles. The GenBank accession numbers of sequences used are as follows: *D. rerio* 12a (NM_200881), *D. rerio* 12b (NM_199613), *S. salar* 12b (BT045689) and *C. batrachus* (JN848590). Protein-BLAST of catfish 17β-hsd 12 showed coenzyme binding motif, active sites, catalytic center and other important motifs specific for SDR superfamily.

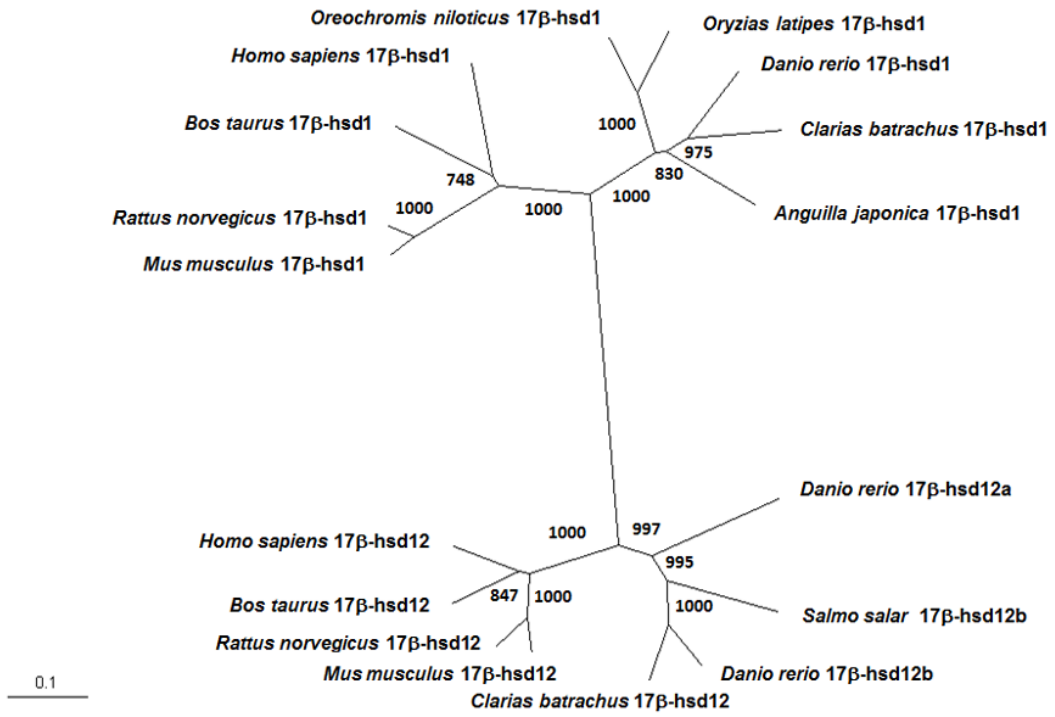

Fig. 3. Phylogenetic analysis of catfish 17β-hsd 1 and 12. The phylogenetic analysis was performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/) and was displayed using TreeView software package version 1.6.6. GenBank accession numbers of sequences used for constructing the phylogenetic tree were given in the section 2.3.

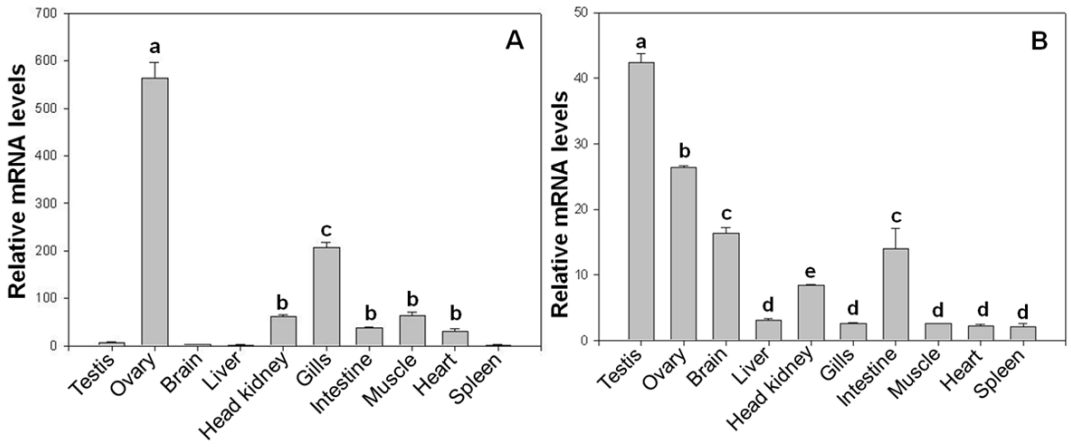

Fig. 4. Relative mRNA levels of (A) *17β-hsd 1* and (B) *17β-hsd 12* in different tissues of adult catfish. All data were expressed as mean ± SEM. Means with different letters differ

significantly ($P < 0.05$; Kruskal-Wallis One Way ANOVA on Ranks followed by SNK post-hoc test).

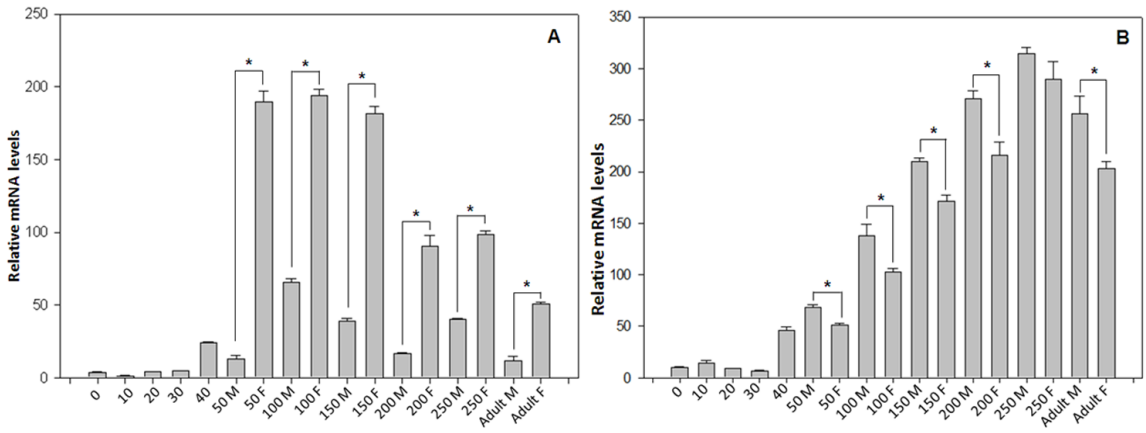

Fig. 5. Relative mRNA levels of (A) *17β-hsd 1* and (B) *17β-hsd 12* in different stages of gonadal development. M-Male and F-Female. All data were expressed as mean ± SEM. * indicates means with significantly higher *17β-hsd 1* and *12* mRNA levels when compared with the corresponding opposite sex of the same age group (*, $P < 0.05$; Mann-Whitney rank sum test).

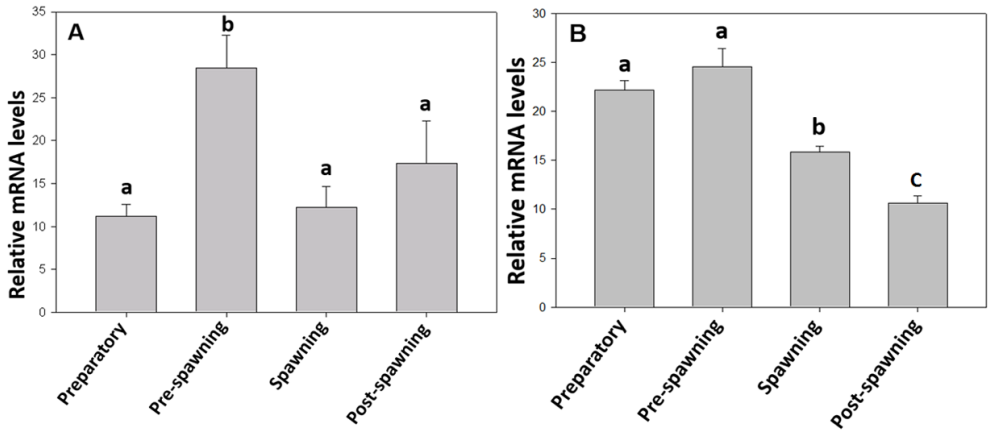

Fig. 6. Relative mRNA levels of (A) *17β-hsd 1* and (B) *17β-hsd 12* in the gonads of catfish during different phases of the reproductive cycle. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; Kruskal-Wallis One Way ANOVA on Ranks followed by SNK post-hoc test).

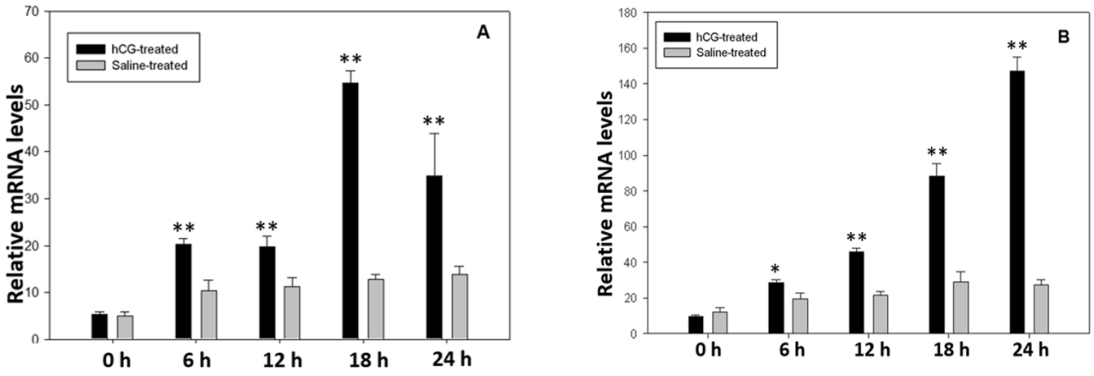

Fig. 7. Relative mRNA levels of (A) *17β-hsd 1* and (B) *17β-hsd 12* in the gonads of catfish at different time intervals after hCG induction. All data were expressed as mean ± SEM. */** indicates means with significantly higher *17β-hsd 1* and *12* mRNA levels compared with the 0 h (**, $P < 0.001$, *, $P < 0.05$, Kruskal-Wallis One Way ANOVA on Ranks followed by SNK post-hoc test).

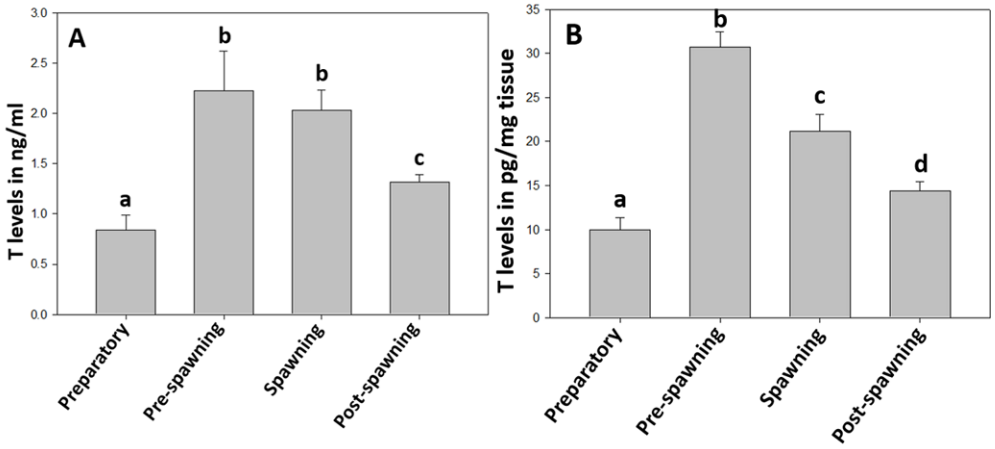

Fig. 8. Levels of T in (A) serum and (B) testicular tissue of catfish during different phases of testicular cycle. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; Kruskal-Wallis One Way ANOVA on Ranks followed by SNK post-hoc test).

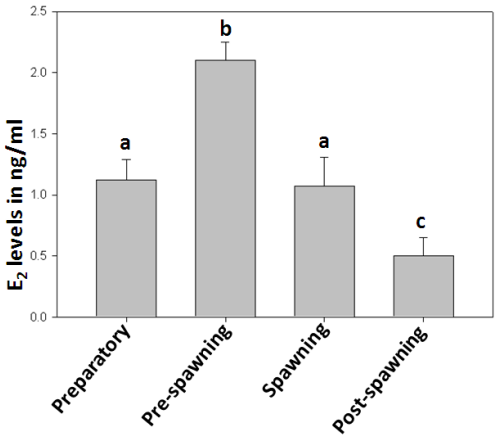

Fig. 9. Levels of E_2 in the serum of catfish during different phases of ovarian cycle. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; Kruskal-Wallis One Way ANOVA on Ranks followed by SNK post-hoc test).

4. Discussion

In this study, we report on the molecular cloning of catfish *17β-hsd 1* and *12* and their expression changes during gonadal development, different phases of the seasonal reproductive cycle and after hCG induction. Besides, modulation of T and E_2 levels during different phases of the seasonal reproductive cycle was also recorded. The deduced aminoacid sequences of *17β-hsd 1* and *12* contains all the important motifs generally observed in the SDR super family and shows high similarity to their respective protein sequences of teleosts. To further confirm that the isolated sequence belongs to *17β-hsd 1* and *12* respectively, phylogenetic analysis was performed with their deduced aminoacid sequences which implied distinct clades with their respective 17β-hsd forms from different teleost counterparts. 17β-hsd 1 and 12 of catfish showed high homology with zebrafish 17β-hsd 1 and 12b, respectively. Two forms of *17β-hsd 12* were cloned from zebrafish (Mindnich et al., 2004a) and BLAST search also retrieved two *17β-hsd*

12-like genes in both fugu and tetraodon genomes. But in the catfish, only one form of *17β-hsd 12* and a variant could be cloned successfully even after varying the reaction conditions in RT-PCR and RACE. Based on the sequence analysis, *17β-hsd 12* (both normal and variant) cloned from catfish showed high homology with *17β-hsd 12b* from other teleosts. The amino acid variation in the variants do not affect important catalytic sites and hence, the variant forms has minimal or no importance. The possible reason for existence of variants might be due to whole genome duplication events in fishes, which resulted in many isoforms and variants of genes (Meyer and Schartl, 1999). In order to gain further insight on the functional role of *17β-hsd 1* and *12*, we analyzed their tissue distribution during pre-spawning phase of catfish reproductive cycle. *17β-hsd 1* was expressed predominantly in the ovary, while a weaker expression was found in the gills, muscle, head kidney, heart and intestine. Similarly, higher *17β-hsd 1* expression was found in the ovary and low levels were seen in tissues like the skin, muscle and eye of zebrafish (Mindnich et al., 2004a). In the Nile tilapia, 17β-hsd 1 is involved in both E_2 and T interconversion and thus suggested to be a multifunctional enzyme that might be involved in many different reactions *in vivo* in the steroidogenic pathway depending on its spatial and temporal expression pattern (Zhou et al., 2005). However, in our present study, expression of *17β-hsd 1* is higher in the ovary followed by gills, and thus suggesting no possible role in testis or other steroidogenic tissues of catfish. Gills are involved in the production of 17α,20α- and 17α,20β-DP and 11-KT in teleosts (Kime and Ebrahimi, 1997). This raises the possibility that the gill of the catfish might produce several steroidal metabolites or E_2. More in depth analyses are required to prove this contention. Expression of *17β-hsd 12* was higher in the testis, ovary, brain, intestine and

head kidney compared to other tissues. This shows the possible involvement of 17β-hsd 12 in gonadal as well as extra-gonadal steroidogenesis together with their involvement in lipid metabolism. The higher expression of *17β-hsd 12* in intestine shows their probable involvement in fatty acid metabolism since at first, human 17β-hsd 12 was characterized as a ketoacylreductase due to its homology with the yeast enzyme YBR159w, which is active in fatty acid elongation (Moon and Horton, 2003). In the Nile tilapia, 17β-hsd 1 is not involved in the early steroidogenesis during sex differentiation while its role in gametogenesis seems essential (Zhou et al., 2005). However, in this study, the expression of both *17β-hsd 1* and *12* was detected much earlier during gonadal differentiation and development and then on sex-specific dimorphic expression was evident, which shows possible involvement of *17β-hsd 1* and *12* in the gonadal differentiation and development.

17β-hsd 1 is primarily involved in E_2 production (Zhou et al., 2005), while 17β-hsd 12 is involved in fatty acid elongation in addition to E_2 production (Moon and Horton, 2003; Luu-The et al., 2006). *17β-hsd 1* expression was higher during pre-spawning phase of ovarian cycle, while *17β-hsd 12* was high during both preparatory and pre-spawning phases of testicular cycle. This shows the involvement of E_2 in oocyte growth and in early stages of gametogenesis. The involvement of 17β-hsd 12 in steroid and lipid metabolism makes it an important candidate in regulation of steroidogenesis through substrate limitation. Further, based on the involvement of 17β-hsd 12 in fatty acid elongation, it can be hypothesized that, the enzyme might also be involved in polyunsaturated fatty acid (PUFA) biosynthesis. A ω-6 PUFA, arachidonic acid (AA) and

its metabolites, eicosanoids are involved in various reproductive processes including gametogenesis, maturation and release (Goetz et al., 1991; Wade et al., 1994; Lenzi et al., 2000; Patiño et al., 2003). A recent study (Rantakari et al., 2010) using embryonic stem cells of knockout mouse showed that *17β-hsd 12* deficiency caused insufficient synthesis of AA suggesting that 17β-hsd 12 is involved in AA synthesis. Wade et al. (1994) reported that AA stimulates T production in goldfish. Such a possibility might exist in catfish, wherein levels of T were increased during pre-spawning phase, the period where expression of *17β-hsd 12* was higher. E_2 in serum was higher during pre-spawning phase of ovarian cycle which corresponds to higher expression of *17β-hsd 1*. T produced by thecal cells is converted into E_2 by Cyp19a1a in the granulosa cells (Nagahama, 1994). It is well known that E_2 and T are involved in gametogenesis and decreased during gamete maturation during which progestin levels might get elevated (Sreenivasulu et al., 2012; Rajakumar and Senthilkumaran, 2013).

Gonadal steroidogenesis can be stimulated with hCG which act more similar to LH by binding to LH receptors present in the gonads of teleosts (Bogerd et al., 2001; Shiraishi et al., 2005; Paul et al., 2010). hCG stimulate the production of E_2, T and 11-KT (Bogerd et al., 2001; Shiraishi et al., 2005; Paul et al., 2010) both directly and indirectly through an increase in the expression of terminal steroidogenic enzyme genes like *11β-h* and *11β-hsd* (Jiang et al., 1996; Rasheeda et al., 2010; Chapter 3; 4). The present study demonstrated that increase in the expression of *17β-hsd 1* and *12* after hCG induction showed the possible regulation of T and E_2 levels by hCG. Androgens including T can stimulate 17α,20β-DP production by down regulating P450c17 activity in the Japanese

eel and catfish (Cavaco et al., 1999; Miura et al., 2006; Rajakumar and Senthilkumaran, 2013).

This may also explain the increased expression of *20β-hydroxysteroid dehydrogenase* (*20β-hsd*) in catfish (Sreenivasulu et al., 2012). PUFAs and its metabolites together with E_2 are involved in various reproductive processes including gametogenesis, gamete maturation, ovulation and spermiation. Hence, there is a possibility of involvement of 17β-hsd 1 and 12 in steroid and lipid metabolism, which might be thereby regulating reproductive processes in teleosts.

5. Conclusions

The present study showed the sexual dimorphic and differential expression of *17β-hsd 1* and *12* during gonadal development and recrudescence in catfish together with E_2 and T levels. Higher levels of E_2 and T together with dynamic expression of *17β-hsd 1* and *12* during gonadal development and reproductive cycle show their possible involvement in gametogenesis and gamete maturation. In addition, the present work also demonstrated a possible involvement of GtH in the regulation of *17β-hsd 1* and *12*.

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Chapter 3

Dynamic expression of *11β-hydroxylase* during testicular development, recrudescence and after *in vivo* and *in vitro* hCG induction

Abstract

11β-h is involved in the production of 11-OHT, an immediate precursor for 11-KT, a potent androgen in teleosts. To understand the role of 11β-h in gonadal development, maturation, function and recrudescence in an annually reproducing teleost, the present study was conducted using *C. batrachus*. Four forms of *11β-h* cDNA, regular type (2253 bp), variant 1 (1290 bp), variant 2 (1223 bp) and variant 3 (1978 bp) were identified from the testis of catfish which expressed ubiquitously with high levels in testis. *11β-h* transcripts were detected as early as 0 dph further, stage- and sex-dependent increase in the *11β-h* transcripts were seen during gonadal differentiation/development. In addition, high expression of *11β-h* (regular type) in pre-spawning phase was detected. Corroboratively, levels of 11-KT in serum and testicular tissue were high during pre-spawning and spawning phases, which might facilitate initiation and normal progression of spermatogenesis. The expression of *11β-h* was upregulated after hCG induction both *in vivo* (all forms) and *in vitro* (regular type). Immunohistochemical and immunofluorescence localization showed the presence of 11β-h in Sertoli and interstitial/Leydig cells of the testis. These results suggest the involvement of 11β-h in late stages of testicular development, together with the regulation of seasonal reproductive cycle in catfish.

1. Introduction

Sex-steroids play an important role in the sex differentiation and development of teleosts. The 11-KT and T are the major sex-steroids which regulate various processes including reproduction (Mayer et al., 1990; Borg, 1994; Devlin and Nagahama, 2002). 11-KT is

essential for inducing male sexual phenotype, secondary sexual characteristics and spermatogenesis in many teleosts which can also cause female-to-male sex reversal (Mayer et al., 1990; Cardwell and Liley, 1991; Miura et al., 1991; Kobayashi and Nakanishi, 1999). In addition, 11-KT triggers Sertoli cells to synthesize activin βB which binds to its type I and II receptors on spermatogonia A leading to the initiation of mitosis, generating spermatogonia B, thereby inducing pre-meiotic spermatogonial proliferation (Ge et al., 1997a; Ge et al., 1997b; Nagahama et al., 1997; Dietrich and Krieger, 2009). In most teleosts, 11-KT was higher than T during all stages of spermatogenesis and is essential for normal progression of spermatogenesis (Koldras et al., 1990; Borg, 1994; Weltzien et al., 2002). Hence, the regulation of enzymes involved in the production of 11-KT are critical for reproductive success of the teleosts.

The biosynthesis of 11-KT requires coordinated action of several steroidogenic enzymes and the final critical steps are catalyzed by the two enzymes, 11β-h (P45011β/P450c11; encoded by *cyp11b1* gene) and 11β-hsd. T is hydroxylated and converted into 11-KT and the whole process occur at testicular Leydig cells in addition to the peripheral conversion in adrenal and liver (Nagahama, 1994; Jiang et al., 1996; Cavaco et al., 1997; Jiang et al., 1998; Kobayashi et al., 1998; Swart et al., 2013). It is also involved in the production of corticosterone and cortisol in the interrenal cells of kidney of several teleosts, while in mammals, the process occurs at adrenal cortex (Miller, 1988). Substantial changes in the mRNA levels of *11β-h* were seen during the reproductive cycle of *O. mykiss* (Liu et al., 2000; Kusakabe et al., 2002) and at spermiation in *S. salar* (Maugars and Schmitz, 2008). Treatment of hCG can induce *11β-h* mRNA in immature eel as well as increase in 11-KT production vis-à-vis spermatogenic process (Miura et al., 1991; Jiang et al., 1996). In the

Nile tilapia, 11-oxygenated androgens are measurable only after the testis differentiation (Nakamura et al., 1998). In *Dicentrarchus labrax*, *O. bonariensis* and *O. mykiss*, *11β-h* expression was detected well before the gonadal differentiation (Liu et al., 2000; Blázquez et al., 2001; Blasco et al., 2010). Evidences for the direct involvement of 11-OHT and 11-KT in testis formation and differentiation along with the regulation of critical enzymes involved in their synthesis are very minimal (Socorro et al., 2007; Wang and Orban, 2007). In addition, the role of 11β-h and 11-oxygenated androgens in spermatogenesis and recrudescence were not studied in detail. Such an attempt to study the ontogeny and recrudescence cohesively in a seasonal breeding teleost like catfish might provide interesting insights. The present study was conducted in catfish, *C. batrachus* to understand the importance of 11β-h in testis development, and how its expression varies during the seasonal reproductive cycle together with the implications of GtH exposure, *in vivo* and *in vitro* in addition to sex steroid analogs treatment.

2. Materials and methods

2.1. Animals, Sampling and Sex steroid analogs exposure

C. batrachus hatchlings at different age groups were obtained by IVF. Sampling for ontogeny, tissue distribution and seasonal reproductive cycle was done as described in the chapter 1. Prior to sacrifice, blood was collected from different stages of catfishes by caudal puncture and the collected blood was centrifuged at 2500 g and 20 °C for 20 min for serum separation. Further, for sex-steroid analog studies, juvenile catfish of 50 dph were divided into three groups of 50 larva each were maintained either with or without

treatment compounds of 1µg/l of MT or 1µg/l EE_2. At end of the treatments sampling was done as described in the chapter 1.

2.2. Molecular cloning of full length *11β-h* cDNA from catfish testis

Total RNA isolation and cDNA synthesis were done as described in the chapter 1. In brief, a set of degenerate primers were designed based on the available *11β-h* nucleotide sequences of different teleosts from the NCBI GenBank database. PCR amplification was performed at 94 ºC (1 min) and 35 cycles of 94 ºC (30 s), 52 ºC (30 s) and 72 ºC (1 min) using degenerate primers (DF and DR; Table 1). The partial cDNA fragment of 402 bp obtained was cloned in pGEM®-T easy vector (Promega), sequenced, analyzed and was confirmed as catfish *11β-h* partial cDNA. RACE strategy was performed to obtain the full length cDNA of *11β-h*. Touchdown PCR reactions were performed using 5P, 5N, 3P and 3N primers (Table 1) as well as anchor primers, universal primer A mix, nested universal primer and the advantage® 2 PCR kit (Clontech) by following manufacturer's universal thermal cycling conditions (Clontech). All the nested PCR amplified fragments were gel purified, cloned in pGEM®-T easy vector (Promega), sequenced, and analyzed using NCBI-BLAST, which showed high similarity with the already known (in other teleosts) *11β-h* cDNA but shorter in length. Hence, a degenerate primer (DR1/Table 1) was designed which along with a specific primer (SpF) were used for RT-PCR, upon cloning, sequence analysis and confirmation, a new set of RACE primers was designed and 3′RACE was repeated, A specific single band was amplified, upon cloning, sequencing and further analysis, it was confirmed as *11β-h*. Further, ClustalW analysis with other teleost *11β-h* showed a gap in the assembled cDNA. Hence a new degenerate

primer (DF1/Table 1) was designed upstream of the 402 bp partial cDNA, which along with a specific primer (SpR/Table 1) amplified PCR fragments, upon subsequent cloning and sequence analysis it was found to be *11β-h*. Upon assembling all the overlapping cDNA fragments, four forms of *11β-h* cDNAs were obtained, which were compared with *11β-h* of other teleosts using the NCBI-BLAST and ClustalW.

2.3. Sequence and Phylogenetic analysis

Multiple alignment of deduced amino acid sequences and phylogenetic analysis were performed based on 11β-h sequences of different teleosts and mammals using ClustalW (DDBJ) as described earlier in the chapter 1. The 3D structures predictions were carried out using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) for all the forms and better models were chosen based on the tm scores. GenBank accession numbers of the sequences used for multiple alignment and /or construction of phylogenetic tree are as follows: *D. labrax* (AF449173), *Micropogonias undulatus* (EU673091), *O. bonariensis* (GQ381267), *Oreochromis niloticus* form 1 (ACY39528), *O. niloticus* form *2* (FJ713104), *D. rerio* (DQ650710), *O. mykiss* form 2 (AF217273), *S. salar* (DQ352841), *Rattus norvegicus 1* (NM_012537), *Ovis aries 1* (OAU78478), *H. sapiens 1* (NM_000497) and *C. batrachus* regular type (KJ475435). *C. gariepinus* 11β-hsd2 (GU220074) was used as an out-group. For clarity of displaying different forms of catfish 11β-h, a different phylogenetic tree (Fig. 3B) was created as mentioned above but by using *C. batrachus* regular type (KJ475435), variant 1 (KJ475436), variant 2 (KJ475437), variant 3 (KJ475438), *O. niloticus* form 1 (ACY39528) and *O. niloticus* form 2 (FJ713104).

2.4. qPCR

Total RNA isolation, reverse transcription and qPCR were done as described in the chapter 1. qPCR primers were designed for the amplicon length of ~250 bp and all reactions were performed in triplicate for three different samples using gene specific primers (Table 1). The changes in the gene expression were calculated by using $2^{-\Delta Ct}$ method. Expression changes after MT and EE_2 treatment were calculated by using the comparative Ct ($2^{-\Delta\Delta Ct}$) method by taking the control as a calibrator.

Table 1. List of primers used for cDNA cloning and qPCR analysis of *11β-h*

S. no.	Primer Name	Primer Sequence 5′ to 3′	Usage
1.	DF	CCTNGGSCCCATWTACAGGSAG	RT-PCR
2.	DR	GTCGTGTCCACYSCYCCBGCCAT	RT-PCR
3.	DF1	GACCGYCTVCWKYTBAACMRKGAG	RT-PCR
4.	DR1	TGCCTSGMSCCRAABCCRAACG	RT-PCR
5.	SpR	TGCCCCAAACCCGAAGGCCAGGGA	RT-PCR
6.	5P	TGTGGTCCCACGCAGTCGCATGGTGAGT	RACE
7.	5N	GAGCAGGGGAGGCGTTGTGTTCAGCA	RACE
8.	5P1	ATCTCGAGCCACCTCGTCCAGCAGC	RACE
9.	5N1	CTCACGGTTCAACAGCAGGCGGTCA	RACE
10.	3P	TGCTGAACACAACGCCTCCCCTGCTC	RACE
11.	3N/SpF	GTCACGCTGATGAGCGAATCCTGCGTGTGT	RACE/ RT-PCR
12.	3P1	GCTGAGAGAGGAGAGAGGCGGGGCA	RACE
13.	3N1	GATCCCTGGCCTTCGGGTTTGG	RACE
14.	ORF F	ATGATCACCTCATCTCTTCG	ORF cloning
15.	ORF R	TCAGGGTGAGAGGGAGGA	ORF cloning
16.	RTF	GGTTCCCCTGCAGTTTGC	qPCR
17.	RTR	TGCACCAGCGTCCCAGCTG	qPCR
18.	11β-hV1RTF	AGGGTCGGGGGGGCAGCA	qPCR
19.	11β-hV1RTR	GACCTGCACACCGACGTAGG	qPCR

20.	**11β-hV2RTF**	**ACAACGCCTCCCCTGCTCTA**	**qPCR**
21.	**11β-hV2RTR**	**TGGACAAGACTGTAGAGGCA**	**qPCR**
22.	**11β-hV3RTF**	**GTCGGGGGGGCAGTGAGG**	**qPCR**
23.	**11β-hV3RTR**	**CATCTTGACTCCGGCCCAG**	**qPCR**
24.	**11β-hV1 ORFR**	**TCACCCTCTCATCCTGCAC**	**ORF cloning**
25.	**11β-hV2 ORFR**	**TTACATACCGTGACTGAAGAT**	**ORF cloning**
26.	**11β-hV3 ORFR**	**TCAAAGACTAAAGGGTCCTG**	**ORF cloning**
27.	**18S rRNAF**	**GCTACCACATCCAAGGAAGGCAGC**	**qPCR**
28.	**18S rRNAR**	**CGGCTGCTGGCACCAGACTTG**	**qPCR**

V1-variant 1; V2-variant 2; V3-variant 3

IUPAC Nucleic acid codes: R- A or G; Y- C or T; S- G or C; W- A or T; K- G or T; M- A or C; B- C or G or T; D- A or G or T; H- A or C or T; V- A or C or G; N- any base

2.5. EIA

Serum and testicular tissue levels of 11-KT were measured by using EIA kit (Cayman Chemical, Ann Arbor, MI) by following the manufacturer's instructions. Serum and testicular tissue collected from catfishes during different stages of reproductive cycle were stored at -80 ºC briefly until assayed. Samples were analyzed following the manufacturer's instructions in a microplate reader (Bio-Rad). The dilutions of series of serum samples and tissue extracts from catfish with differing hormone concentrations were linear with standards of 11-KT. Intra- and inter-assay variations were within the limits specified by the manufacturer's protocol. 11-KT antisera supplied in the EIA kit showed cross reactivity with 4-Androsten-11β,17β-diol-3-one (0.01%), T (<0.01%), 5α-Androstan-17β-ol-3-one (<0.01%) and 5α-Androsten-3β-diol (<0.01%).

2.6. Immunohistochemistry (IHC) and Immunofluorescence (IF)

IHC was carried out to detect 11β-h protein in catfish testis using anti-11β-h polyclonal antibody (Life Span Biosciences, Seattle, WA, USA), which was raised against the C-terminal region of *O. latipes* 11β-h which showed ~70% similarity with the C-terminal region of catfish 11β-h. Western blot analysis was carried out prior to IHC to check the specificity and suitability of the antibody to catfish. The western blot analysis using catfish testicular tissue homogenate showed a single band of ~62 kDa (Fig. 12A), which corresponds to the theoretical molecular weight of deduced regular type catfish 11β-h. While the western blot analysis using antibody, pre-adsorbed with catfish 11β-h partial peptide showed no band (Fig. 12B). Testis of adult catfish (pre-spawning phase) was fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) at 4 ºC overnight and cryosection (7 μm) were prepared on to a Poly-L-Lysine coated slides using a cryostat (Leica CM1850, Leica Microsystems, GmbH, Nussloch, Germany). Anti-11β-h polyclonal antibody (1:1000) or pre-adsorbed antibody (for negative control) was incubated for overnight at 4 ºC in a humid chamber. Following incubation, the sections were incubated with horse radish peroxidase (HRP) conjugated secondary antibody (Merck Bangalore Genei, Bengaluru, India) for 2 h at room temperature (RT). Sections were then incubated with VECTASTAIN® Elite ABC reagent (Vector Laboratories, Burlingame, Calif., USA) for 30 min at room temperature. The slides were washed with PBS and developed using commercially supplied 3′, 3′-diaminobenzidine (DAB) as chromogen and H_2O_2 (Vector Laboratories) as substrate. The sections were counterstained with hematoxylin (Qualigens fine chemicals, Worli, Mumbai, India), dehydrated using a graded ethanol series and mounted using DPX mountant. For IF, FITC-conjugated anti-rabbit secondary antibody (Vector Laboratories) was used. The slides were counterstained and mounted using

VECTASHIELD® mounting media containing DAPI (Vector Laboratories). For IHC, images were acquired using Q capture Pro 6 software (Quantitative Imaging Corporation, BC, Canada) with Micropublisher 3.3 RTV-CCD camera in a CX41 Olympus microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). While for IF, Carl Zeiss LSM 710 confocal microscope containing Zen 2010 software (Carl Zeiss AG, Oberkochen, Germany) together with IX81 Olympus Microscope (Olympus Corporation) containing Cell Sens dimension software were used.

2.7. hCG induction, *in vivo* and *in vitro*

In vivo and *in vitro* hCG inductions were carried out during the mid-preparatory phase (March) of the reproductive cycle as described in the chapter 1. Samples were collected at different time intervals and *11β-h* expression changes were analyzed by using qPCR. The change in the gene expression was calculated by using $2^{-\Delta Ct}$ method.

2.8. Statistical analysis

All data were expressed as the mean ± SEM and all statistical analyses were performed using SigmaPlot 11.0 software (Systat Software Inc.). All data passed homogeneity and normality tests which were compared by one-way ANOVA followed by SNK post-hoc test. A probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning of *11β-h* cDNA from catfish testis

A partial cDNA of 402 bp of *11β-h* was cloned from catfish testis by RT-PCR using degenerate primers DF and DR (Table 1). The full length cDNA of *11β-h* was isolated

from catfish testis using 5′ and 3′RACE strategies with GSP designed based on the partial cDNA of *11β-h*. The cDNA fragments obtained through 5′RACE had been cloned and confirmed as *11β-h* using NCBI-BLAST and ClustalW. However, 3′RACE reaction yielded specific amplification which upon cloning and sequencing showed high similarity with the already known *11β-h* cDNA but shorter in length when compared to the *11β-h* of other teleosts. Repeated attempts by varying the reaction conditions often resulted in the similar sized 3′RACE products without any new longer amplicons. Hence, to search for the other form, which corresponds to the already known *11β-h* cDNA of other teleosts**,** a degenerate primer (*11β-h* DR1/Table 1) was designed at the 3′ side of the partial cDNA using the known *11β-h* cDNA sequence of different fish species which along with a specific primer (SpF) were used for RT-PCR, which yielded a partial cDNA of 344 bp. After sequence analysis and confirmation, a new set of RACE primers was designed based on the 344 bp partial cDNA and used for 3′ RACE, which yielded a specific amplification upon cloning, sequencing and further analysis, it was confirmed as *11β-h*. Upon assembling all the cDNA fragments, ClustalW analysis was carried out using the known *11β-h* cDNA of different teleosts to check the completion of catfish full length cDNA, but a gap of 272 bp was observed. To check, whether that 272 bp exists in the catfish *11β-h* or not, a degenerate primer (DF1) was designed upstream of the 402 bp partial cDNA, which along with a specific primer (SpR) was used for RT-PCR, which yielded a band of 1002 bp which upon cloning, sequencing and further analysis showed the presence of 272 bp and a 3 bp addition in the aligned full length cDNA of catfish. The full length cDNAs of catfish *11β-h* were obtained by aligning the overlapping sequences of 5′, 3′RACE and partial cDNA sequences. In total, 4 forms of *11β-h* full length cDNA

were obtained from catfish testis which were 2253 bp (regular type), 1290 bp (variant 1), 1223 bp (variant 2) and 1978 bp (variant 3) in length which had ORF of 1641 bp (regular type), 1065 bp (variant 1), 867 bp (variant 2) and 1080 bp (variant 3) that encodes putative proteins of 546, 354, 288, 359 amino acid residues respectively. The sequence identity of the *11β-h* cDNA was confirmed by comparing with *11β-h* of other teleosts using NCBI-BLAST and ClustalW. The nucleotide sequence of *11β-h* (all forms) of catfish were submitted to GenBank and the accession numbers are as follows: *11β-h* regular type (KJ475435), variant 1 (KJ475436), variant 2 (KJ475437) and variant 3 (KJ475438). The 3′ untranslated region of *11β-h* (regular type) was 500 bp long which contained a variant polyadenylation signal sequence, AAGAAA with poly-A^+ tail.

3.2. Sequence and phylogenetic analysis

The deduced amino acid sequence of *11β-h* (regular type) in comparison with already characterized 11β-h of other species revealed considerable similarity with *D. rerio* (69%), *O. niloticus* (65%), *H. sapiens* (41%) and *R. norvegicus* (37%). The multiple alignment of 11β-h (regular type) with other teleosts revealed that 11β-h was conserved through teleosts evolution (Fig. 1) specifically at five characteristic regions of P450 enzymes, like steroid binding site, oxygen-binding site, Ozols'region, aromatic and heme-binding region. Except steroid binding site, all the other regions are absent in all the 11β-h variant forms (Fig. 2A). These variants might not have hydroxylase activity but it can regulate substrate availability for the functional/regular type of 11β-h in steroidogenic tissues. 3D structures of all the 11β-h forms predicted using I-TASSER was given in fig. 2B. Better models were chosen for all the 11β-h forms based on the tm

scores. The phylogenetic analysis of 11β-h from different teleosts and mammals showed that all teleosts including *C. batrachus* formed a single clade, while the mammals formed a separate clade (Fig. 3A). A different phylogenetic tree was created using all forms of 11β-h of catfish with *O. niloticus* form 1 and 2 to clearly display the similarity and variation between the different forms of catfish 11β-h (Fig. 3B).

3.3. Tissue distribution of *11β-h* in the catfish

The expression of all forms of *11β-h* was ubiquitous in all the tissues analyzed during the pre-spawning phase of adult catfish. The expression of *11β-h* (regular type and variant 1) showed maximum expression ($P < 0.05$) in the testis when compared to other tissues analyzed (Fig. 4A & B). The expression of *11β-h* (variant 2) showed high expression ($P < 0.05$) in the testis, kidney and spleen, while, *11β-h* (variant 3) showed high expression ($P < 0.05$) in the testis, brain, kidney, muscle and spleen compared to other tissues analyzed (Fig. 4C & D).

3.4. Dimorphic expression of *11β-h* during gonadal ontogeny

qPCR analysis of *11β-h* at different stages of gonadal developmental showed its first sign of expression from 0 dph. Starting from 50 dph, *11β-h* expressed in both testis and ovary differentially throughout gonadal development till maturity. The *11β-h* expression in males is much higher ($P < 0.05$) compared to females during gonadal development and at maturity (Fig. 5).

3.5. Expression of *11β-h* during different phases of testicular recrudescence

The qPCR analysis during testicular reproductive cycle showed that the expression of *11β-h* (regular type) were significantly high ($P < 0.05$) during pre-spawning phase followed by preparatory and spawning phases (Fig. 6A), while, *11β-h* (variant 1) expression was high during preparatory, pre-spawning and spawning phases (Fig. 6B) when compared to post-spawning phase. The expression of *11β-h* (variant 2 and 3) was significantly higher ($P < 0.05$) during preparatory and pre-spawning phases (Fig. 6C & D) when compared to spawning and post-spawning phases. This indicates that the *11β-h* expression was abundant during early and active spermatogenesis and decreased at sperm maturation and spermiation.

3.6. Levels of 11-KT in serum and testicular tissue

The levels of 11-KT was significantly high ($P < 0.05$) during pre-spawning and spawning phases in the serum (Fig. 7A) and in the testicular tissue (Fig. 7B).

3.7. Localization of 11β-h in catfish testis

IHC and IF analyses revealed the presence of 11β-h immunoreactivity (Fig. 8A-E) in the Sertoli and interstitial/Leydig cells of testis. No immunoreactivity was observed in the negative control (Fig. 8F).

3.8. Changes in the expression of *11β-h* in testis after *in vivo* and *in vitro* hCG induction

The hCG induction, *in vivo*, in the mid-preparatory phase of catfish testis significantly enhanced *11β-h* expression (all forms) compared to 0 h. The *11β-h* (regular type and variant 3) expression increased significantly ($P < 0.05$) at 6 , 12 and 18 h, and peaked at

24 h after hCG induction (Fig. 9A & D). The *11β-h* (variant 1 and 2) expression was significantly high ($P < 0.05$) at 12 h followed by 6 and 18 h after hCG induction (Fig. 9B & C). *In vitro* cultures of testicular slices (preparatory phase) revealed significant increase ($P < 0.05$) in *11β-h* expression compared to 0 h at all the time points studied, with maximum at 12 h followed by 24 and 6 h (Fig. 10).

3.9. Changes in the expression of *11β-h* in the testis of MT and EE_2 treated catfish

MT and EE_2 treatments decreased the expression of *11β-h* significantly ($P < 0.05$) to 56 and 22.6% (Fig. 11) respectively, during gonadal development of catfish.

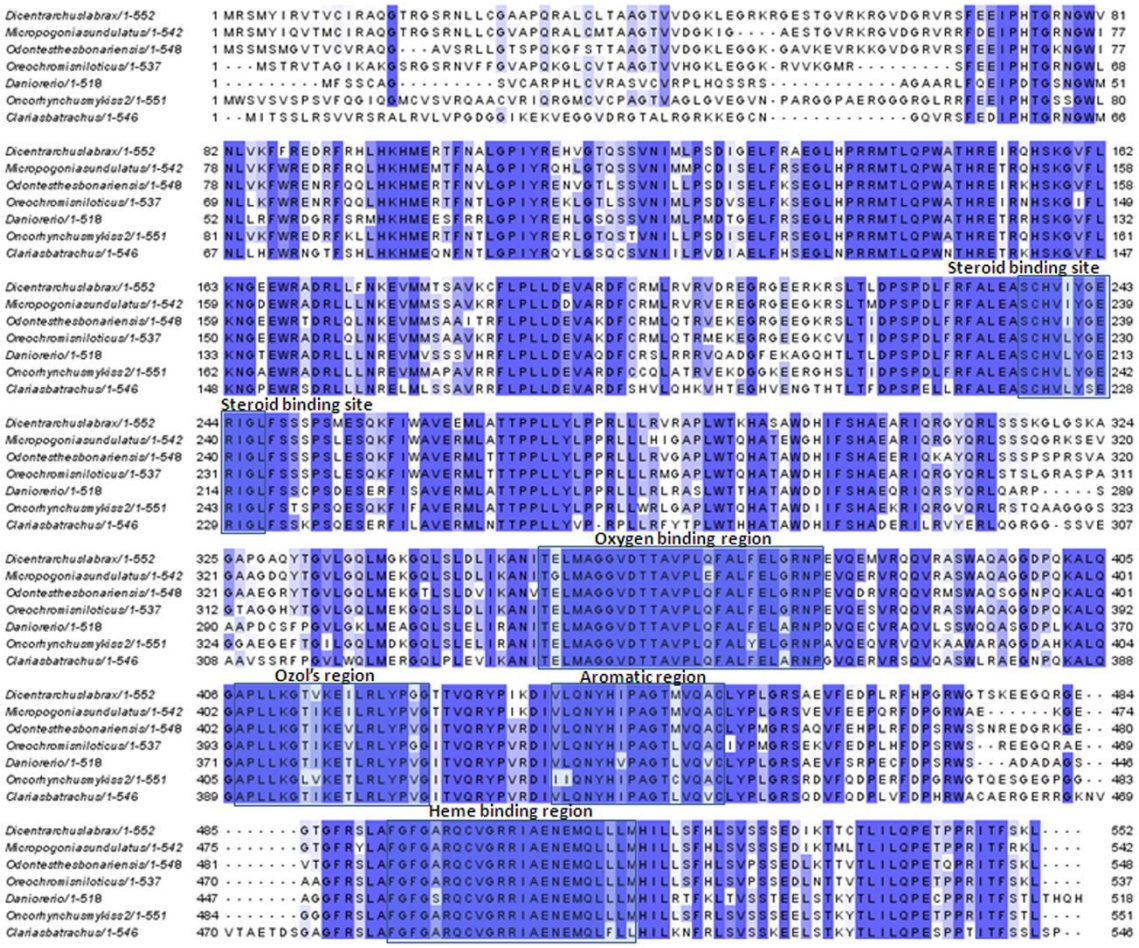

Fig. 1. Multiple alignment of the aminoacid sequences of 11β-h from catfish and other teleosts. The multiple alignment was created using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and high homologous regions are shaded using Jalview 2.8 and the conserved domains are shown as rectangles. GenBank accession numbers of sequences used for the multiple alignment are given in the section 2.3.

Fig. 2A. Multiple alignment of catfish 11β-h (all forms), *C. batrachus* (regular type; KJ475435), variant 1 (V1; KJ475436), variant 2 (V2; KJ475437), variant 3 (V3; KJ475438). The other details are as in fig. 1.

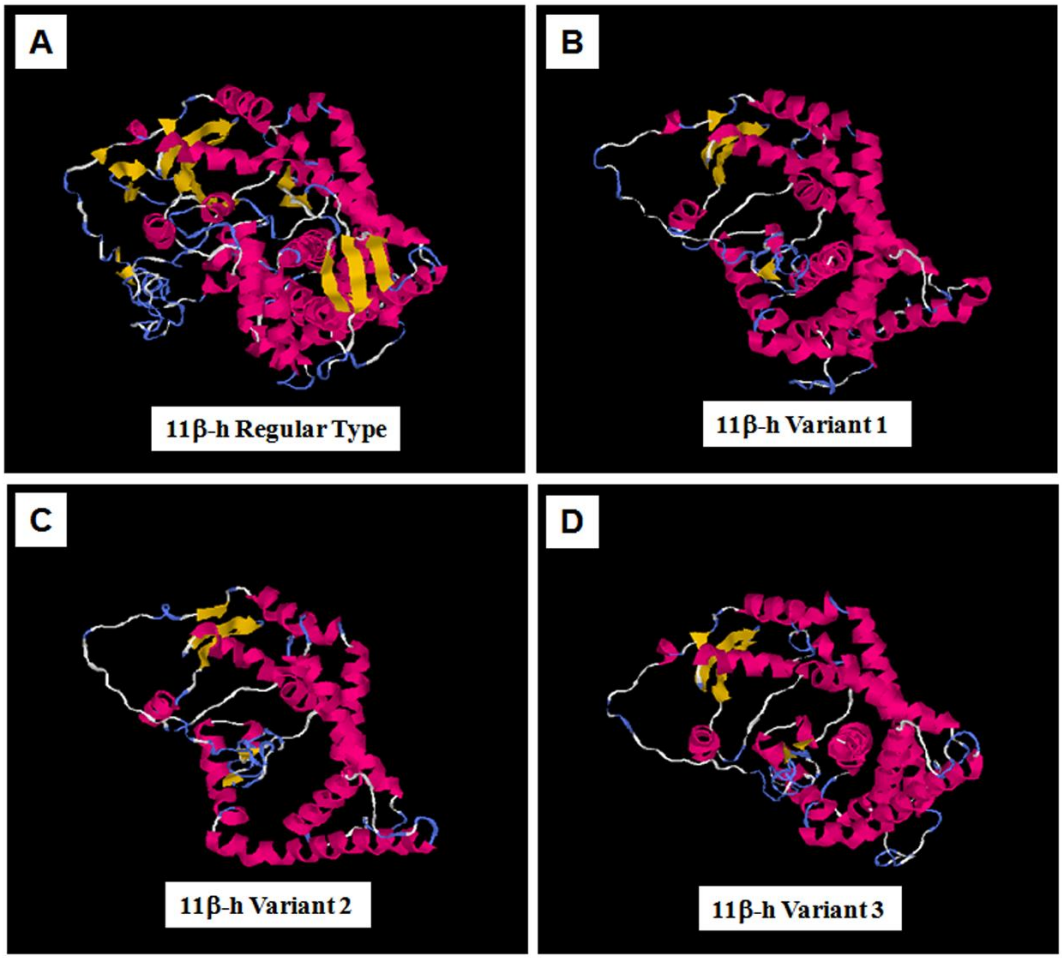

Fig. 2B. Structure prediction of all forms of catfish 11β-h (I-TASSER). Better models were chosen for all the forms based on the tm scores. A) 11β-h Regular type (TM-score = 0.61±0.14; RMSD = 9.4±4.6Å), B) Variant 1 (TM-score = 0.62±0.14; RMSD = 8.2±4.4Å), C) Variant 2 (TM-score = 0.58±0.14; RMSD = 8.5±4.5Å) and D) Variant 3 (TM-score = 0.63±0.14; RMSD = 8.1±4.4Å).

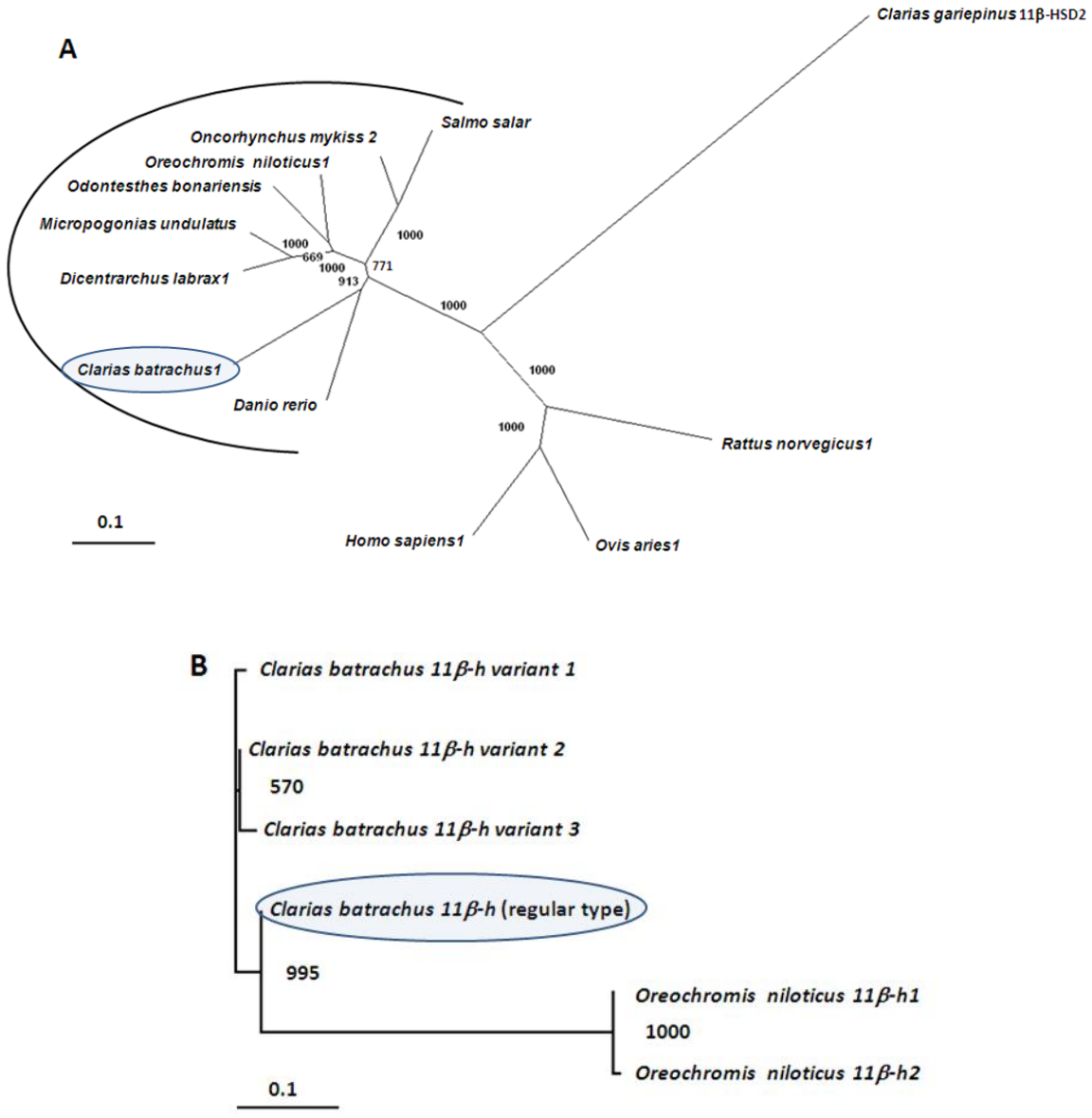

Fig. 3. Phylogenetic analysis of 11β-h of catfish. (A) Catfish 11β-h (regular type) with other vertebrates and (B) Catfish 11β-h (all four forms) with *O. niloticus 1* and *O. niloticus 2*. The phylogenetic analysis was performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/) and displayed using Treeview software 1.6.6. GenBank accession numbers of sequences used for constructing the phylogenetic tree were given in the section 2.3.

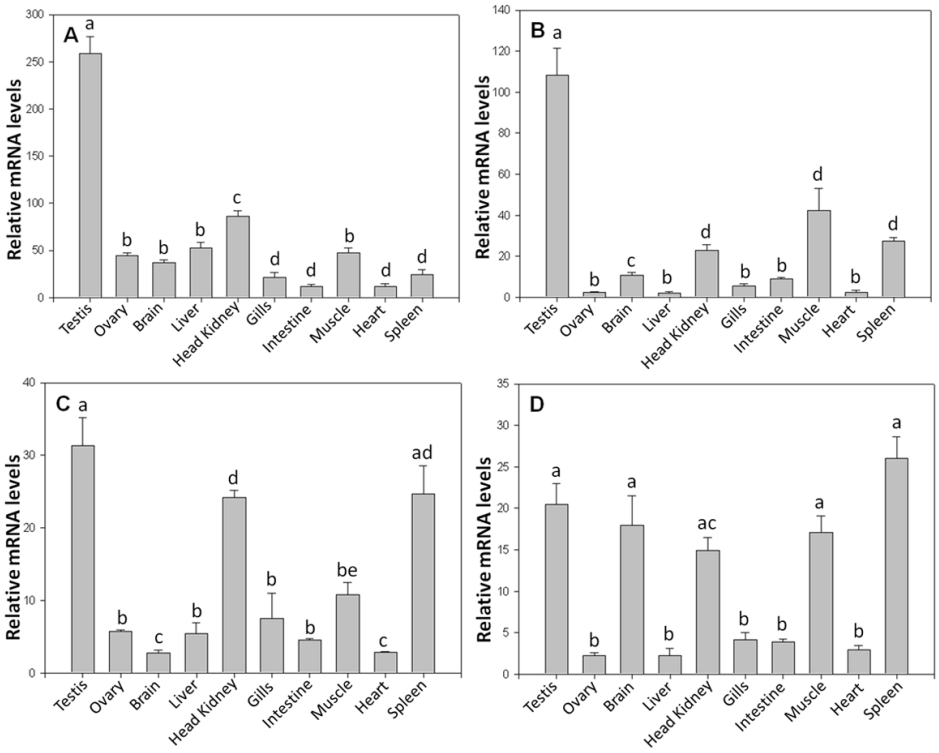

Fig. 4. Relative mRNA levels of *11β-h* (all forms) in different tissues of adult catfish. (A) *11β-h* (regular type), (B) *11β-h* (variant 1), (C) *11β-h* (variant 2) and (D) *11β-h* (variant 3). All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

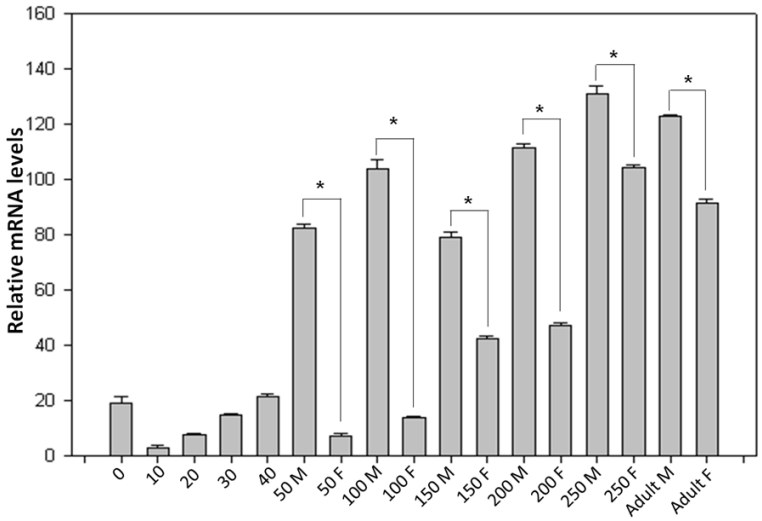

Fig. 5. Relative mRNA levels of *11β-h* (regular type) in different stages of gonadal development. M-Male and F-female. All data were expressed as mean ± SEM. *****indicates means with significantly higher *11β-h* mRNA levels compared with the corresponding females of the same age group (*, $P < 0.05$; ANOVA followed by SNK post-hoc test).

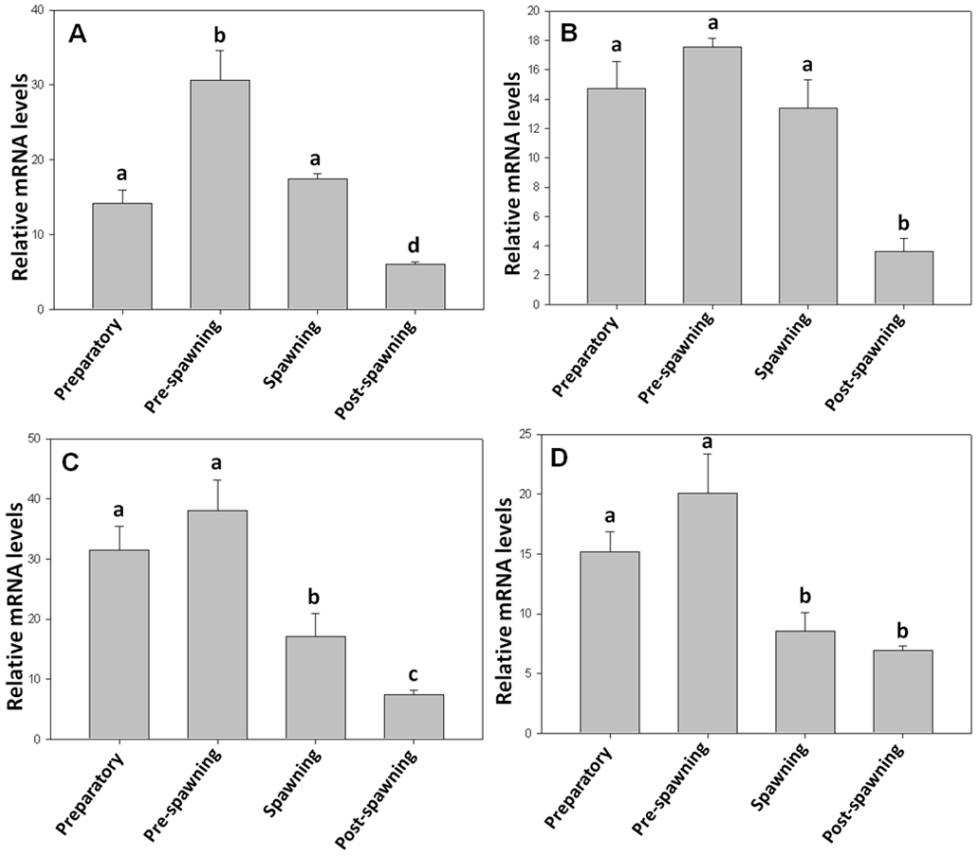

Fig. 6. Relative mRNA levels of *11β-h* in testis of catfish during different phases of reproductive cycle. (A) *11β-h* (regular type), (B) *11β-h* (variant 1), (C) *11β-h* (variant 2) and (D) *11β-h* (variant 3). All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

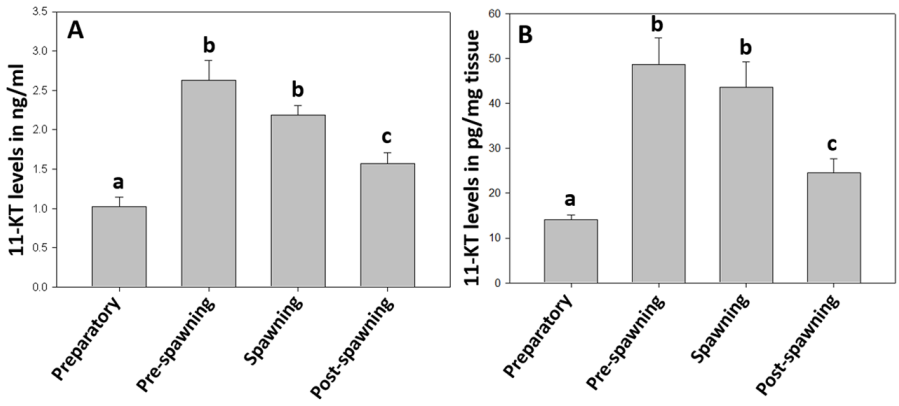

Fig. 7. Levels of 11-KT in (A) serum and (B) testicular tissue of catfish during different phases of reproductive cycle. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

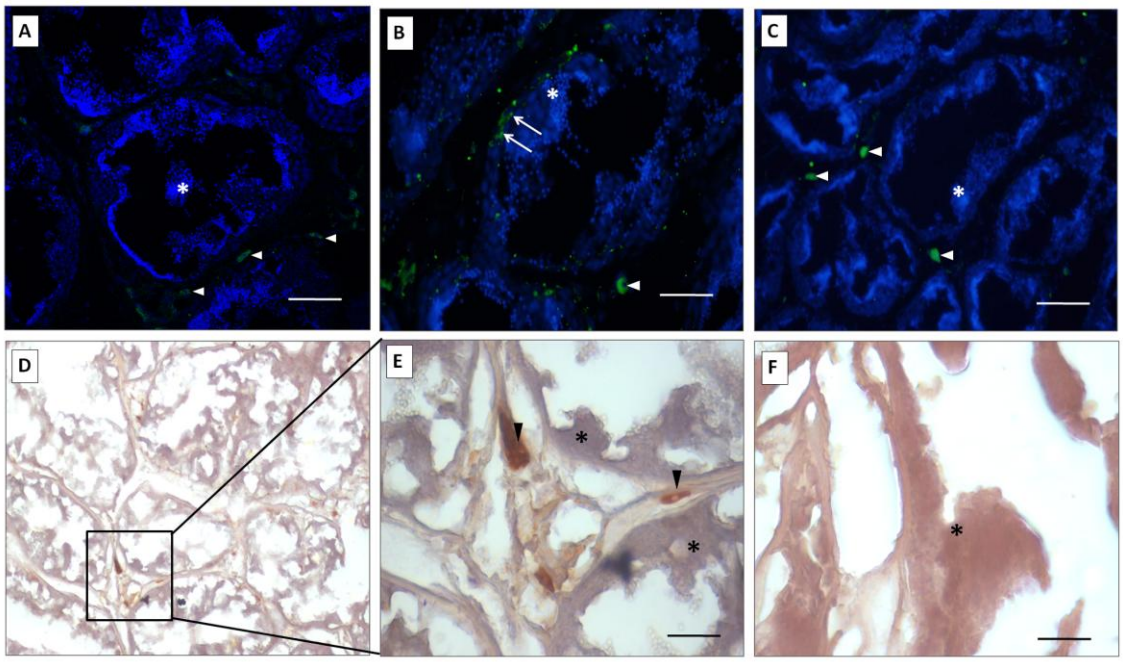

Fig. 8. Localization of 11β-h in the adult catfish testis. (A & B) Testis sections showing immunoreactivity in interstitial/Leydig cells (arrow heads), (C) Leydig cells (arrow heads) showing immunoreactivity near the periphery of a spermatogenic cyst, (D and E) Low and high magnification images showing Leydig cells (arrow heads), and (F)

Negative control (pre-adsorbed antibody) showing no immunoreactivity. Asterisk represents spermatids/sperm. Scale bars in all the panels indicate ~30 μm.

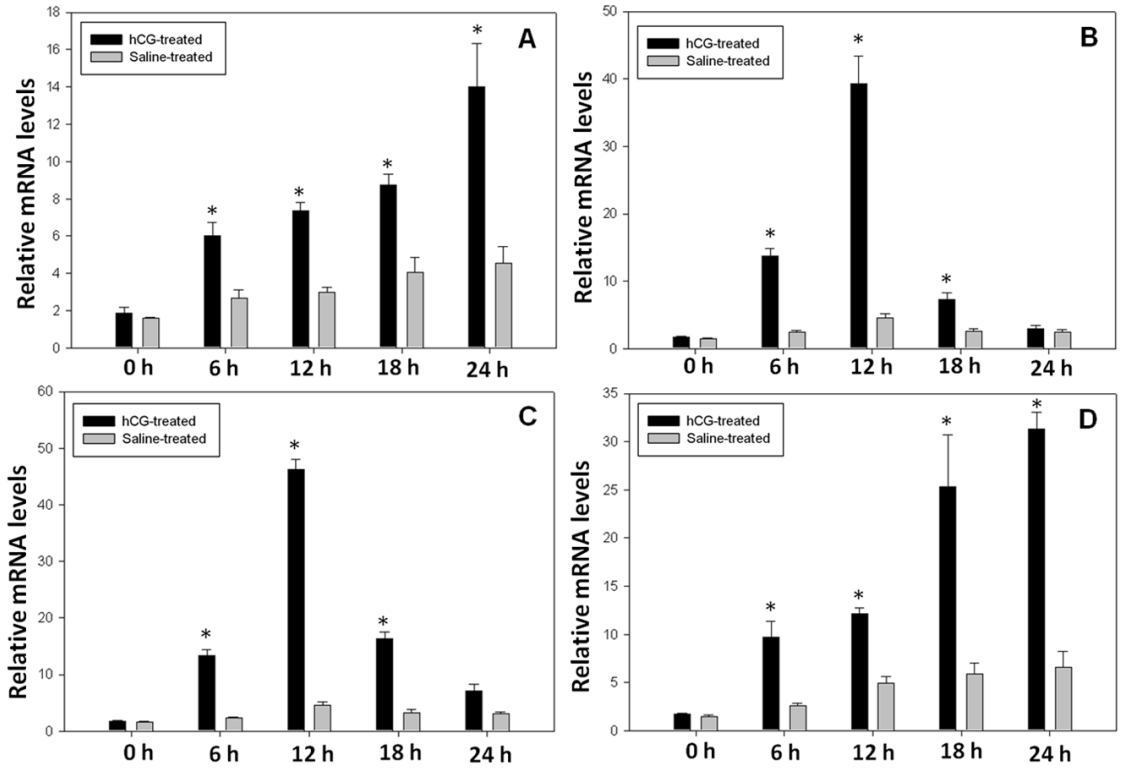

Fig. 9. Relative mRNA levels of *11β-h* in the testis of catfish at different time intervals after *in vivo* hCG induction. (A) *11β-h* (regular type), (B) *11β-h* (variant 1), (C) *11β-h* (variant 2) and (D) *11β-h* (variant 3). All data were expressed as mean ± SEM. *****indicates means with significantly higher *11β-h* mRNA levels compared with the 0 h (*, $P < 0.05$, ANOVA followed by SNK post-hoc test).

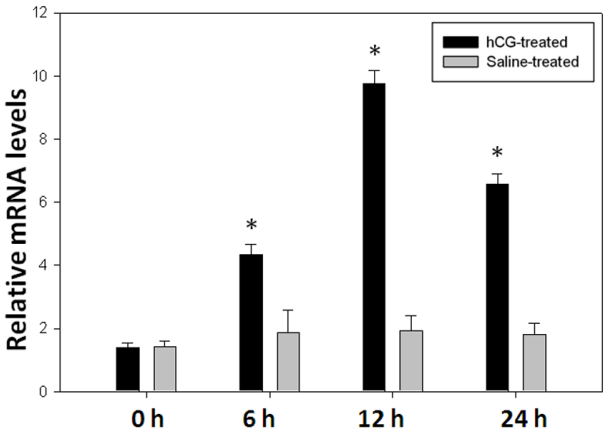

Fig. 10. Relative mRNA levels of *11β-h* (regular type) in the testis of catfish at different time intervals after hCG induction, *in vitro*, in the testicular slices. All data were expressed as mean ± SEM. *****indicates means with significantly higher *11β-h* mRNA levels compared with the 0 h (*, $P < 0.05$, ANOVA followed by SNK post-hoc test).

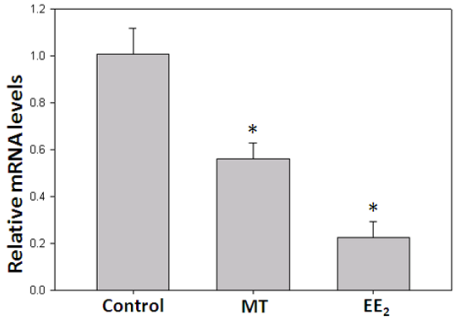

Fig. 11. Relative mRNA levels of *11β-h* (regular type) in the testis of catfish after MT and EE_2 treatments. All data were expressed as mean ± SEM. *****indicates means with significantly lower *11β-h* mRNA levels compared to control (*, $P < 0.05$, ANOVA followed by SNK post-hoc test).

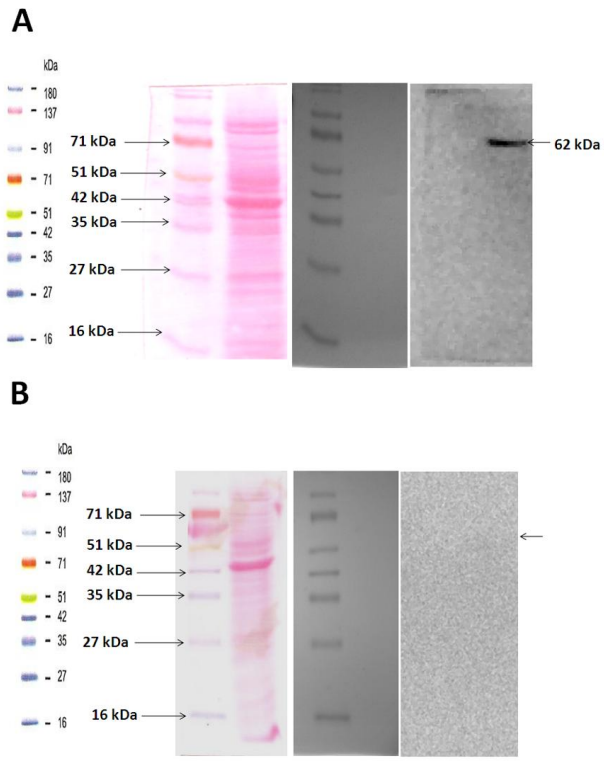

Fig. 12. Western blot analysis demonstrating the antibody characteristics of 11β-h. (A) Testicular lysate detected with 11β-h antibody showed signal [~62 kDa] and (B) Testicular lysate detected with pre-adsorbed antibody showed no signal.

4. Discussion

In this study, we report on the molecular cloning of *11β-h* from catfish and its expression pattern during early stages of gonadal development, different phases of the seasonal reproductive cycle, after hCG induction *in vivo*, *in vitro* and after MT and EE_2 exposure during gonadal development. In addition, measurement of 11-KT levels during different reproductive phases together with 11β-h localization in testis was also done in this study. To our knowledge, the present study was first of its kind to show four forms of *11β-h* transcripts in a teleost.

In the present study, we isolated four cDNAs of *11β-h* using RT-PCR and RACE strategy from catfish testis. In *O. mykiss* and *O. niloticus* the presence of two non-allelic genes and/or isoforms were reported (Liu et al., 2000; Zhang et al., 2010). However, in the present study, three variants of *11β-h* were cloned from *C. batrachus* in addition to a regular type. 11β-h forms from catfish share high similarity (>95%) within themselves, but loss and/or variation at end of the coding region shows that, they may be non-allelic genes. We assume that the presence of different *11β-h* forms with high similarity might be due to recent mutations and/or genomic duplication events in fishes (Meyer and Schartl, 1999). Based on the deduced aminoacid sequence variation in C-terminal region, we presume that the different transcripts identified represent different genes. Homology study of catfish 11β-h (regular type), with that of *D. rerio* (69%) and *O. niloticus* (65%) showed that more amino acid identity exists between these three species suggesting common functions of this enzyme in teleosts. 11β-h (variant 1) shared high similarity with *D. rerio* (59.6%), *O. niloticus* (58.47%) and *H. sapiens* (33.6%). 11β-h (variant 2) shared high similarity with *D. rerio* (60.4%), *O. niloticus* (59.7%) and *H. sapiens* (35.7%). 11β-h (variant 3) shared high similarity with *D. rerio* (59%), *O. niloticus* (57.3%) and *H. sapiens* (33.1%). The deduced amino acids sequence of *11β-h* showed that only 11β-h (regular type) possesses all five region characteristics for P450 enzymes. The 11β-h variant 1, 2 and 3, only possesses complete steroid binding site, however, they lost Ozol's and aromatic region, and oxygen and heme-binding sites. Without these important regions, binding of substrates to the steroid binding site of the enzyme might occur, but the enzyme lacks hydroxylase activity due to the loss of the redox centers which are present in the heme-binding region. However, we could not perform enzyme

activity due to technical limitations to detect 11-OHT specifically. In another study, Zhang et al. (2010) showed that 11β-h isoform 2 of *O. niloticus* lacks important regions. It was hypothesized that the function of 11β-h isoform 2 (non-functional) is to act as a steroid binding protein to regulate the substrate concentration in the steroid producing cells (Zhang et al., 2010). The presence of 3 variant forms in addition to the regular 11β-h type in catfish also indicates competitive binding of precursor substrates. The presence of all domains solely in 11β-h (regular type) indicates that as an essential enzyme for the production of 11-KT. Further studies are necessary to show the cumulative regulation of all steroidogenic enzyme genes, in supplying substrate to 11β-h, which in turn regulate 11-KT production indirectly. The phylogenetic tree shows two distinct clades, where one clade includes all teleost 11β-h and other clade includes mammalian 11β-h. The catfish 11β-h shares high similarity with *D. rerio* 11β-h, forming a separate clade within the teleosts.

Tissue distribution analysis of *11β-h* (all forms) in adult catfish revealed that the expression of *11β-h* was ubiquitous in various tissues with abundance in testis. The increase in the expression of *11β-h* in kidney (regular type, variant 2 and 3) and brain (variant 4) might be due to the extra-gonadal/peripheral conversion in these organs. The spleen (in variant 2 and 3) and muscle (variant 4) also showed increase in *11β-h* expression. This kind of ubiquitous expression of *11β-h* was seen in *D. rerio* (Wang and Orban, 2007) and catfish, *C. gariepinus* (unpublished data). Presence of moderate expression of *11β-h* (regular type) in liver and kidney indicates its importance in contributing peripheral production of 11-KT (Cavaco et al., 1997; Swart et al., 2013). The

expression of *11β-h* during gonadal development of catfish is similar to studies in several teleosts, such as *O. mykiss* (Liu et al., 2000; Kusakabe et al., 2002). The role of androgens in testis differentiation of fish was mainly inferred from results obtained from the *in vivo* steroid treatments. However, the regulation of steroids by steroidogenic enzyme genes is very minimal. In few teleosts, *11β-h* expression was detected well before and during testicular differentiation/development (Liu et al., 2000; Vizziano-Cantonnet et al., 2007), while in the present study, *11β-h* expression was detected as early as 0 dph and showed high expression from 50 dph onwards to adult, indicating active role for 11-KT during mid stages of testicular differentiation/development but not determination. In pejerrey, 11-KT is produced in undifferentiated gonads which are exposed to masculinizing temperatures previous to morphological sex differentiation (Blasco et al., 2013). Dimorphic expression of *11β-h* correlates with the testis development and expression of other testis specific steroidogenic enzyme and transcription factor genes in catfish, *C. gariepinus* during this stage of development (Raghuveer et al., 2011). Further, binding sites for dmrt family of transcription factors were known to exist in *11β-h* promoter (unpublished data). Hence, there might be a possibility that dmrt1 in addition to its direct role in testis differentiation/development, it could activate *11β-h* expression at later stages and in turn increase 11-KT to augment testis growth.

Serum and tissue 11-KT levels were found to be high during the pre-spawning phase of seasonal reproductive cycle correlative to high *11β-h* expression demonstrates the possible importance of 11-KT and indirectly *11β-h* in early spermatogenesis. In the present study, the mRNA level of *11β-h* in testes during the seasonal cycle, was in

accordance with the increased steroid levels, which implies that 11β-h is one of the essential enzymes involved in the enzymatic conversion of T to 11-OHT, a precursor for 11-KT, and 11-KT was shown to be involved in spermatogenesis of teleosts (Schulz and Miura, 2002). Further, the results were comparable to other fish species like *O. mykiss* wherein, *11β-h* expression was high at the beginning of spermatogenesis (Liu et al., 2000) and follows a seasonal pattern similar to that of plasma androgens (Kusakabe et al., 2002), further, in *S. salar*, highest levels of *11β-h* was found when, high 11-KT levels were recorded (Maugars and Schmitz, 2008). Changes in the expression of *11β-h* during sex change in the Nile tilapia correlated with the change in serum level of 11-KT (Nakamura and Nagahama, 1989). The plasma levels of T and 11-KT are high during initial stages of spermatogenesis in many teleosts and declined at sperm maturation, when maturation-inducing steroid, 17α,20β-DP increases considerably (Miura et al., 1991; Borg, 1994; Liu et al., 2000; Scott et al., 2010; Sreenivasulu et al., 2012). Localization analysis using IHC and IF during pre-spawning phase of catfish revealed the presence of 11β-h in the Sertoli and interstitial/Leydig cells of testis. However, no 11β-h could be localized in the germ cells of catfish testis. Sertoli and interstitial/Leydig cell identification were confirmed by previous studies (Schulz et al., 2005; Pfennig et al., 2012) and also from our laboratory with markers such as Sox9 (Raghuveer and Senthilkumaran, 2010) and 20β-hsd (Sreenivasulu et al., 2012).

Previous reports in teleosts, showed that 11β-h was found to be present in the male gonads mainly in Leydig cells (Devlin and Nagahama, 2002; Zhang et al., 2010), however, the present study showed its localization in Sertoli cells for the first time in any teleost. The significance of 11β-h localization in Sertoli cells needs to be evaluated as

these cells are predominantly contributing for sperm nourishment rather than steroidogenesis in teleosts. A recent study (Shima et al., 2013) demonstrated that both Leydig and Sertoli cells are required for T synthesis in the fetal mouse testis. In chondrichthyes, steroid producing Sertoli cells were reported earlier (Sourdaine and Garnier, 1993; Prisco et al., 2008). In the spotted ray testis, steroidogenic enzymes, 3β-hsd and 17β-hsd were localized in Sertoli and Leydig cells and these cells were shown to be differently involved in the hormonal control of spermatogenesis (Prisco et al., 2008).

To understand the regulation and seasonal effects of GtHs on the expression of *11β-h*, hCG induction was carried out during the mid-preparatory phase of catfish, both *in vivo* and *in vitro* on testicular slices. Treatment of hCG up regulated the expression of *11β-h* by several folds indicating that in addition to local control of steroidogenesis in testis, the process was regulated at the levels of GtH. In immature eel, hCG induced *11β-h* mRNA expression in Leydig cells (Jiang et al., 1996). In the present study, the sex steroid analog, MT and EE_2 decreased *11β-h* expression during gonadal development much like androgen receptor blocker, flutamide in the same species, wherein the expression of *11β-hsd* (11-KT production) and *17β-hsd12* (T production) were found to be decreased (Chapter 6). Further, EE_2 exposure repressed the activity of 11β-h similar to E_2 suppression of 11β-h in *O. mykiss* (Govoroun et al., 2001). The down regulation of *11β-h* expression by MT or EE_2 has occurred directly because of feedback intervention and indirectly by limitation of precursor substrate which eventually diminishes *11β-h* expression.

5. Conclusions

The present study demonstrated the expression changes of *11β-h* during gonadal development, maturation and different stages of reproductive cycle in catfish. Interestingly, the study showed the presence of four forms of *11β-h* in catfish, yet a single form might be involved competitively to produce 11-OHT and in turn, 11-KT. Further, changes in the levels of 11-KT and *11β-h* expression during different phases, together with 11β-h localization in Sertoli and interstitial/Leydig cells indicate its possible involvement in spermatogenesis. In addition, the present work demonstrated the GtH dependency of *11β-h* using hCG. Sexual dimorphic and differential expression, together with 11-KT levels can further corroborate the importance of 11β-h in testicular development and function including reproductive cycle of catfish.

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Chapter 4

Isolation of 11β-hsd in catfish: localization, expression changes, promoter motif analysis and regulation by transcription factor Sox3

Abstract

In teleosts, the expression of steroidogenic enzyme genes and their related transcription factors (TFs) are critical for the regulation of steroidogenesis and gonadal development. In fishes, 11-KT is the potent androgen and hence, 11β-hsd, enzyme involved in the conversion of 11-OHT to 11-KT is important. Expression of *11β-hsd* together with its promoter level regulation is critical for sex-, stage- and tissue-specific expression which was never studied in any teleosts. Hence to understand the importance of *11β-hsd* and its promoter level regulation, the present study was conducted using catfish as a teleost model. At first the ORF of *11β-hsd* was cloned from catfish testis and expression changes were analyzed during gonadal development and maturation together with the regulation of seasonal reproductive cycle in catfish. *11β-hsd* was expressed ubiquitously with high levels in testis and liver. *11β-hsd* expression starts very early during development in addition, stage- and sex-dependent increase were seen in the ontogenic studies. Further, *11β-hsd* expression was higher during spawning phase of reproductive cycle and was found to be GtH inducible both *in vivo* and *in vitro*. IHC and IF analysis revealed the presence of 11β-hsd immunoreactivity in the interstitial/Leydig cells and Sertoli of testis. To clone the 5′ upstream region of *11β-hsd*, genomic DNA library was prepared from catfish testis and used for two rounds of PCR reactions. In total, ~2kb of 5′ upstream region was cloned and *in silico* promoter and potential TF binding site prediction were done using various promoter prediction and TF binding site algorithms. Sox3, Wt1, Pax2, Dmrt1 and Ad4BP/SF-1 binding sites were found to exist in the cloned *11β-hsd* promoter region. Luciferase reporter assay using the sequential deletion constructs of the promoter in Human embryonic kidney and Chinese hamster ovary cells revealed the high promoter

activity of the constructs containing Sox3 sites, but not with other sites. Site-directed mutagenesis, Sox3 over expression and electrophoretic mobility shift assay with Sox3 binding sites further substantiated the importance of Sox3. These results suggest that Sox3 binds to the *11β-hsd* promoter and activates its transcription and thus it appears to be a critical factor that regulates 11β-hsd expression during gonadal development, maturation and seasonal cycle in *C. batrachus*.

1. Introduction

Steroidogenesis entails processes by which cholesterol is converted to biologically active steroid hormones with the help of several intermediate enzymes. In fishes, T and 11-KT are principal androgens and their production is regulated at multiple levels during steroidogenesis (Borg, 1994). 11-KT is produced in testicular Leydig cells in addition to their peripheral conversion in adrenal and liver, and the final two steps are catalyzed by 11β-h and 11β-hsd (Jiang et al., 1998; Swart et al., 2013). The ability of fish 11β-hsd to convert 11-OHT into 11-KT was first demonstrated using recombinant 11β-hsd in rainbow trout (Kusakabe et al., 2003). T is hydroxylated at carbon 11 by 11β-h and the resulting 11-OHT is oxidized into 11-KT by 11β-hsd (Nagahama, 1994; Cavaco et al., 1997; Jiang et al., 1998; Kobayashi et al., 1998). In most teleosts, 11-KT was higher than T during all stages of spermatogenesis and was suggested to play important role in the normal progression of spermatogenesis (Koldras et al., 1990; Borg, 1994; Weltzien et al., 2002). 11-KT has been shown to activate all stages of spermatogenesis, in addition to their involvement in inducing male sexual phenotype, secondary sexual characteristics and female-to-male sex-reversal (Mayer et al., 1990; Cardwell and Liley, 1991; Miura et al., 1991; Kobayashi and Nakanishi, 1999). 11-KT promotes activin βB synthesis by

Sertoli cells and thereby inducing pre-meiotic spermatogonial proliferation (Ozaki et al., 2006; Dietrich and Krieger, 2009). Regulation of *11β-hsd* expression at the promoter level seems to be critical as it is directly involved in the production of 11-KT, a potent androgen in fishes (Borg, 1994), in addition to their role in inactivating glucocorticoids (Jiang et al., 2003; Ozaki et al., 2006). Inhibition of this enzyme may enhance glucocorticoid levels, which are known to affect testicular development (Consten et al., 2001) and down regulate/inhibit *cyp11a1*, *3β-hsd* and *star* to diminish androgen biosynthesis (Xiao et al., 2010). Hence, *11β-hsd* is important for maintaining the overall steroidogenesis in addition to their direct role in 11-KT production. *11β-hsd* expression and enzyme activity was shown to be upregulated by LH in rat Leydig cells *in vitro* (Wang et al., 2009) and also by hCG induction in catfish, *C. gariepinus in vivo* (Rasheeda et al., 2010), which shows the GtH regulation of *11β-hsd* function.

Sex steroids/sex steroid analogues administered at the time of sex determination can strongly influence the course of sex differentiation in fishes. Thus, the role of sex steroids and expression of steroidogenic enzyme genes seems to be critical for teleostean reproduction. Differential expression of specific steroidogenic enzyme genes during different stages of gonadal development, maturation and seasonal cycle results in the production of specific sex-steroids required for specific processes. However, only very little is known about their promoter regulation and how they are regulated both at mRNA and protein levels which needs to be explored in detail. Taken together, *11β-hsd* seems to be an ideal candidate to study the promoter level regulation, as it has been never studied in any teleost species.

In silico/functional analysis on various steroidogenic enzyme genes in teleosts showed the transcription binding sites for NR5A, Creb, Wt1 and Sox family of TFs (Hu et al., 2001; Parker et al., 2002; Wang et al., 2007; Sreenivas et al., 2012; Sridevi et al., 2012; personal communication). Owing to the conserved nature of these transcription factors and steroidogenic enzyme genes, it is worthwhile to study the possible involvement of these families of TFs in *11β-hsd* regulation at the promoter level which might provide valuable insight into the physiological roles of specific upstream factors in eliciting their effects on steroidogenesis and spermatogenesis. Hence, to understand these processes, the present study was conducted using catfish, *C. batrachus* an annual breeder, which might provide basic understanding on the implication of steroidogenic enzyme gene with the regulation by TFs cohesively during gonadal development, maturation and seasonal reproductive cycle in male teleosts.

2. Materials and methods

2.1. Animals

IVF, sampling for ontogeny, tissue distribution and seasonal reproductive cycle studies using catfish were done as described earlier in the chapter 1.

2.2. Cell culture

Human embryonic kidney (HEK 293) and Chinese hamster ovary (CHO) cell lines were procured from the National Center for Cell Science (Pune, India). Usage of various cell lines has been taken up to test the efficacy of the promoter in a broad way in addition to their availability. Cells were grown in either 100mmx20mm culture dishes or 24 well plates in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotic and antimycotic solution (Gibco-

BRL, Invitrogen, Carlsbad, CA,USA) at 37 °C, 5% CO_2. Passage number of both the cell lines was maintained at less than 10 for all the experiments.

2.3. Molecular cloning of *11β-hsd* cDNA and functional analysis

Total RNA isolation and cDNA synthesis were done as described in the chapter 1. ORF primers of *C. gariepinus 11β-hsd*, was used to amplify the ORF of *11β-hsd* from *C. batrachus*. The nucleotide sequence variations at the primer region of *11β-hsd* were confirmed by using RACE. Based on the sequence data, a new set of *C. batrachus* specific *11β-hsd* ORF primers (Table 1) were designed and used to clone it into pcDNA 3.1(+) mammalian expression vector (Invitrogen) and the sequence integrity of the insert was verified by nucleotide sequence analysis. Transfection was carried out with the pcDNA-*11β-hsd* ORF plasmid in HEK 293 cells and later supplemented with substrate (11-OHT) as described previously (Rasheeda et al., 2010). After 24 h incubation, culture medium was collected, extracted with diethyl ether and used for 11-KT EIA (Cayman) according to the manufacturer's protocol. Cross reactivity, intra- and inter-assay variations were within the limits as specified in the manufacturer's instructions.

2.4. Phylogenetic analysis

Multiple alignment of deduced amino acid sequences were performed using ClustalW (DDBJ) and TreeView software 1.6.6 was used to display the phylogenetic tree as described in the chapter 1. GenBank accession numbers of the sequences used for the construction of phylogenetic tree are as follows: *D. rerio* (AAH65613), *G. rarus* (AGJ70766), *A. japonica* (BAF35260), *O. niloticus* (NP_001266686), *C. gariepinus* (ADI60062), *H. sapiens* (AAH64536), *M. musculus* (NP_032315), *O. aries* (AAB50810) and *C. batrachus* (Submitted to GenBank).

2.5. qPCR

Total RNA isolation, cDNA synthesis and qPCR analysis were done as described in the chapter 1. qPCR primers were designed for the amplicon length of ~250 bp and all reactions were performed in triplicate for three different samples using gene specific primers (Table 1). The change in the gene expression was calculated by using $2^{-\Delta Ct}$ method.

2.6. IHC and IF

IHC was carried out to detect 11β-hsd protein in catfish testis using anti-11β-hsd polyclonal antibody, which was raised in rabbit against C-terminal region of *C. batrachus* 11β-hsd. Testis of adult catfish (pre-spawning phase) was fixed in 4% PFA in PBS (pH 7.4) at 4 ºC overnight and cryosection (7 μm) were prepared on to a Poly-L-Lysine coated slides using a cryostat (Leica CM1850, Leica Microsystems). Anti-11β-hsd polyclonal antibody (1:1000) or pre-adsorbed antibody (for negative control) was incubated for overnight at 4 ºC and proceeded further as described in the chapter 3. The IHC slides were counterstained with hematoxylin (Qualigens) and mounted using DPX mountant. For IF, FITC-conjugated anti-rabbit secondary antibody (Vector Laboratories) was used. The slides were counterstained and mounted using VECTASHIELD® mounting media containing DAPI (Vector Laboratories). For IHC, images were taken with CX41 Olympus microscope (Olympus Corporation) fitted with Micropublisher 3.3 RTV-CCD camera through Q capture Pro 6 software (Quantitative Imaging Corporation). While, for IF, Carl Zeiss LSM 710 confocal microscope containing Zen 2010 software (Carl Zeiss AG) was used.

2.7. hCG induction, *in vivo* and *in vitro*

In vivo and *in vitro* hCG inductions were carried out during the mid-preparatory phase (March) of reproductive cycle as described in the chapter 1. Samples were collected at different time intervals and expression changes were analyzed by using qPCR. The change in the gene expression was calculated by using $2^{-\Delta Ct}$ method.

2.8. Isolation of 5′ upstream region of *11β-hsd* by genome walking strategy

Genomic DNA was isolated from the catfish testis using manufacturer's protocol (Qiagen, GmbH, Germany). After checking the integrity of the prepared genomic DNA using agarose gel electrophoresis, it was utilized for the construction of genome walking library using Universal Genome Walker kit (Clontech). At first, aliquots of genomic DNA (25 µg) were digested overnight with EcoRV, PvuII, DraI or StuI and were ligated separately to the Genome Walker adaptor. Primary and secondary PCR amplification were carried out with the prepared libraries and primers, AP1, 5PW and AP2, 5NW respectively (Table 1). High fidelity advantage 2 polymerase (Clontech) was used for PCR amplifications in a 2720 thermal cycler (Applied Biosystems). Secondary PCR amplicons were cloned into pGEM®-T Easy vector (Promega) and sequenced bi-directionally using dye terminator cycle sequencing method in an ABI 3730 DNA analyzer (Applied Biosystems). Promoter prediction was carried out using the neural network promoter prediction and McPromoter programs. Putative TF binding sites were predicted using the MatInspector matrix family library version 9.0 of MatInspector professional 8.06 (Genomatix Software Suite, München, Germany). The parameters were set to predict general core promoter elements for vertebrates with maximum score of 1.0 and minimum of 0.75. The program output generated a table of matrices with several predicted transcription factor binding sites but only those with high core similarity

together with possible importance in reproductive/steroidogenic processes were chosen for further functional analysis.

2.9. *11β-hsd* plasmid constructs and site directed mutagenesis (SDM)

Progressive deletion constructs of *11β-hsd* 5′ upstream region were prepared by using PCR with restriction site flanked gene specific primers (Table 1). The resulting PCR amplicons were double digested with XhoI and KpnI and subsequently cloned into XhoI and KpnI sites of pGL3-basic vector (Promega). The identity of each construct was verified by double digestion and the absence of cloning artifacts were verified using plasmid DNA sequencing. SDM (Q5 NEB) was carried out to mutate the specific TF binding sites. Luciferase plasmid constructs bearing mutation in either one or both Sox3 binding motifs were constructed by PCR-based mutagenesis using primers (NEB base changer) containing the mutated bases (Table 1). ORF of catfish Sox3 was cloned into pcDNA3.1 using ORF primers (Table 1) and was used for over expression studies by co-transfection with deletion constructs, while, reverse locked pcDNA-Sox3 ORF was used as negative control.

2.10. Transient transfection and luciferase reporter assay

HEK 293 and CHO cells were grown in DMEM supplemented with 10% FBS and 2 mM L-glutamine in 24-well plates. At around 70-80% confluence promoter plasmid constructs (500 ng) and pcDNA-Sox3 plasmids (100 ng) were transfected using Lipofectamine 2000 reagent (Invitrogen) as per manufacturer's protocol. Promoter activity analysis was performed as per the method described earlier (Sreenivasulu et al., 2012). In brief, pRL-TK plasmid (10 ng) expressing *Renilla* luciferase under herpes

simplex virus thymidine kinase promoter was used as an internal control. Plasmids were prepared with an endotoxin free plasmid isolation kit (Qiagen) and used for transfections. After transfection (~36 hour), cells were washed once with PBS and lysed with 120 µl (per well) of passive lysis buffer (Promega) for 20 min at 37 ºC in a rocker. Cell lysates were collected from the plates, vortexed and briefly spun at 4 °C (12,000g, 30s) and the supernatant was used to check promoter activity. The level of reporter gene expression was quantified by using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol with a GloMax 20/20 single tube luminometer (Promega). Results are presented as the average of firefly luciferase activity expressed as the ratio to the *Renilla* luciferase activity. All experiments were conducted for three independent samples in triplicate. Plasmid constructs used for transfection are as follows: 1) 500 ng of different *11β-hsd* sequential deletion constructs (normal as well as mutated) inserted in the pGL3-basic luciferase reporter plasmid. 2) 100 ng of pcDNA3.1 plasmid expressing Sox3 protein (forward/ reverse locked). 3) 10 ng of pRL-TK vector (Promega) was employed as an internal control.

2.11. Electrophoretic mobility shift assay (EMSA)

Nuclear extract preparation and EMSA were performed by following the method of Smith and Delbary-Gossart (2001) and Sreenivasulu et al., (2012) with minor modifications. In brief, oligonucleotides corresponding to the Sox3 site (A) was synthesized (Table 1) and annealed into double strands. Radiolabelled probes were generated by incubation of 250 ng annealed oligonucleotides with 20 µCi [γ-^{32}P] dATP in the presence of T4 Polynucleotide Kinase (Fermentas) for 30 min at 37°C and were subsequently separated from free nucleotides using G-25 column purification (GE-

Healthcare, Amersham Biosciences, Uppsala, Sweden). Nuclear extract (25 µg) was incubated with ~20 fmol of [γ-^{32}P] end labelled wild or mutated Sox3 oligonucleotides (Table 1) in the presence of 0.5 µg of poly(dI-dC) in binding buffer (20 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.4 mM DTT and 7.5% glycerol) for 30 min at 37°C. The DNA-protein complex formed was separated on a 6% native polyacrylamide gel with 0.5X Tris-borate EDTA running buffer. For cold competition, different concentrations of unlabelled/cold (ds) oligos were incubated with the nuclear extract in binding reaction prior to the addition of radiolabelled probe. For supershift, binding reaction was subsequently incubated with 3 µg of Sox3 antibody for 30 min at RT. The gels were exposed to a phosphorimager cassette and scanned with a typhoon trio+ variable mode imager (GE-Healthcare, Amersham biosciences).

Table 1. List of primers used for cloning, qPCR and promoter motif analysis

S.no.	Primer Name	Primer Sequence 5′ to 3′	Usage
1.	Fr	ATGATGGATGCCTATGCCCTG	pcDNA ORF cloning
2.	Rv	CTATAAAAGATGTACTCCATC	pcDNA ORF cloning
3.	5PW	GAAGACGACGAGTGCCAGCACAGCAGG	Walking
4.	5NW	GCACGACACTGACATGGGTCGCCAGGAAC	Walking
5.	5PW1	GAGAGAGCGTATGGGGGGATACAATTGT	Walking
6.	5NW1	CCTATGAACAGTGTATTCAGCACCAGGT	Walking
7.	RTF	TCATCACAGGGTGTGACTCCG	qPCR
8.	RTR	GAGCCCACAGTCCTTTCATG	qPCR
9.	18S rRNAF	GCTACCACATCCAAGGAAGGCAGC	qPCR
10.	18S rRNAR	CGGCTGCTGGCACCAGACTTG	qPCR
11.	D1F	CGGGGTACCCCGCTGATGATGGGTAAGCCTGT	Del. Construct
12.	D2F	CGGGGTACCCCGCACAAGAGTATAGAGAAATC	Del. Construct
13.	D3F	CGGGGTACCCCGCATTAGTTTGAGCTGCTTCA	Del. Construct
14.	D4F	CGGGGTACCCCGCTCTCTCTTTTAATGGTGAC	Del. Construct
15.	D5F	CGGGGTACCCCGCTTAGTGACACCTGCCCATC	Del. Construct

16.	**D6F**	**CGGGGTACCCCGCTGATTAGATCCGTCCCTGA**	**Del. Construct**
17.	**TATAF**	**CGGGGTACCCCGTATAGATCTAAAGTGTAGA**	**Del. Construct**
18.	**DR**	**CCGCTCGAGCGGACAGGGCATAGGCATCCATCTA**	**Del. Construct**
19.	**SDMF1**	**cttctcctCAATAACTTTCATCATTTCATGTC**	**SDM (Sox3-A site)**
20.	**SDMR1**	**gggtgcggCATAGGTGTTTAATGCAGG**	**SDM (Sox3-A site)**
21.	**SDMF2**	**tttccttcAAGAGAATATAGATCTAAAGTGTAG**	**SDM (Sox3-B site)**
22.	**SDMR2**	**gggagagcTCTTTTTTTTCTTCTTCTTCTTCTG**	**SDM (Sox3-B site)**
23.	**EMSA N**	**ACCTATGAATACAAAAGGAGAAGCAATAACTTTCATC**	**Normal EMSA probe**
24.	**EMSA M**	**ACCTAGTCCGCACCCCTTCTCCTACCGAACTTTCATC**	**Mutant EMSA probe**
25.	**Sox3pcF**	**ATGATGGATGCCTATGCCCTG**	**pcDNA ORF**
26.	**Sox3pcR**	**TTACTCCATCGTGGTGATGT**	**pcDNA ORF**

2.12. Statistical analysis

All data were expressed as the mean ± SEM and all statistical analyses were performed using SigmaPlot 11.0 software (Systat Software Inc.). All data passed homogeneity and normality tests which were compared by one-way ANOVA followed by SNK post hoc test. A probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning of *11β-hsd* cDNA and *in vitro* functional analysis

A cDNA of 1236 bp of *11β-hsd* obtained was cloned from *C. batrachus* testis and confirmed as *11β-hsd2* by comparing with *11β-hsd* of other teleosts using NCBI-BLAST and ClustalW and were submitted to GenBank. Further, Catfish *11β-hsd* ORF cloned into pcDNA 3.1(+) mammalian expression vector could convert ~40% of the substrate (11-OHT) when transfected into HEK 293 cells. Based on these we conclude the cloned *11β-*

hsd form from catfish is indeed belongs to type 2. However for ease of expression, it was named simply as "*11β-hsd*" throughout this chapter and dissertation.

3.2. Sequence and phylogenetic analysis

The multiple alignment of deduced amino acid sequence of catfish 11β-hsd in comparison with other vertebrate counterparts revealed high homology in NAD binding domain and catalytic site (Fig. 1). The phylogenetic analysis of 11β-hsd from different teleosts showed that 11β-hsd of *C. batrachus* showed more homology with 11β-hsd of *C. gariepinus* (89%) and *D. rerio* (74%), while all the mammals formed a separate clade (Fig. 2).

3.3. Tissue distribution of *11β-hsd* in the catfish

The expression of *11β-hsd* was ubiquitous in all the tissues analyzed with higher levels ($P< 0.05$) in the testis and liver than the other tissues analyzed (Fig. 3).

3.4. Dimorphic expression of *11β-hsd* during gonadal ontogeny

qPCR analysis of *11β-hsd* at different stages of gonadal developmental showed its first sign of expression from 0 dph. *11β-hsd* was expressed differentially in both testis and ovary from 50 dph, throughout gonadal development till maturity. The *11β-hsd* expression in testis is much higher ($P< 0.05$) compared to ovary during gonadal development and at maturity (Fig. 4).

3.5. Expression of *11β-hsd* in comparison with *sox3* during recrudescence

Expression of *11β-hsd* was significantly higher ($P < 0.05$) during spawning phase (Fig. 5) compared to other phases of testicular seasonal cycle more similar to the transcription factor *sox3* (Fig. 5).

3.6. Changes in the expression of *11β-hsd* in testis after *in vivo* and *in vitro* hCG induction

hCG induction significantly enhanced the expression of *11β-hsd* when compared to 0 h. The *11β-hsd* expression increased significantly ($P < 0.05$) at 6 h after hCG induction both *in vivo* (Fig. 6A) and *in vitro* (Fig. 6B) when compared to other time points.

3.7. Localization of 11β-hsd in catfish testis

IHC and IF analyses in the pre-spawning phase of adult testis revealed the presence of 11β-hsd immunoreactivity in the Sertoli and interstitial/Leydig cells of testis (Fig. 7A & B). No immunoreactivity was observed in the pre-adsorbed negative control (Fig. 7C).

3.8. Cloning and promoter organization of 5′ upstream region of *11β-hsd* gene

To find and understand the functional regions in the promoter, genomic sequence upstream of the *11β-hsd* cDNA sequence is required. As a first step, genomic DNA was isolated from the catfish testis and used for library construction and the prepared libraries were used for cloning of 5′ upstream region of *11β-hsd* (Fig. 8A). Two rounds of PCR amplifications were carried out using the libraries and the products (~1.4 and 0.9 kb) were cloned, which corresponds to the 5′ upstream region of *11β-hsd* which was submitted to GenBank. *In silico* promoter motif analysis revealed the presence of binding motifs of Dmrt1, Wt1, Ad4BP/SF-1, Sox3, glucocorticoid receptor (GR) binding elements and TATA box (Fig. 8B & C). The putative transcription start site (+1) of *11β-hsd* was predicted based on the 5′RACE data.

3.9. Identification of functional Sox3 binding sites in the upstream region of *11β-hsd*

To assess the functionality of these identified binding motifs, a series of PCR based deletion constructs (Fig. 8B) were prepared in pGL3-basic vector and were transfected into HEK 293 and CHO cells. The constructs, which contained both the Sox3 binding sites showed a significantly high ($P < 0.05$) promoter activity in both the cell lines. The construct with TATA box showed only minimal activity. Whether the other TATA box, which is present upstream of Sox3 (site-B) involved in the basal activity is not clear. The presence of other sites, Wt1, Dmrt1 and Ad4BP/SF-1 did not alter the promoter activity significantly further in both the cell lines (Fig. 9A & B). These results depict that both the Sox3 binding motifs are functional in the *11β-hsd* promoter region.

3.10. Promoter activity after SDM of Sox3 sites

A significant decrease ($P < 0.05$) in the promoter activity was observed in both the cell lines, when either one or both of the Sox3 sites were mutated in the whole promoter construct (D1). The decrease was maximal when the second Sox3 site (site-A) was mutated than the first one (site-B). Further, when both the Sox3 sites (A and B) are mutated, appreciable decrease in the promoter activity was found than the constructs where, either one of the Sox3 sites (A or B) were mutated individually in both the cell lines used (Fig. 10A & B). This shows that both the Sox3 sites (A and B) are important for the *11β-hsd* promoter activity.

3.11. Sox3 mediated activation of *11β-hsd*

To further confirm, the role of transcription factor, Sox3 in the activation of *11β-hsd* promoter, both HEK 293 and CHO cells were co-transfected with wild or mutant (for Sox3 site) whole *11β-hsd* promoter construct (D1) with Sox3 expression plasmids

(pcDNA-Sox3Fr). When, Sox3 was co-transfected with the whole promoter construct (D1), the promoter activity was upregulated significantly ($P < 0.05$) both in HEK 293 (Fig. 11A) and CHO cells (Fig. 11B), which emphasizes the importance of Sox3 in the transcriptional activation of *11β-hsd*. The promoter activity did not alter when the reverse locked Sox3 (pcDNA-Sox3R) co-transfection was carried out in both the cell lines (Fig. 11A & B). When, over expression studies were carried out with D1 and SDM constructs, promoter activity upregulated appreciably in D1 where both the Sox3 sites (A and B) are functional in both the cell lines (Fig. 11A & B) compared to the SDM constructs (A,B and AB) which showed that, both the sites are important for functional activity. When compared between two Sox3 sites (A and B), D1 construct with functional Sox3 site (A) and mutant Sox3 site (B) showed higher activity than the D1 construct with mutant Sox3 site (A) and functional Sox3 site (B), which shows the relative importance of Sox3 site (A) over (B) in the transcriptional activation of *11β-hsd* and hence, Sox3 site (A) was chosen for EMSA. The promoter activity did not alter when, over expression of Sox3 was carried out using D1 construct with mutated Sox3 sites (A and B) in both the cell lines (Fig. 10A & B, 11A & B), which shows the specificity of the transcription factor, Sox3 for the given binding sites on the *11β-hsd* promoter.

3.12. Confirmation of Sox3 binding to *11β-hsd* promoter

To further confirm the Sox3 binding, EMSA with supershift was carried out using both the normal and mutated Sox3 binding sites of the *11β-hsd* promoter as the probes. EMSA with testicular nuclear extracts demonstrated a DNA-protein complex formation (Fig. 12) with ^{32}P end labeled-ds oligomeric probe containing normal Sox3 sites (lane no. 2),

while, no significant complex formation was observed with mutated ^{32}P end labeled ds oligomeric probe (lane no. 3). Depletion of binding in the lanes 4 to 7 due to the presence of increasing concentration of cold competitor ds oligomer indicates the specificity of the probe binding (Fig. 12). Further, Sox3 antibody addition resulted in the mild mobility shift of protein-DNA complex (lane no.8). The results further confirm the specificity of the Sox3 binding on the *11β-hsd* promoter.

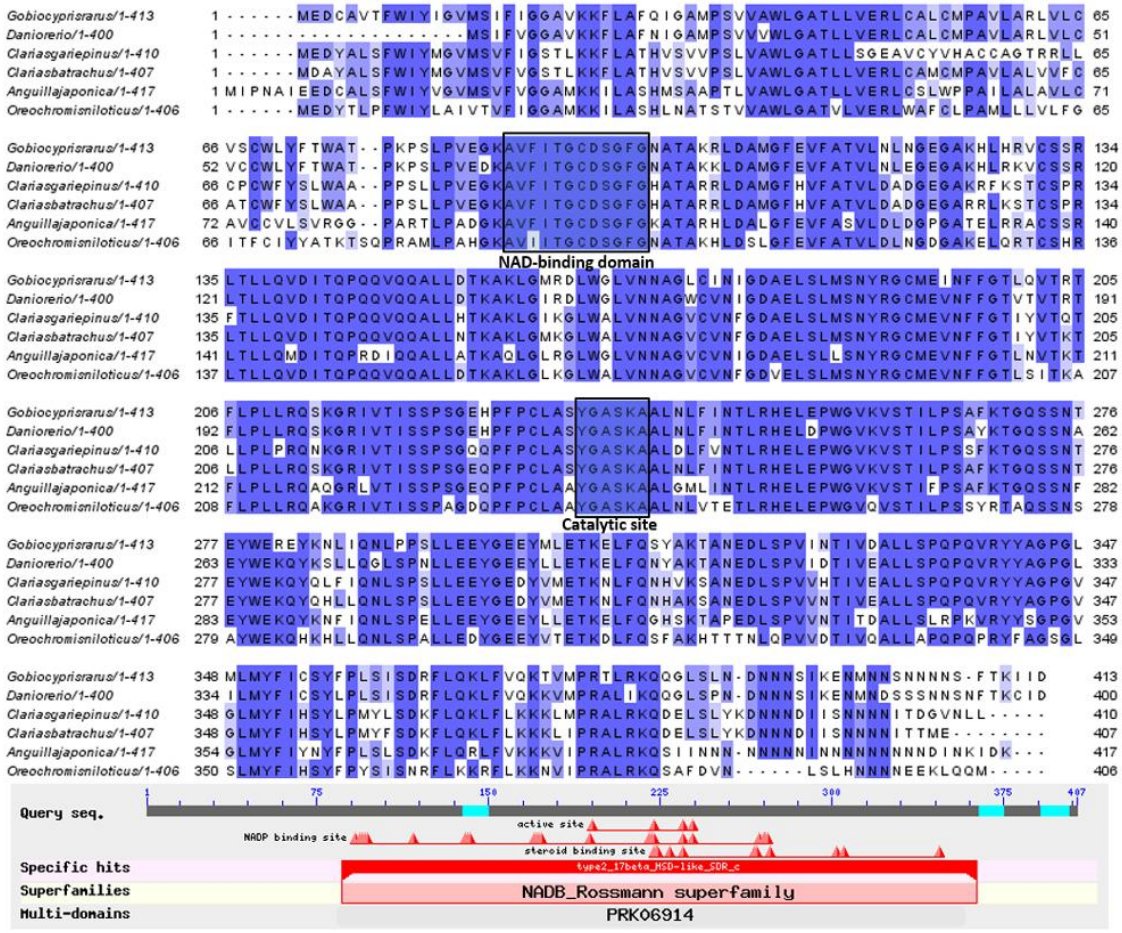


Fig. 1. Multiple alignment of catfish 11β-hsd with other teleosts. The multiple alignment was created using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and high homologous regions are shaded using Jalview 2.8 and the conserved domains are shown as rectangles. Protein-BLAST of catfish *11β-hsd* showed active site, NADP binding and

steroid binding sites. GenBank accession numbers of sequences used for the multiple alignment were given in the section 2.4.

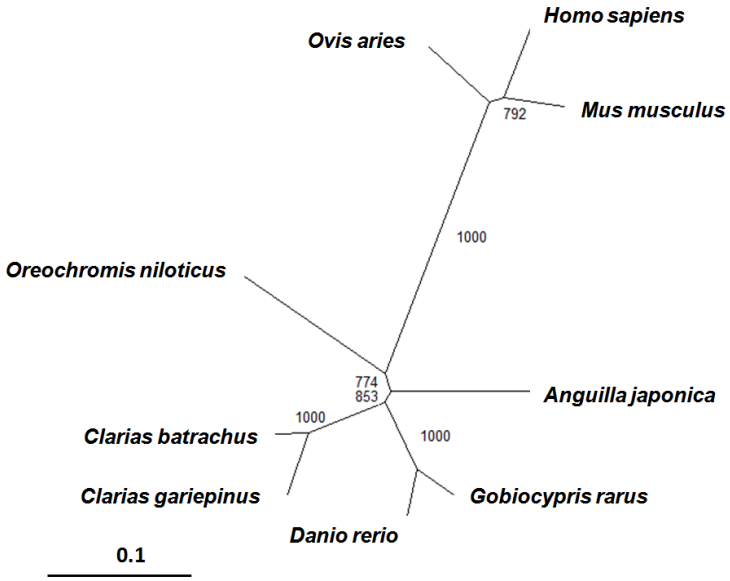


Fig. 2. Phylogenetic analysis of 11β-hsd of catfish. Please refer section 2.4 for the GenBank accession numbers of sequences used for the construction of the phylogenetic tree.

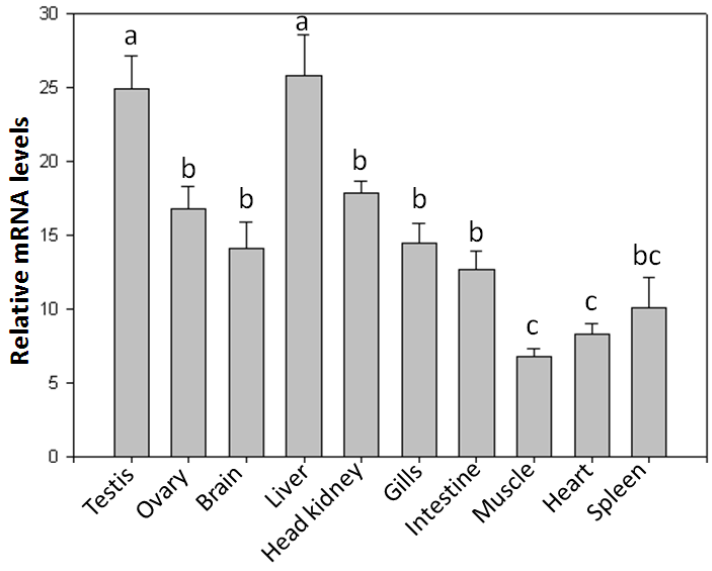


Fig. 3. Relative mRNA levels of *11β-hsd* in different tissues of adult catfish. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

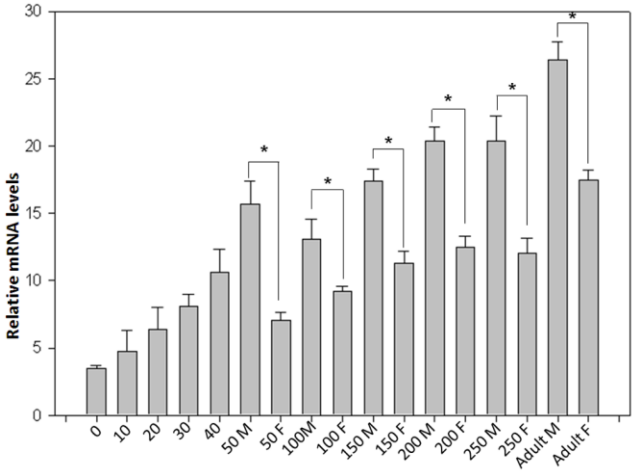


Fig. 4. Relative mRNA levels of *11β-hsd* in different stages of gonadal development. M-Male and F-female. All data were expressed as mean ± SEM. *****indicates means with significantly higher *11β-hsd* mRNA levels compared with the corresponding females of the same age group (*, $P < 0.05$; ANOVA followed by SNK post-hoc test).

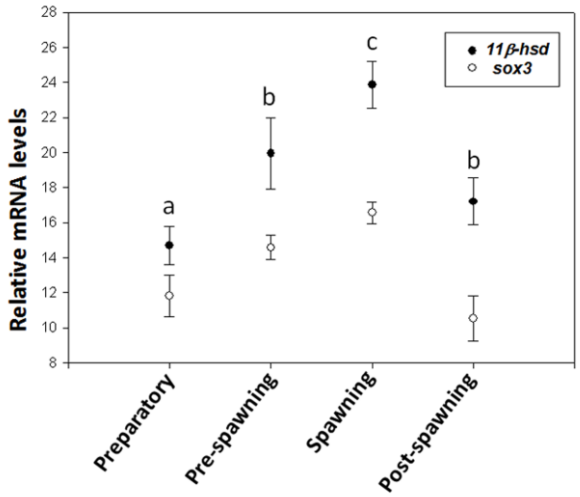


Fig. 5. Relative mRNA levels of *11β-hsd* in comparison with *sox3* in the testis of catfish during different phases of reproductive cycle. All data were expressed as mean ± SEM.

Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

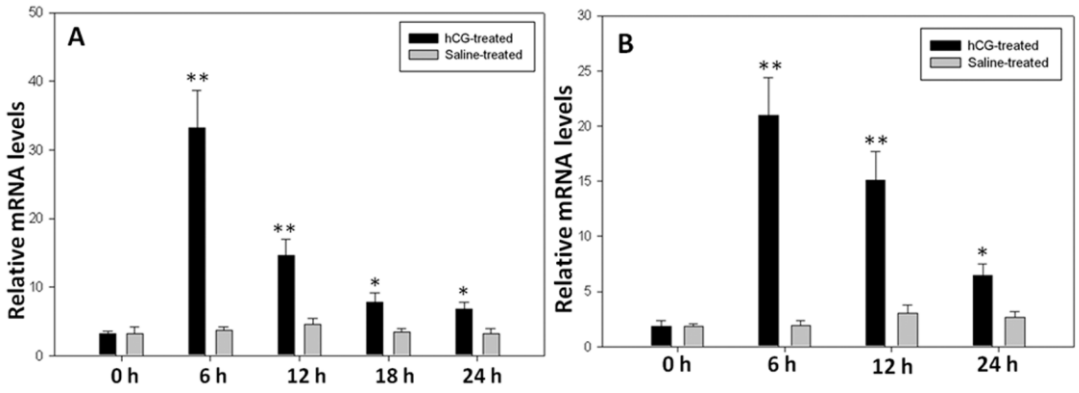


Fig. 6. Relative mRNA levels of *11β-hsd* in the testis of catfish at different time intervals after hCG induction. (A) *in vivo* and (B) *in vitro*. All data were expressed as mean ± SEM. *indicates means with significantly higher *11β-hsd* mRNA levels compared with the 0 h (**, $P < 0.001$; *, $P < 0.05$, ANOVA followed by SNK post-hoc test).

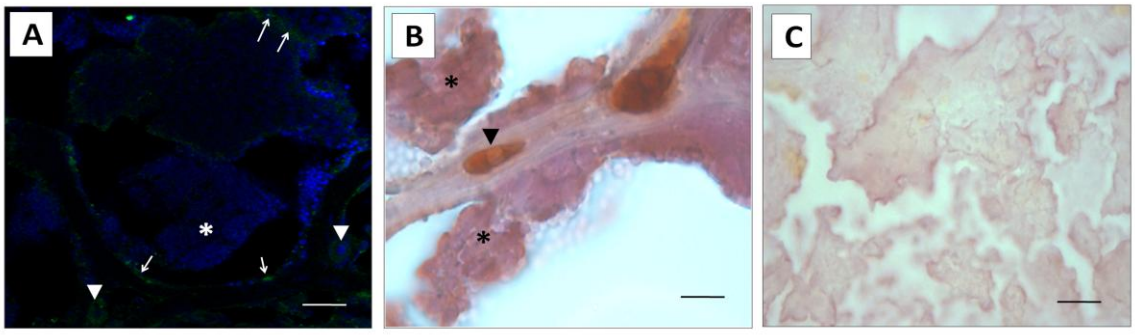
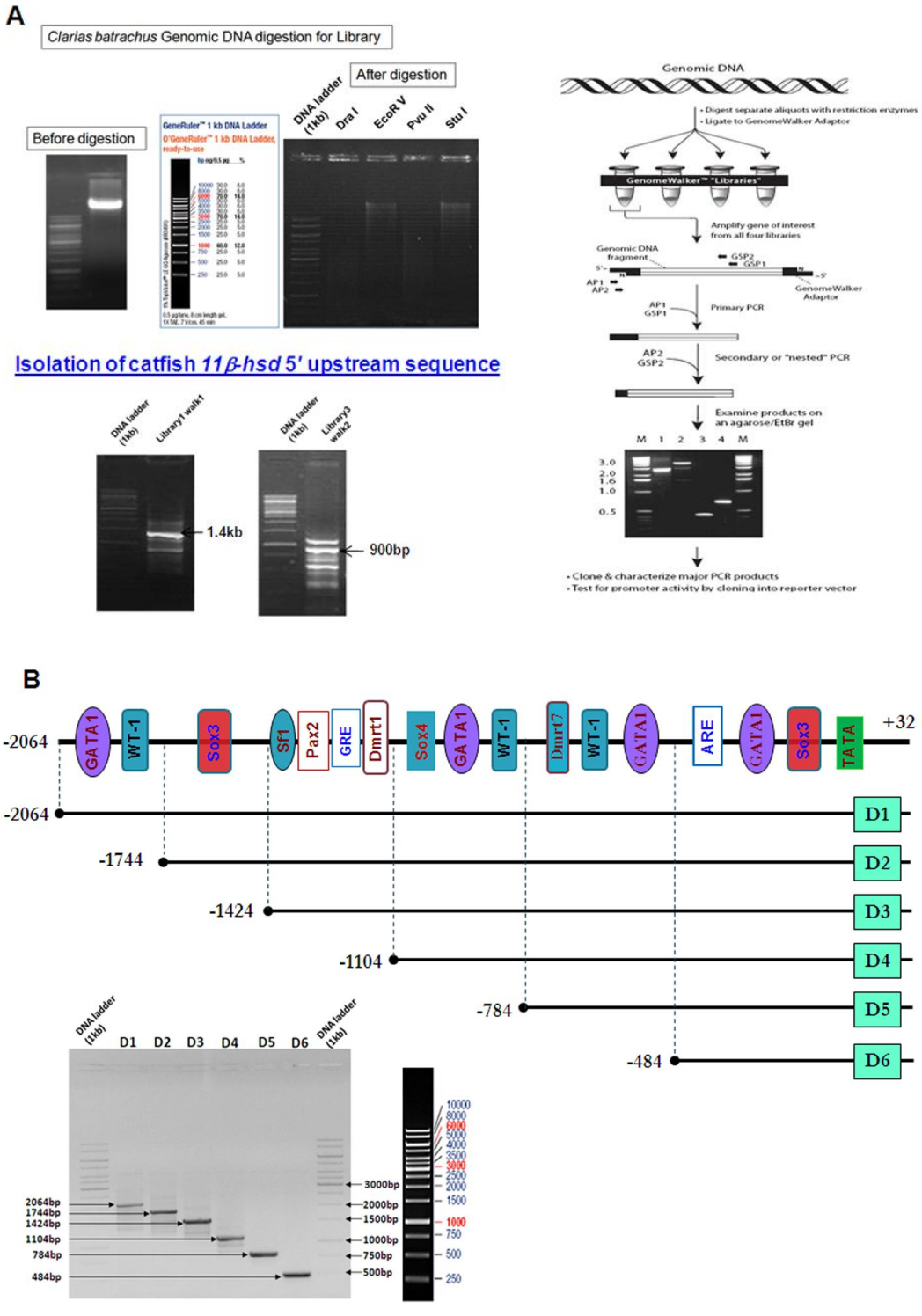


Fig. 7. Localization of 11β-hsd in the adult catfish testis. (A) Testis sections showing immunoreactivity in the interstitial/Leydig cells (arrow heads) and Sertoli cells (arrows) (B) Interstitial/Leydig cells (arrow heads) were present in between two fused spermatogenic cysts and (C) Negative control (pre-adsorbed antibody) showing no

immunoreactivity. Asterisk represents spermatids/sperms. Scale bars in all panels indicate ~30 μm.



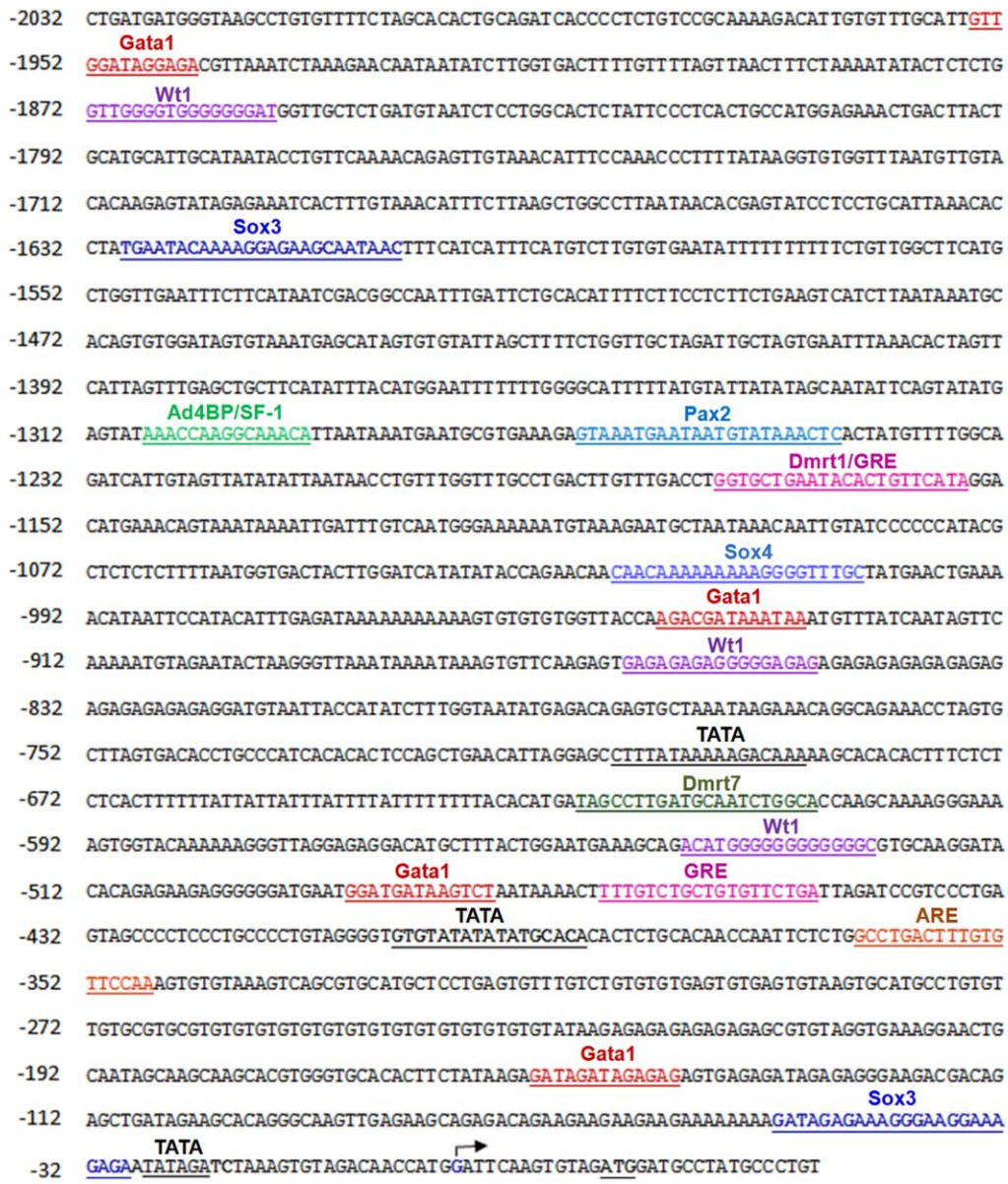


Fig. 8. Schematic representation of genome walking strategy, deletion constructs PCR and 5′ upstream nucleotide sequence of *11β-hsd* with transcription factor binding sites. A) Preparation of Genomic DNA libraries and PCR amplification of 5′ upstream region of *11β-hsd*, B) PCR amplification of different deletion constructs of 11β-hsd promoter and C) Nucleotide sequence of *11β-hsd* 5′ upstream region with its potential transcription factor binding sites. Putative transcription start site (+1) is depicted by an arrow and the

nucleotide sequence is numbered on the left. The potential transcription binding sites are underlined and labeled.

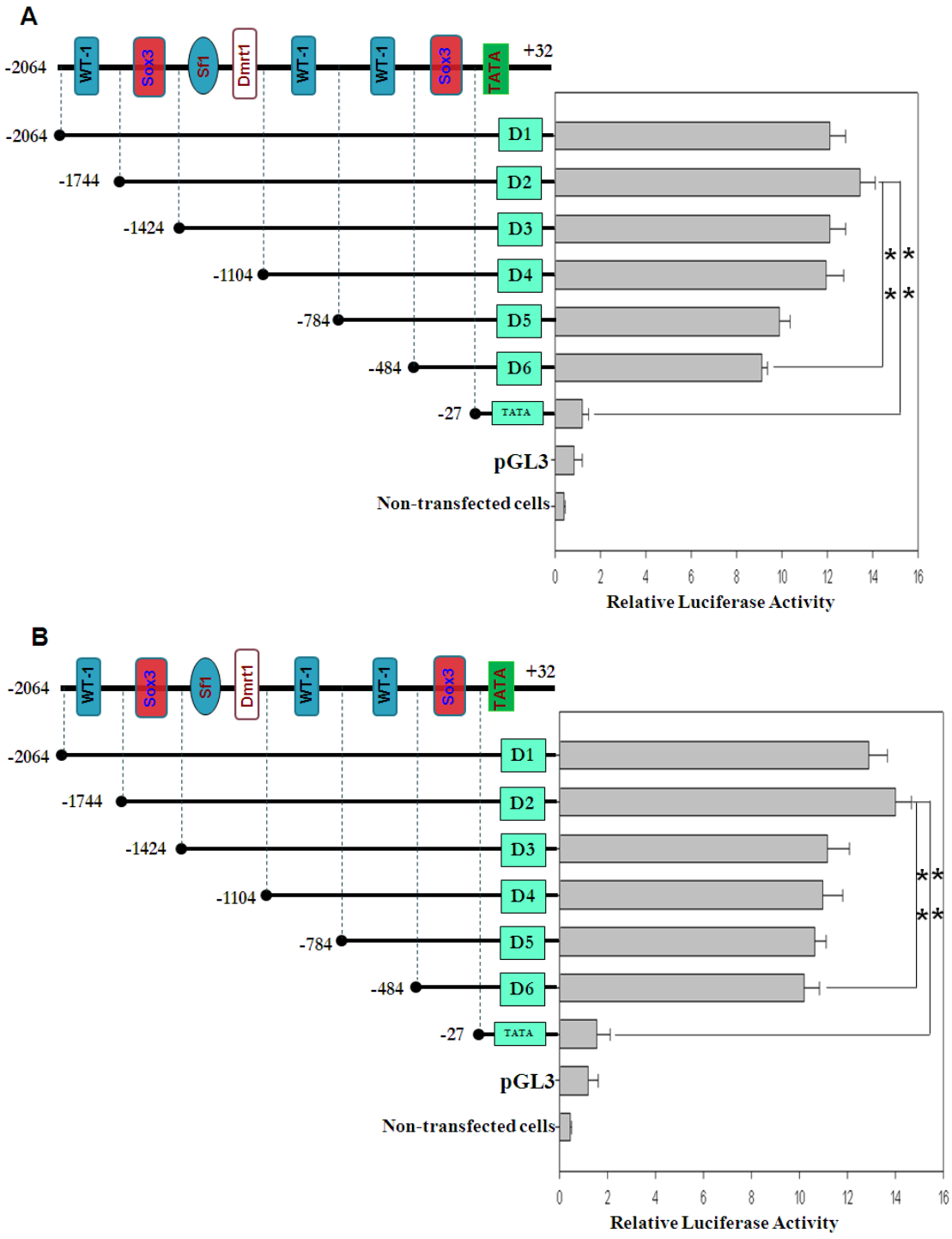


Fig. 9. Functional activity of Sox3 binding sites in A) HEK 293 and B) CHO cells. Progressive *11β-hsd* promoter deletion constructs in pGL3 basic plasmid (500 ng each) were transfected into the cell lines. Luciferase activity is presented as fold change relative to the activities measured for *Renilla* luciferase. The data represent mean ± SEM from three independent experiments performed in triplicate. *indicates means with significantly higher luciferase activity compared to -27/+36 construct (**, $P < 0.01$; ANOVA followed by SNK post-hoc test).

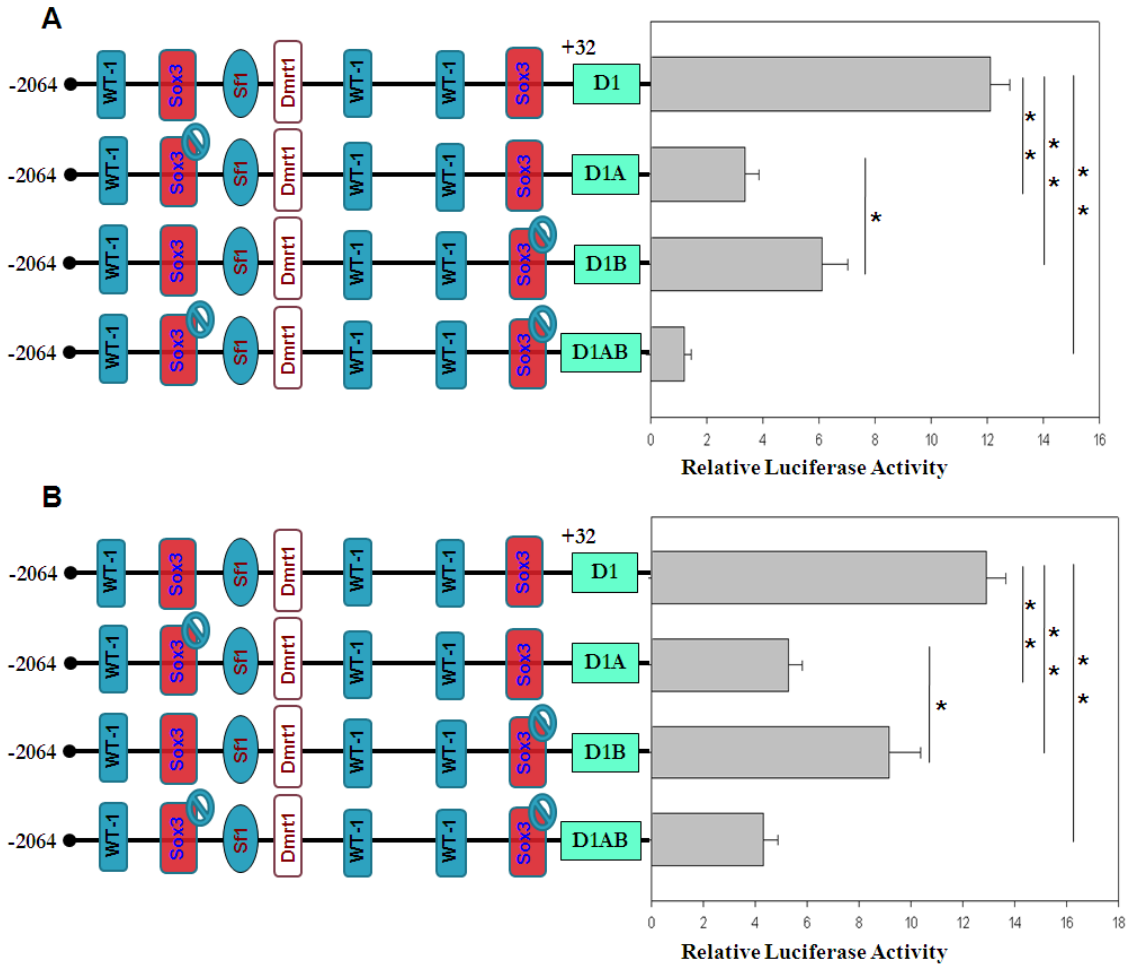


Fig. 10. Functional activity of wild and mutated Sox3 binding sites in A) HEK 293 and B) CHO cells. Wild and mutated *11β-hsd* (D1) whole promoter constructs (500 ng each)

were transfected into the cell lines. ⊘ indicates mutated site. Luciferase activity is presented as fold change relative to the activities measured for *Renilla* luciferase. The data represent mean ± SEM from three independent experiments performed in triplicate. *indicates means with significantly different luciferase activity compared to the wild *11β-hsd* whole promoter construct (**, $P < 0.01$; *, $P < 0.05$; ANOVA followed by SNK post-hoc test).

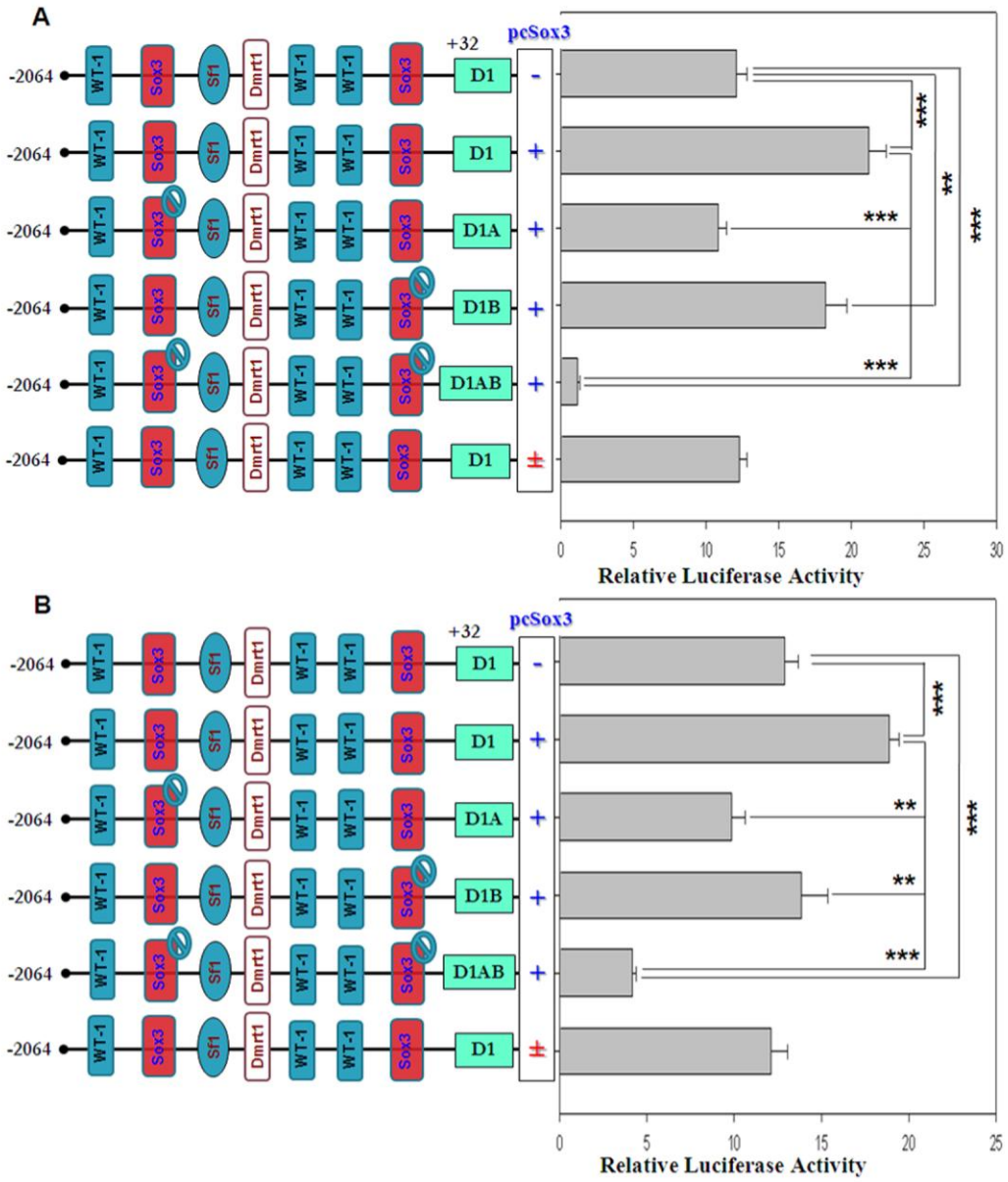


Fig. 11. Functional activity of wild and mutated Sox3 binding sites with Sox3 over expression in A) HEK 293 and B) CHO cells. pGL3 basic *11β-hsd* whole promoter (D1) wild or mutated constructs (500 ng each) co-transfected with Sox3 expression vector (100 ng each) into the cell lines. ⊘ indicates mutated site. ± indicates pCDNA-Sox3 reverse locked ORF plasmid co-transfection. Luciferase activity is presented as fold change relative to the activities measured for *Renilla* luciferase. The data represent mean ± SEM from three independent experiments performed in triplicate. *indicates means with significantly different luciferase activity (***, $P < 0.01$; **, $P < 0.05$; ANOVA followed by SNK post-hoc test).

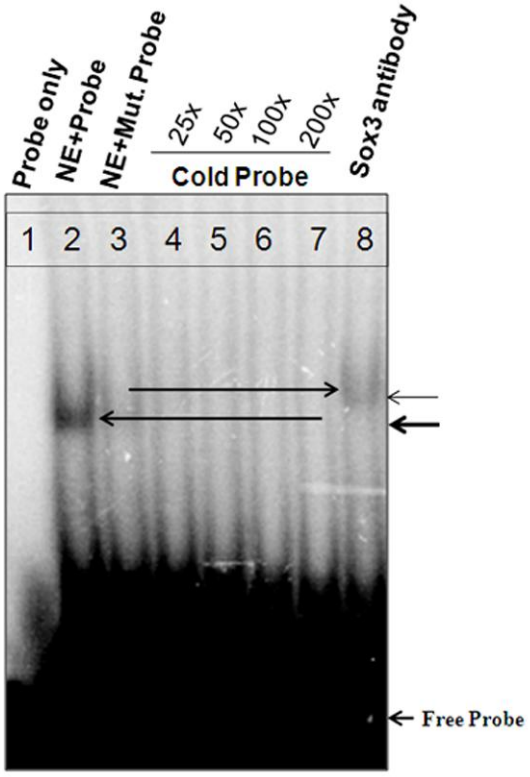


Fig. 12. EMSA with cold competition and super-shift. Gel shift assay showing the binding of Sox3 (in the nuclear extract) to the oligomeric ^{32}P probe. The probe was synthesized from the upstream nucleotide sequence corresponding to Sox3 site (A) of

11β-hsd. Thick arrow depicts shift (DNA-protein complex), while thin arrow indicates the super-shift (DNA-protein-Sox3 antibody complex). The figure represents one of the two independently performed assays. Abbreviations: NE- Nuclear extract and Mut.- Mutant.

4. Discussion

In this study, we report expression changes of *11β-hsd* during early gonadal development, different phases of the seasonal reproductive cycle and after hCG induction. In addition, promoter motif analysis of *11β-hsd* was also carried out. 11β-hsd is important for maintaining the overall steroidogenesis in teleosts in addition to their direct role in 11-KT production. Though 11β-hsd has been identified in many teleosts earlier (Jiang et al., 2003; Kusakabe et al., 2003; Ozaki et al., 2006; Rasheeda et al., 2010), none of the previous reports attempted to describe the regulation of 11β-hsd at the promoter level to correlate expression analysis. There is a strong correlation in the expression of *11β-hsd* and *sox3* during testicular development, maturation, seasonal reproductive cycle and after hCG induction in catfish, *C. batrachus* (Fig. 5; Chapter 5).

The deduced amino acid sequence of 11β-hsd revealed higher homology to human 11β-hsd type 2 (42.72%) than to 11β-hsd type 1 (17.47%). Further, it showed high homology with putative catalytic and NAD-binding domains of mammalian 11β-hsd type 2. In addition, recombinant 11β-hsd could convert the substrate 11-OHT into 11-KT similar to other teleostean 11β-hsds and hence, the cloned *11β-hsd* form indeed belongs to type 2. Phylogenetic analysis revealed that all teleosts formed a single clade wherein, 11β-hsd of *C. batrachus* showed more similarity with 11β-hsd of *C. gariepinus* and *D.rerio* than the

rest of the teleosts, which also justify the genetic relation of Siluriformes and Cypriniformes. Expression of *11β-hsd* is ubiquitous in teleosts however, no solid reasons defined for its tissue specific expression except for the gonads. One possible reason might be its involvement in deactivating cortisol (the primary teleost glucocorticoid), however, detailed studies are needed to further ascertain this. In contrast to the previous study (Rasheeda et al., 2010), first sign of *11β-hsd* expression starts from 0 dph in *C. batrachus* and sex specific differential expression was seen from 50 dph to adult. Further, high *11β-hsd* expression was seen in spawning phase of testicular seasonal cycle more similar to *sox3* expression which shows the possible inter-relation of *11β-hsd* and *sox3* in male catfish. In the case of Japanese eel, immature testes have the ability to produce 11-KT, if the substrate, 11-OHT, is present and was suggested that 11-KT synthesis in immature testis is arrested earlier in the steroidogenic pathway other than the step from 11-OHT to 11-KT (Ozaki et al., 2006). Hence, cohesive study of other steroidogenic enzyme genes and their stringent control might provide novel leads in unraveling the "check points of testicular steroidogenesis". It is also important to analyze the role of LH, as it directly stimulates Leydig cells to produce steroids and/or through the upregulation of steroidogenic enzyme genes. hCG can bind to LH receptor and activate both adenylate cyclase and phospholipase c mediated pathways (Bogerd et al., 2001; Choi and Smitz, 2014). hCG up regulates the expressions of both *11β-hsd* and *sox3* (Chapter 5) both *in vivo* and *in vitro* in catfish, which shows the GtH control on transcription factor and steroidogenic enzyme gene expression together with the availability of specific sex-steroids for spermatogenesis. Interestingly, 11β-hsd immunoreactivity was observed in Sertoli cells of catfish in addition to their localization in interstitial/Leydig cells similar to

11β-h (Chapter 3). In the fetal mouse testis, Sertoli cells are involved in T synthesis (Shima et al., 2013). Even in chondrichthyes, Sertoli cells were shown to produce sex-steroids (Sourdaine and Garnier, 1993; Prisco et al., 2008). Further studies are necessary to unravel its possible importance in reproduction. Having found the importance of *11β-hsd* expression and function, it is also essential to understand the regulation of *11β-hsd* expression by transcription factors.

Sox3 is a member of the Sox family of transcription factors, which encode a protein with a conserved DNA binding high mobility group (HMG) box/domain. In addition to Sox3, the other HMG box protein, Sox9 is also important. Sox9 is involved in gonadal development in mammals and fishes (Sekido and Lovell-Badge, 2008; Raghuveer and Senthilkumaran, 2010). Mammals have *Sry* gene which codes for DNA binding HMG box protein, SRY, which binds to DNA, acting as a transcription factor and regulate testis development (Koopman, 1999; Sekido and Lovell-Badge, 2008). However, there is no Sry in other non-mammalian vertebrates like fishes or even in prototherian mammals like the platypus (Wallis et al., 2007). Instead, few fishes have analog of *sry* like dmy, gsdfy and amhr2 (Matsuda et al., 2002; Takehana et al., 2014), but sry like molecules are not present in catfishes however, Sox3 has been identified (Chapter 5). Sox3 is considered as the ancestral precursor of sry and is highly conserved during evolution (Collignon et al., 1996; Graves, 1998). Hence, there is a high possibility that Sox3 might have a role in testicular development. Further, in mammals, Sox3 was shown to be important for gonadal development and gametogenesis but not in the sex determination (Weiss et al., 2003; Raverot et al., 2005; Sutton et al., 2011). Recent studies in mice have shown that the loss of Sox3 causes germ cell depletion in developing testis (Laronda and Jameson,

2011) and Sox3 can activate male pathway more similar to Sry in the absence of Y chromosome and can facilitate testicular development in mice and human (Sutton et al., 2011; Vetro et al., 2014). Hence, it is plausible that Sox3 might be involved in testicular development and function through the regulation of steroidogenic enzyme genes like *11β-hsd* vis-à-vis sex steroid levels. Considering these, it is essential to understand the involvement of Sox3 on *11β-hsd* regulation at the promoter level which might provide valuable insights with the possible inter relation between these two correlates to understand their importance in teleost reproduction.

Expression of *sox3* and *11β-hsd* in male catfish testis at 250 dph and during spawning phase correlated well with the initiation and progression of spermatogenesis in catfish, which warranted for an involvement of Sox3 in the regulation of *11β-hsd* expression. However, no evidence exists in the recent past implicating the role of Sox3 in teleost testis function. The involvement of Sox3 in mammalian reproduction is relatively well understood (Weiss et al., 2003; Raverot et al., 2005; Laronda and Jameson, 2011; Sutton et al., 2011). Interestingly, in case of amphibians, Sox3 binds and activates Cyp19a1a promoter and was suggested to play an important role in ovarian differentiation (Oshima et al., 2009). In Orange spotted grouper fish, Sox3 was shown to play more important roles in oogenesis than in the spermatogenesis (Yao et al., 2007). However, Orange spotted grouper being a protogynous hermaphroditic fish, it can be assumed that, the germ and supporting cells display similar phenomenon with respect to sex specific markers. Hence, this might not be the case in catfish, as it is a gonochoristic fish, showing higher *sox3* expression in the developing as well as mature testis than the ovary of

corresponding stages (Chapter 5), more similar to the results obtained in black porgy (Shin et al., 2009).

Transient transfection studies using two different cell lines (HEK293 and CHO) demonstrated that Sox3 can bind to two of its sites present in the *11β-hsd* promoter and can activate its transcription appreciably. The confirmation of binding of Sox3 to its respective binding sites demonstrated through the EMSA with super-shift provides substantial proof for the Sox3 regulation of *11β-hsd* transcription. Progressive deletion and/or mutant construct reporter assay experiments involving Sox3 over expression further authenticate the effect of Sox3 on *11β-hsd* transcription, which has been shown for the first time in any vertebrates.

As 11β-hsd is also involved in glucocorticoid metabolism it is also important to analyze the role of GRE, which is identified in between the two Sox3 sites. Based on our luciferase reporter assay results, the influence of GRE might be minimal considering the significant upregulation in the Sox3 induced promoter activity of *11β-hsd*. The other two important sites present in catfish *11β-hsd* promoter which might play important roles include Ad4BP/SF-1, Dmrt1 and Wt1.

Ad4BP/SF-1, an orphan nuclear receptor, is important for gonadal development, function and steroidogenesis (Parker et al., 2002). Several studies have highlighted the importance Ad4BP/SF-1 in the transcriptional regulation of steroidogenic enzyme genes (Hu et al., 2001; Parker et al., 2002; Yoshiura et al., 2003; Wang et al., 2007). The other transcription factor is Dmrt1, which contain a highly conserved DNA binding DM domain across different phyla (Raymond et al., 1998). Dmrt1 was suggested to play an

important role in testicular development in catfish (Raghuveer and Senthilkumaran, 2009). Ad4BP/SF-1 and Dmrt1 sites are present in catfish *11β-hsd* promoter, in between Sox3 and dmrt1 sites. However, no appreciable increase in the luciferase reporter activity was observed in the deletion constructs containing Ad4BP/SF-1 and Dmrt1 sites, which indicates no plausible involvement of Ad4BP/SF-1 and Dmrt1 at least in *11β-hsd* transcription. The other potential site present in the upstream promoter is Wt1. *Wt1* gene encodes a zinc finger transcription factor and RNA-binding protein which plays an important role in the maintenance of Sertoli cells and seminiferous tubules in the developing mice testis (Discenza and Pelletier, 2004; Gao et al., 2006). Transcription factors involved in male sex determination, Sry and AD4BP/SF-1 use Wt1 as a transcriptional co-activator (Nachtigal et al., 1998; Wilhelm et al., 2002; Matsuzawa-Watanabe et al., 2003). In the present study, no appreciable increase in the promoter activity was seen. However, a marginal increase is seen in D4 in HEK 293 might be purely because of the nature of the cell line and expression of Wt1 isoforms in the kidney. As the same D4 construct could not give significant promoter activity in CHO cells. Further studies on these lines might provide more insights to understand the role of Wt1, if any in 11β-hsd transcription.

5. Conclusions

We demonstrate for the first time that Sox3 is a major transcriptional activator of the *11β-hsd* gene by binding to two of its specific promoter motifs in catfish. These results provide evidence of the regulatory mechanisms that control the expression of *11β-hsd* in testis and this will form the basis for understanding of the regulation of testicular

steroidogenesis, development and spermatogenesis in teleosts. Furthermore, detailed studies are needed to identify the factors that up regulate *sox3* expression in the testis which might provide the detailed mechanisms of *11β-hsd-sox3* inter-relation in gonadal development and maturation in teleosts.

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Chapter 5

Expression analysis of sox3 during testicular development, recrudescence and after hCG induction

Abstract

In teleosts, the expression of steroidogenic enzymes and related transcription factor genes occurs in a stage- and tissue specific manner causing sexual development. However the role of *sox3*, an evolutionary ancestor of *Sry*, has not been studied in detail. Therefore, full length cDNA of *sox3* (1197 bp) was cloned from catfish testis, and mRNA expression was analyzed during gonadal development, seasonal reproductive cycle, and after hCG induction. Tissue distribution analysis showed that *sox3* expression was higher in testis, ovary, and brain compared to other tissues analyzed. Developing and mature testis showed higher *sox3* expression than the ovary of corresponding stages. *Sox3* transcripts were found to be higher during the spawning phase of the seasonal reproductive cycle. Furthermore, the expression of *sox3* was upregulated by hCG after *in vivo* and *in vitro* induction, suggesting that GtHs might stimulate its expression. *In situ* hybridization and IHC showed the presence of *sox3* mRNA and protein in somatic and interstitial cell layers of the testis. Sox3 could also be localized in the zona radiata of developing and mature oocytes. Exposure of MT (1 µg/l) and EE_2 (1 µg/l) for 21 days during testicular development showed lower *sox3* expression levels in the testis and brain, indicating a certain feedback intervention. These results suggest a possible role for Sox3 in the regulation of testicular development and function.

1. Introduction

Gonadal development and maturation are complex processes involving various factors or genes which undergo highly coordinated events of regulation. *Sry* gene has been found to be highly conserved in mammals, specifically at the HMG box (Sinclair et al., 1990).

After the discovery of *Sry* in mammals, a vast number of *sox* genes were isolated and classified from a wide variety of organisms (Wright et al., 1993; Bowles et al., 2000). One such member is *Sox3* which is considered as the ancestral precursor of *Sry* and is highly conserved during mammalian evolution (Collignon et al., 1996; Graves, 1998). *Sox3* is a single-exon gene located on the X chromosome in most vertebrates and is expressed in the brain and gonads (Stevanovic et al., 1993; Collignon et al., 1996; Weiss et al., 2003). It plays a pivotal role in early neural development and maintenance of stemcellness in the brain of most vertebrates (Wood and Episkopou, 1999; Dee et al., 2008). *Sox3* is also shown to play an important role in gonadal development and gametogenesis but not in the sex determination of mammals (Weiss et al., 2003; Raverot et al., 2005; Sutton et al., 2011). Recent studies showed that the loss of *Sox3* causes germ cell depletion in developing mouse testis (Laronda and Jameson, 2011) and can activate the male pathway in the same way as *Sry* in the absence of a Y chromosome and facilitate testicular development in mice and human (Sutton et al., 2011). Such a possibility may exist in lower vertebrates, wherein no Sry-like molecules were found, except for the existence of dmy, gsdfy, amhr2 and other Y-chromosome specific molecular markers in few teleosts (Devlin and Nagahama, 2002; Matsuda et al., 2002; Kirankumar et al., 2003; Takehana et al., 2014). Hence, Sox3 alone or in combination with other transcription factors can drive the male pathway either directly or through the regulation of steroidogenic enzymes vis-à-vis sex-steroid levels. In addition, Sox3 directs the formation of the H-H-G- axis (Rizzoti et al., 2004) and can regulate gonadal function indirectly through brain development or through hypothalamic innervations of the pituitary and thus maintaining the critical function of the H-H-G- axis (Camper, 2004).

However, the specific role of Sox3 in gonadal development and reproductive function is not clearly understood in teleosts. Hence, we used the catfish *C. batrachus* as a teleost model. Teleosts exhibit a broad range of sexual plasticity, ranging from hermaphroditism to gonochorism and from genetic to environmental sex determination (Devlin and Nagahama, 2002). *C. batrachus* is an annual breeder which undergoes a seasonal cycle with gonadal attenuation and recrudescence that may provide interesting insights to understand the role of Sox3 not only during early development but also in recrudescence.

2. Materials and methods

2.1. Animals sampling and sex-steroid analogs exposure

C. batrachus hatchlings at different age groups were obtained by IVF. Sampling for ontogeny, tissue distribution and seasonal reproductive cycle were done as described in the chapter 1. Further, for sex-steroid analog studies, juvenile catfish of 50 dph were divided into three groups of 50 larva each and were maintained either with or without treatment compounds of 1µg/L of MT or 1µg/L of EE_2. At end of the treatments sampling was done as described in the chapter 1.

2.2. Molecular cloning of full length sox3 from catfish testis

Total RNA isolation and cDNA synthesis were done as described in the chapter 1. In brief, a set of degenerate primers were designed based on the available *sox3* nucleotide sequences of teleosts from the NCBI GenBank database. PCR amplification was performed at 94 °C (1 min) and 35 cycles of 94 °C (30 s), 54 °C (30 s), 72 °C (1 min), and 72 °C (10 min) using degenerate primers DF and DR (table 1). The partial cDNA

fragment (669 bp) obtained was cloned into the pGEM®-T easy vector (Promega) and sequenced bi-directionally. RACE strategy was performed to obtain the full length cDNA of *sox3* using SMARTer™ RACE (Clontech) according to the manufacturer's protocol. Touchdown PCR reactions were performed to obtain 5′ and 3′ ends using the advantage® polymerase kit (Clontech). All nested PCR fragments were gel purified, cloned into the pGEM®-T easy vector (Promega), and sequenced bi-directionally. All overlapping DNA fragments were assembled, and the full length cDNA (1197 bp) with an ORF of 912 bp was obtained. *Sox3* ORF was cloned into pcDNA 3.1(+) mammalian expression vector (Invitrogen) and used for over expression studies (Chapter 4).

2.3. Sequence and phylogenetic analysis

Multiple alignment of deduced aminoacid sequences were performed based on Sox3 sequences of different teleosts and mammals using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and Jalview 2.8. Phylogenetic tree was created using ClustalW and displayed using TreeView 1.6.6 as described earlier in the chapter 1. All Sox3 amino acid sequences used for the multiple alignment and/or phylogenetic analysis were obtained from GenBank, which are as follows: *Carassius auratus* (EF174418), *D. rerio* (AB242330), *Amphiprion melanopus* (EU908060), *O. latipes* (NM_001104764), *Acanthopagrus schlegelii* (EF605272), (AY277953), *H. sapiens* (BC093865), and *C. batrachus* (KJ475439). Sox1 [*D. rerio* (NM_001002483), *O. latipes* (NP_001158337), and *Astyanax mexicanus* (XP_007255632)] and Sox2 [*D. rerio* (AB242329), *Acipenser schrenckii* (AAU08213), and *S. salar* (NP_001135190)] sequences of different teleosts were also used in the phylogenetic analysis for comparison.

2.4. qPCR

Total RNA isolation, reverse transcription and qPCR were done as described in the chapter 1. qPCR primers were designed for the amplicon length of ~220 bp and all reactions were performed in triplicate for three different samples using *sox3* specific primers (Table 1). Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification and *sox3* expression was normalized against the expression of *18S rRNA* to generate a ΔCt value (Ct of *sox3* - Ct of *18S rRNA*). The change in the gene expression was calculated by using $2^{-\Delta Ct}$ method. For MT and EE_2 treatments, relative expression was calculated by using the comparative Ct ($2^{-\Delta\Delta Ct}$) method by taking the control as a calibrator.

Table 1. List of primers used for cDNA cloning and qPCR analysis

S. No.	Primer Name	Primer Sequence 5′ to 3′	Usage
1.	DF	ATGAAYGCBTTYATGGTVTGG	RT-PCR
2.	DR	CATGCTKATCATRTCBCKCAGG	RT-PCR
3.	5P	ACGGCCGCTTTTCAGCGTCGGTGA	RACE
4.	5N	TGTGCATCTTGGGGTTCTCCTGAG	RACE
5.	3P	GCCTCGGCTCAGTGGCCTCAGTG	RACE
6.	3N	ACCTGCCATCGCCTCCCACTCAC	RACE
7.	ORF F	ATGTATAGCATGATGGAGA	ORF cloning
8.	ORF R	TTAGATGTGGGTTAGGGGTAG	ORF cloning
9.	RTF	ATCGCCTCACACTCACAG	qPCR
10.	RTR	CTCCATAGCCCTGTACTACC	qPCR
11.	18S rRNA F	GCTACCACATCCAAGGAAGGCAGC	qPCR
12.	18S rRNA R	CGGCTGCTGGCACCAGACTTG	qPCR

IUPAC nucleic acid codes are: B = C or G or T; D = A or G or T; H = A or C or T; K = G or T; M = A or C; N = any base; R = A or G; S = G or C; V = A or C or G; W = A or T; Y = C or T.

2.5. hCG induction, *in vivo* and *in vitro*

In vivo and *in vitro* hCG inductions were carried out during the mid-preparatory phase (March) of reproductive cycle as described in the chapter 1. Samples were collected at different time intervals and expression changes of *sox3* were analyzed by using qPCR. The change in the gene expression was calculated by using $2^{-\Delta Ct}$ method.

2.6. *In situ* hybridization (ISH)

Testes of adult catfishes (pre-spawning phase) were dissected and fixed in 4% PFA in PBS (pH 7.4) at 4 °C overnight. After fixation, testes were washed with PBS for 4 times with the cycle of 10 min each at 4 °C and embedded in cryomedium (OCT compound medium, Leica Microsystems) onto a cryomold (Tissue-Tek, Sakura Finetek Europe B.V., AJ Alphen aan den Rijn, The Netherlands) for storage at -80 °C until sectioning by using a cryostat. Later, the cross-sections of OCT embedded testis tissues were cut at 7 µm on to poly-L-Lysine coated slides using a cryostat (Leica CM1850, Leica Microsystems) and were allowed to dry at 42 °C overnight on a hotplate. The pGEM®-T easy- *sox3* cDNA (669 bp) was linearized using restriction enzymes (zero cutters for insert). Based on the sequence data of pGEM®-T- *sox3* cDNA either T7 or SP6 RNA polymerase was used for sense and antisense 'cRNA' probe preparation using digoxigenin (DIG; Roche Diagnostics GmbH, Mannheim, Germany) based *in vitro* transcription. The prepared slides were incubated in PBS with 0.1% Tween 20 (PBT)-

DEPC for 10 min, and the sections were permeabilized with 1 µg/ml proteinase K in PBT at RT for 20 min and washed twice with PBT for 5 min each. The slides were fixed further with 4% PFA in PBT for 5 min at RT and washed twice with PBT for 5 min each. PapPen was used to encircle the periphery of the section before being overlaid with 200 µl of hybridization buffer (50% formamide, 5x SSC, 5 mM EDTA, 1% SDS, 0.1% CHAPS, 50 µg/ml yeast tRNA, 50 µg/ml heparin) and incubated for 1 h at 50 °C. A volume of 0.2 µg/ml cRNA probe (sense or antisense) was mixed separately in 200 µl hybridization buffer heat denatured at 80 °C for 5 min, added to the sections, covered with parafilm, and incubated overnight at 50 °C in a sterile RNase free incubator. The next day, slides were washed with wash buffers (SSC, 50% formamide, 0.1% Tween 20) with decreasing concentrations of SSC 5 times and 5 min each at 50 °C. The slides were first incubated with Solution A (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl; pH 8.0, 0.1% Tween 20) for 5 min at RT and then with 20 µg/ml of RNase A in Solution A for 20 min at RT. Washes were carried out using Solution A and wash buffer (1xSSC, 0.1% Tween 20) for 5 min each at 50 °C. The slides were incubated with maleic acid buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5, 0.1% Tween 20) for 5 min at RT and blocked using 10% FBS with maleic acid buffer for 1.5 h. Antibody reaction was carried out using anti-DIG-ALP antibody (Roche) in maleic acid buffer (1: 2000) overnight at 4 °C. The following day, slides were washed with DIG washing buffer (Roche) for 4 times 15 min each and incubated for 15 min at RT with detection buffer (Roche). The slides were developed using BCIP-NBT (Roche) in detection buffer at RT. The slides were then washed with detection buffer and counterstained with nuclear red (Vector Laboratories), incubated with TE buffer (Ambion, Life Technologies, Carlsbad, Calif., USA) for 5 min,

and further washed with PBT buffer. The slides were then dehydrated with a graded ethanol series and mounted using DPX mountant. All images were taken using a CX41 Olympus microscope (Olympus Corporation) fitted with a Q capture Pro 6 software controlled Micropublisher 3.3 RTV-CCD camera (Quantitative Imaging Corporation).

2.7. Western blot analysis

Western blot analysis was carried out to detect Sox3 protein in catfish testis using anti-Sox3 polyclonal antibody (Life Span Biosciences) raised against the C-terminal region of human Sox3 which showed 70% homology with the C-terminal region of catfish Sox3. Adult testis and liver tissue homogenates were prepared using homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, and ProteoBlock^{TM} protease inhibitor cocktail (Thermo Scientific). 100 µg of protein from each tissue homogenates were separated on a 12% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane (Pall Life sciences, Port Washington, N.Y., USA), and blocked with 5% skimmed milk in Tris-buffered saline (TBS) for 1 h at RT. After blocking, the membrane was washed 7 times for 7 min each in TBS with 0.1% Tween 20 (TBST), and incubated with a 1: 1,000 dilution of polyclonal anti-Sox3 antibody overnight at 4 °C. For negative control, liver tissue homogenate protein was used. β-tubulin was used as an equal loading control. After washing, membranes were incubated with alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody (Merck Bangalore Genei) for 1 h at RT. Following washing, blots were developed with BCIP-NBT (Roche).

2.8. IHC and IF

Testes of adult catfishes (pre-spawning phase) were dissected and fixed in 4% PFA in PBS (pH 7.4) at 4 °C overnight, and cryosections were prepared as described earlier in the chapter 3. In brief, the sections were hydrated and later blocked with 10% normal goat serum (Merck Bangalore Genei) for 1 h at RT. Anti-Sox3 polyclonal antibody (1: 1,000) or pre-adsorbed antibody (for negative control) was incubated overnight at 4 °C in a humid chamber. Following incubation, the sections were incubated with HRP-conjugated secondary antibody (Merck Bangalore Genei) for 1 h at RT. Sections were then incubated with ABC reagent provided in VECTASTAIN® Elite ABC kit (Vector Laboratories) for 30 min at room temperature. The slides were washed with PBS and developed using commercially supplied DAB as chromogen and H_2O_2 (Vector Laboratories) as substrate for HRP. The sections were counterstained with hematoxylin (Qualigens), dehydrated using a graded ethanol series, and mounted using DPX mountant. For IF, FITC-conjugated anti-rabbit secondary antibody (Vector Laboratories) was used and further counterstained and mounted using VECTASHIELD® mounting media with DAPI (Vector Laboratories). Images were taken with a CX41 Olympus microscope (Olympus Corporation) fitted with a Micropublisher 3.3 RTV-CCD camera through Q capture Pro 6 software (Quantitative Imaging Corporation) for DAB staining, while for IF, an IX81 Olympus Microscope (Olympus Corporation) with Cell Sens dimension software was used.

2.9. Statistical analysis

All data are presented as the mean ± SEM which passed homogeneity and normality tests. All data were compared by one-way ANOVA followed by SNK post hoc test, and

all statistical analyses were performed using SigmaPlot 11.0 software (Systat Software Inc.). A probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning of sox3 from catfish testis

A partial cDNA of 669 bp of *sox3* was cloned from catfish testis and based on sequence data, gene specific RACE primers were designed and used for 5′ and 3′RACE. The cDNA fragments obtained through 5′ (204 bp) and 3′RACE (458 bp) were cloned and verified by comparing with *sox3* of other teleosts using NCBI-BLAST and ClustalW. After sequence analysis, confirmation and assembling of all cDNA fragments yielded a 1197 bp full length cDNA with an ORF of 912 bp which was submitted to GenBank (KJ475439). The *sox3* ORF of 912 bp was cloned into pcDNA 3.1(+) mammalian expression vector (Invitrogen), sequenced and used of over expression studies (Chapter 4). The 3 ′ UTR of *sox3* was 265 bp long with poly (A) tail.

3.2. Sequence and phylogenetic analysis

Multiple alignment of the deduced Sox3 amino acid sequence in comparison with other species (Fig. 8) revealed considerable homology with *C. auratus* (86%) and *D. rerio* (85%) and a conservation through evolution in many teleosts specifically at the HMG box (Fig. 8). The phylogenetic comparison of Sox3 with Sox1 and Sox2 showed that the cloned cDNA from catfish belongs to the Sox3 clade than to any other subgroup (Fig. 1). When Sox3 sequences from different teleosts and *H. sapiens* were compared it was

shown that catfish Sox3 was more similar to *D. rerio* than to the others that had been analyzed (Fig. 1).

3.3. Tissue distribution of sox3 in different tissues of adult catfish

Maximum expression of *sox3* was seen in the testis, brain and ovary while the expression was negligible in other tissues analyzed (Fig. 2).

3.4. Expression of sox3 during gonadal ontogeny

The qPCR analysis of *sox3* at different stages of gonadal developmental showed its first sign of expression as early as 0 dph. It was expressed in both male and female gonads throughout gonadal development till maturity (Fig. 3). The expression was higher ($P < 0.05$) in males compared to females from 200 dph during gonadal development and at maturity.

3.5. Expression of sox3 during different phases of the seasonal cycle

The qPCR analysis during the seasonal cycle showed that the expression of *sox3* was significantly higher ($P < 0.05$) during spawning phase followed by pre-spawning, preparatory and post-spawning phases (Fig. 4).

3.6. Changes in the expression of *sox3* in testis after *in vivo* and *in vitro* hCG induction

The hCG induction during the mid-preparatory phase (March) of the catfish reproductive cycle resulted in significantly enhanced *sox3* expression in testis during the entire duration of the study when compared to 0 h. The *sox3* expression increased significantly ($P < 0.05$) at 6, 12, and 18 h and peaked at 24 h after hCG induction (Fig. 5A). In *in vitro*

cultures of testicular slices, a significant increase ($P < 0.05$) in *sox3* expression was observed at 6 h followed by 12 and 24 h (Fig. 5 B).

3.7. Changes in the expression of *sox3* in testis and brain of MT and EE_2 treated catfish

MT and EE_2 treatments (50-71 dph) decreased the expression of *sox3* significantly ($P < 0.05$) to 5 and 8%, respectively in the testis (Fig. 6 A) and to 8 and 10%, respectively in the brain (Fig. 6 B) during gonadal development of catfish.

3.8. Localization of *sox3* mRNA and protein and western blot analysis

ISH, IHC, and IF analyses revealed the presence of *sox3* mRNA (Fig. 7A, B) and protein (Fig. 7D, E, G, H) in the somatic and interstitial cell layer of testis. No probe signal or immunoreactivity was observed in the respective negative controls (Fig. 7C, F). Western blot analysis revealed an expected band of ~33 kDa (Fig. 7I) in the testicular homogenates of catfish which corresponds to the theoretical molecular weight of the catfish Sox3 putative protein. No band (Fig. 7I) was observed in the negative control. Sox3 could also be localized in the zona radiata of developing and mature oocytes of the catfish ovary (Fig. 7 J-L).

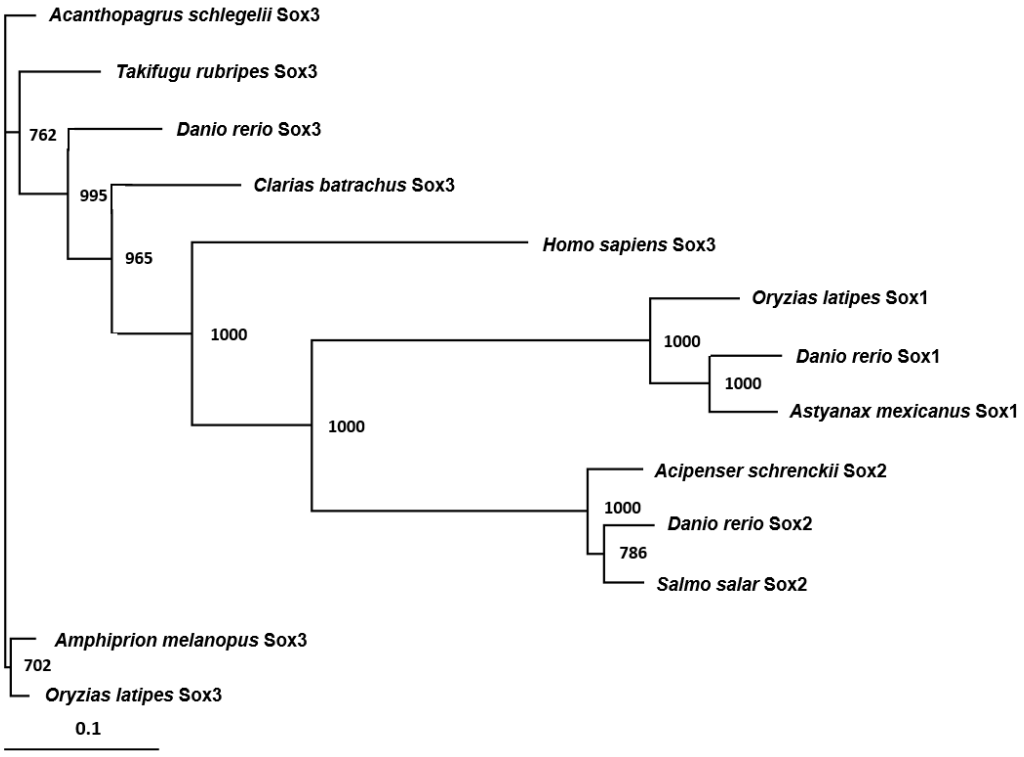

Fig. 1. Phylogenetic analysis of catfish Sox3 with Sox1 and Sox2 of other vertebrates. The phylogenetic analysis was performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/) and was displayed using TreeView software package version 1.6.6. GenBank accession numbers of sequences used for constructing the phylogenetic tree are given in the section 2.3.

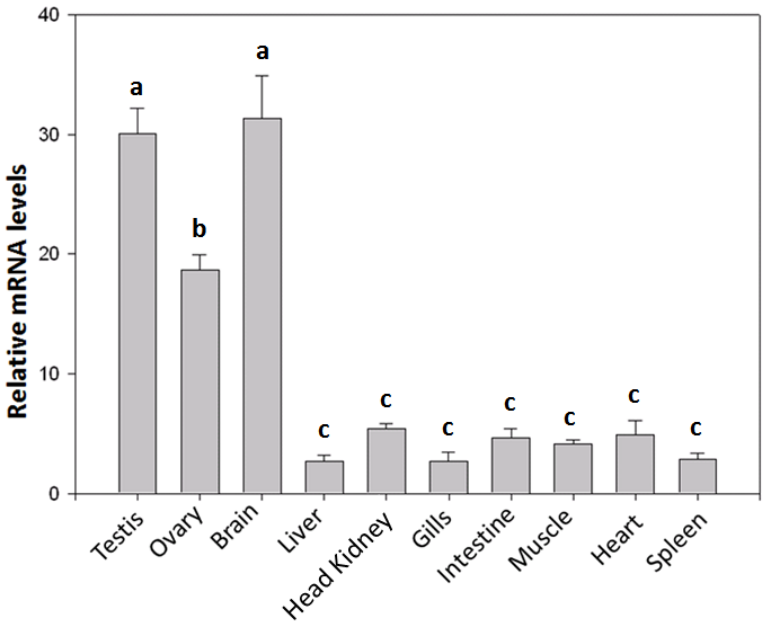

Fig. 2. Relative mRNA levels of *sox3* in different tissues of adult catfish. M-Male and F-Female. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

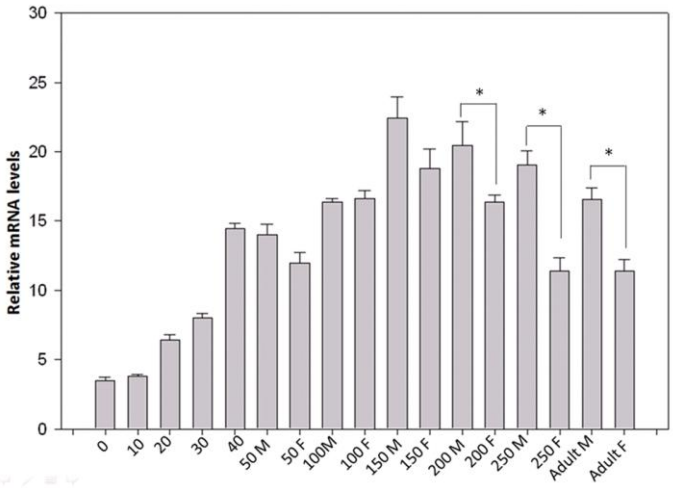

Fig. 3. Relative mRNA levels of *sox3* in different stages of gonadal development. All data were expressed as mean ± SEM. *****indicates means with significantly higher *sox3* mRNA levels when compared with the corresponding females of the same age group (*, $P < 0.05$; ANOVA followed by SNK post-hoc test).

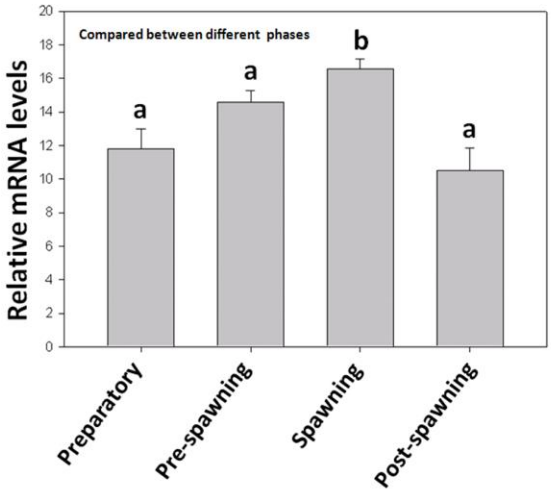

Fig. 4. Relative mRNA levels of *sox3* in testis of catfish during different phases of reproductive cycle. All data were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

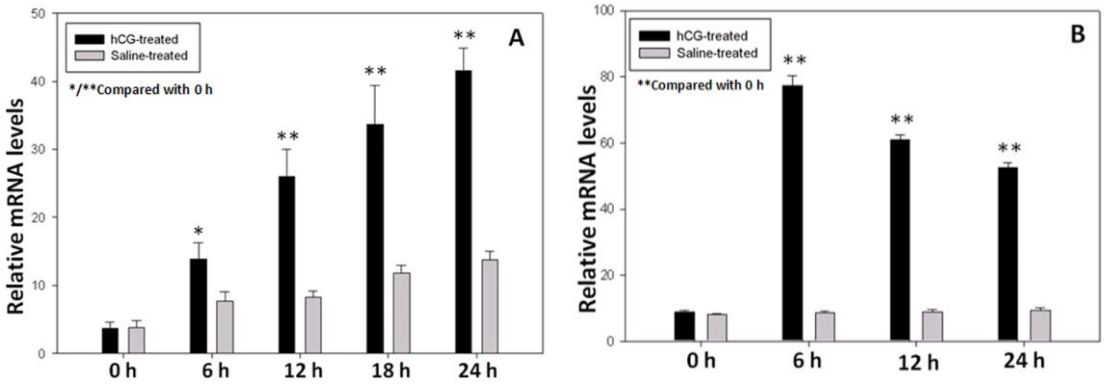

Fig. 5. Relative mRNA levels of *sox3* in the testis of catfish at different time intervals after hCG induction, (A) *in vivo* and (B) *in vitro*. All data were expressed as mean ± SEM. *indicates means with significantly higher *sox3* mRNA levels compared with the 0 h (**, $P < 0.001$, *, $P < 0.05$, ANOVA followed by SNK post-hoc test).

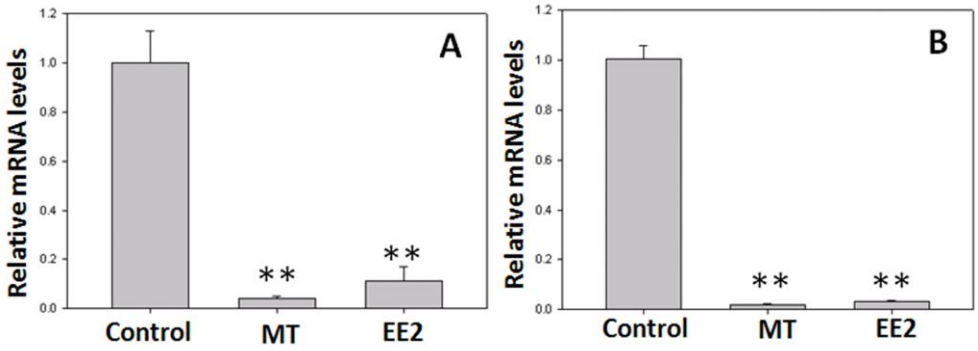

Fig. 6. Relative mRNA levels of *sox3* after MT and EE_2 treatments (50-71 dph) in the (A) testis and (B) brain of catfish. All data were expressed as mean ± SEM. *indicates means with significantly lower *sox3* mRNA levels compared to control (**, $P < 0.001$, ANOVA followed by SNK post-hoc test).

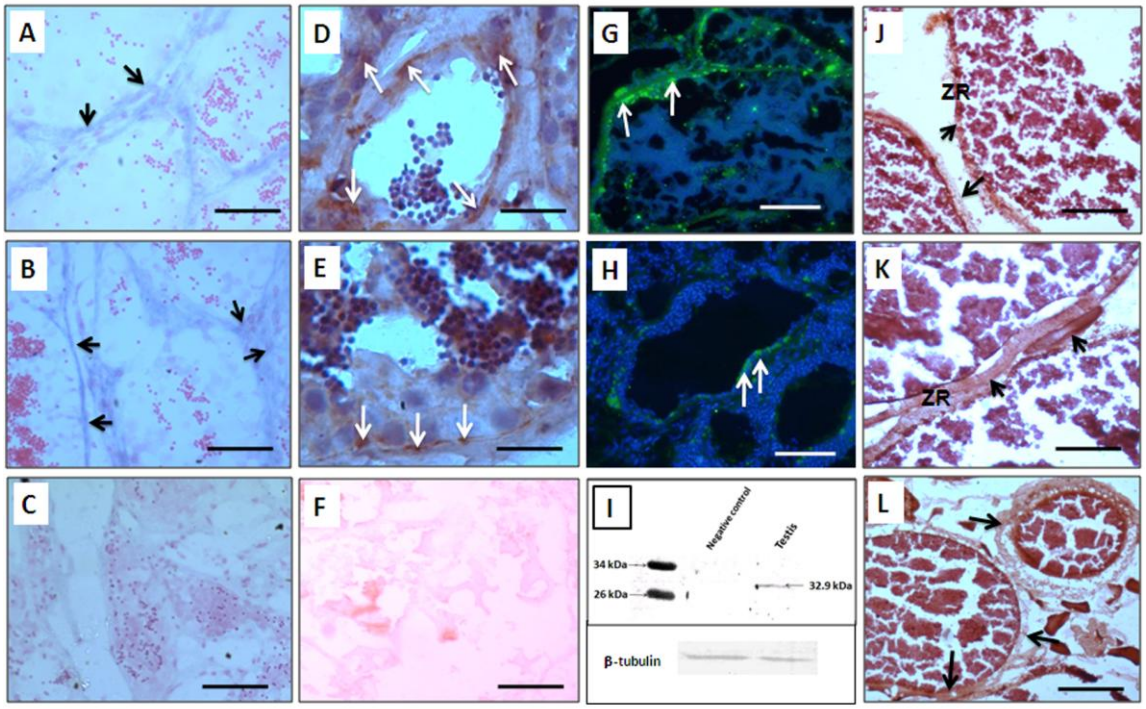

Fig. 7. Localization of *sox3* mRNA and protein, and western blot analysis in adult catfish. (A & B) ISH, anti-sense [testis] (C) ISH, sense [testis] (D and E) IHC [testis], (F) IHC [testis], Negative control-pre-adsorbed antibody, (G & H) immunofluorescence [testis],

(I) western blotting, (J, K and L) IHC [Ovary]. ISH signal and immunoreactivity was shown as arrows. ZR- Zona radiata. Scale bars in all the panels indicate 50 μm.

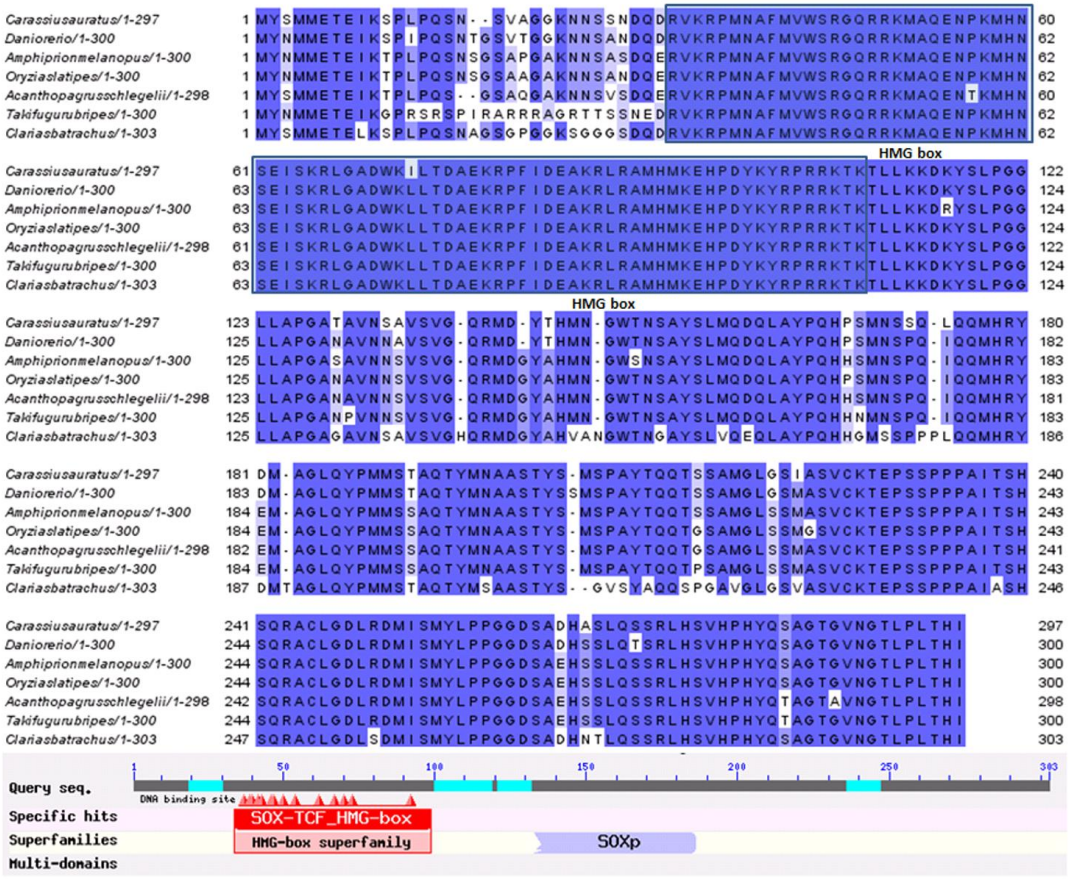

Fig. 8. Multiple alignment of catfish Sox3 with other teleosts. The multiple alignment was created using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and high homologous regions are shaded using Jalview 2.8 and the conserved domains are shown as rectangles. Protein-BLAST of catfish Sox3 showed DNA binding sites, HMG-box super family and multi-domains. GenBank accession numbers of sequences used for the multiple alignment are given in the section 2.3.

4. Discussion

In this study, we report on the molecular cloning of catfish sox3 and its dimorphic expression during early gonadal development and expression changes during different phases of the seasonal reproductive cycle after hCG induction *in vivo* and *in vitro* and after MT and EE_2 exposure. The present study was the first to describe sexual differences in sox3 expression during the gonadal development of *C. batrachus*. In addition, sox3 mRNA and protein was localized in the somatic and interstitial cell layer of adult testis. Furthermore, differential expression of sox3 during gonadal ontogeny, different phases of the seasonal reproductive cycle and after hCG induction suggested an important role for Sox3 in catfish testicular development, maturation and perhaps function. Heterologous Sox3 antibody used in the present study was more specific to catfish Sox3, which could be ascertained from the single band (33 kDa) obtained in the Western blot and which corresponds to the theoretical molecular weight of catfish Sox3. Further, pre-adsorbed antibody in IHC showed no immunoreactivity which further substantiated the specificity of the antibody to catfish Sox3 protein.

Sox3 might function as an analog of Sry in testis similar to mammalian testis differentiation, wherein the threshold of Sox9 activity in supporting cell precursors, once exceeded, promotes testis differentiation by suppressing the pro-ovarian differentiation (DiNapoli and Capel, 2008; Sekido and Lovell-Badge, 2009; Sutton et al., 2011). Despite the fact that *sox3* was expressed exclusively during testicular differentiation and development in the present study, the evidences are minimal to support the notion that Sox3 functions as an analog of Sry to initiate testicular differentiation in catfish. A recent study (Takehana et al., 2014) in the Indian medaka has shown that, Sox3 initiates testicular differentiation by upregulating the expression of Gsdf, which is highly

conserved in fish sex differentiation. But such a phenomenon exists in other fishes globally needs to be elucidated. Tissue distribution analysis showed the presence of abundant *sox3* transcripts in the testis, ovary, and brain, while other tissues showed a negligible expression which is similar to *Epinephelus coioides* (Yao et al., 2003), indicating less significance for *sox3* in these tissues. Differential expression (at the protein level) of Sox3 was seen during gametogenesis in the protogynous hermaphrodite fish *E. coioides*, wherein gonads undergo transition from ovary to an intersexual gonad and then to testis (Yao et al., 2003; 2007). However, the present study showed the dimorphic expression of *sox3* during gonadal development and differential expression during the seasonal cycle in a gonochoristic fish model. *Sox3* expression was noticed in both male and female developing gonads in *C. gariepinus* (Raghuveer et al., 2011), while the present study showed expression changes during gonadal development in comparison to adult fishes. In catfish, the immature testis and ovary showed higher *sox3* expression than the mature testis during ontogeny, which confirms the more important role of Sox3 in gonadal development and maturation during early stages than in the adult fish.

Similar to the present study, Yao et al. (2003) showed a higher expression of Sox3 in immature than mature ovaries of *E. coioides*. Furthermore, Sox3 protein expression was higher in mature ovaries than mature testes of *E. coioides*, wherein Sox3-positive primordial germ cells develop into oogonia and then to oocytes. When Sox3 expression is stopped, the Sox3 positive primordial germ cells develop into spermatogonia (Yao et al., 2007). In contrast, the present study showed higher *sox3* expression in the testis than the ovary during later stages of gonadal development and in adult fishes. In addition, *sox3* expression was higher at the spawning phase where spermatogenesis and spermiation

processes occur. Similar to the present study, *sox3* expression was higher in mature testis than in the ovary of *A. schlegeli* (Shin et al., 2009). The role of Sox3 in neural development and function has been well established (Wood and Episkopou, 1999; Dee et al., 2008) and has been shown to be a permissive factor for sensory placode formation, which plays an important role in sensory organ development in *O. latipes* (Koster et al., 2000). Involvement of Sox3 in brain sex differentiation and H-H-G- axis regulation is an interesting aspect to explore, and it might give some more valuable inputs on the brain regulation of gonadal function. Sex-steroids are known to play a crucial role in gonadal differentiation in teleosts, but the complete mechanism involved in sexual maturation of male teleosts is poorly understood. *Sox3* expression was found to be higher during the spawning phase than in other phases, much like steroidogenic enzyme genes in testis, which corresponds to spermiogenesis, spermiation and sperm maturation in catfish. Further, Sox3 binding sites are present in the *11β-hsd* promoter, wherein Sox3 can bind and transactivate the transcription of *11β-hsd* (Chapter 4). Based on these findings, we are hypothesizing that Sox3 in addition to its direct supportive role in testicular development and spermatogenesis might also control sex-steroid levels by regulating certain important steroidogenic enzyme genes like *11β-hsd* during critical stages of gonadal development and maturation. Whether high *sox3* expression causes an increase in sex-steroid levels during the spawning phase needs to be explored in detail. However, the levels of endogenous sex-steroids have no impact on *sox3* expression, which can be ascertained by the higher *sox3* expression during the spawning phase of the catfish reproductive cycle.

To understand the regulation at the brain level and the seasonal effect of GtHs on the expression of *sox3*, hCG induction was carried out during the preparatory phase of the catfish reproductive cycle. Expression of *sox3* increased after hCG administration both *in vivo* and *in vitro* to maximum levels at 24 and 6 h, respectively. The hCG-induced *sox3* expression in catfish was more similar to the gonadotropin-releasing hormone (GnRH) analog-induced expression in *A.* schlegeli (Shin et al., 2009), indicating GtH dependency. Sox3 protein was expressed in germ cells (Raverot et al., 2005; Yao et al., 2007; Laronda and Jameson, 2011) and Sertoli cells of testes in *M. musculus* and *A. schlegeli* (Weiss et al., 2003; Yao et al., 2007). Further, Sox3 prevents the differentiation of spermatogonia in pre-pubertal mice (Laronda and Jameson, 2011). The present study revealed the presence of *sox3* mRNA and Sox3 immunoreactivity by IHC and IF in the somatic and interstitial cell layer. However, no *sox3* mRNA or protein could be localized in germ cells of catfish testis. Previous studies from our laboratory clearly showed the localization of Sertoli cell marker Sox9 (Raghuveer and Senthilkumaran, 2010) and interstitial/Leydig cell markers like steroidogenic enzyme (Sreenivasulu et al., 2012) in testicular sections of catfish. Sox3 could also be localized in the ovary which may warrant further studies. In addition to natural sex-steroids, MT and EE_2 are commonly used sex-steroid-analogs during the critical period of gonadal development for sex reversal studies (Piferrer, 2001; Orn et al., 2003; Pawlowski et al., 2004; Sudhakumari and Senthilkumaran, 2013). In the present study, MT treatment down regulated *sox3* expression in catfish. A previous study from our laboratory has shown that the exposure of MT during gonadal development skewed the cell population towards the male pathway (Raghuveer and Senthilkumaran, 2009) possibly through the down regulation of steroidogenic enzyme genes like *11β-h*

and *11β-hsd* (Chapter 4). However, the present study could not detect any skewing in sex populations in catfish, which might be due to the low dose of MT as well as the duration and age of the catfish used in the present study. *Sox3* expression in catfish was down regulated by EE_2 treatment during testicular development. EE_2 acts similar to E_2 and modulates the expression of essential steroidogenic enzyme genes and enzyme activity (Govoroun et al., 2001; Filby et al., 2007; Sridevi et al., 2015). Naturally, it may be assumed that the sex-steroid-analog treatments might increase *sox3* expression based on our ontogeny results which depict a surge from 50 dph onwards. However, to our surprise, sex-steroid-analog treatments down regulated *sox3* expression which might reveal a feedback intervention, and this is also justified by the localization of *sox3* mRNA and protein in the interstitial cell layer. Further studies are necessary to substantiate the detailed mechanism involved in the *sox3* regulation at the level of promoter motifs of steroidogenic enzyme genes or others during gonadal development and maturation in both gonads and brains of teleosts.

5. Conclusions

The present study showed the importance of *sox3* in testicular development and maturation during recrudescence in catfish. The study also showed the GtH regulation of *sox3* expression using hCG both *in vivo* and *in vitro*. Decreased expression of *sox3* after the treatment of sex-steroid-analogs warrants feedback-mediated target action and its dependency on GtH. Sexual dimorphic expression of *sox3* during gonadal development can substantiate the significance of *sox3* in testicular development. In addition, *sox3* mRNA and protein localization in the somatic and interstitial cell layer of catfish testis

implicates a role for Sox3 in catfish reproduction, more specifically in gonadal steroidogenesis.

6. References

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Chapter 6

Endosulfan and flutamide impair testicular development in juvenile catfish

Abstract

Sex steroid production and expression/regulation of transcription factors are highly dynamic and sensitive. Therefore analyzing the impact of hormone analogs and endocrine disruptors on gonadal development of fishes can provide new insights on regulatory aspects. On this perspective, the impact of few endocrine disruptors like endosulfan and flutamide on catfish gonadal development were analyzed. Endosulfan and flutamide, a widely used pesticide and a prostate cancer/infertility drug, respectively, have an increased risk of causing endocrine disruption if they reach water bodies. Though many studies are available on neurotoxicity/bioaccumulation of endosulfan and receptor antagonism of flutamide, very little is known about their impact on testicular steroidogenesis at molecular level. As sex steroids play an important role in sex differentiation of lower vertebrates including fishes, a small change in their levels caused by endocrine disruptors can affect the gonadal development of fishes significantly. The aim of this study was to evaluate the effects of endosulfan and flutamide on testis related transcription factor and steroidogenic enzyme genes with a comparison on the levels of androgens during critical period of catfish testicular development. We also analyzed the correlation between the above mentioned genes and catfish gonadotropin-releasing hormone (cfGnRH)-tryptophan hydroxylase2 (tph2). *C. batrachus* males of 50 dph were exposed to very low dose of endosulfan (2.5 μg/l) and flutamide (33 μg/l), alone and in combination for 50 days. The doses used in this study were far less than those used in the previous studies of flutamide and reported levels of endosulfan in surface water and sediments. Sampling was done at end of the treatments (100 dph) to perform testicular germ cell count (histology), measurements of T and 11-KT by EIA and transcript

quantification by qPCR. In general, treatments down regulated the expression of several genes including testis-related transcription factors (Dmrt1, Sox9a and Wt1]), steroidogenic enzymes (Cyp11a1, P450c17, 17β-hsd12, 11β-h and 11β-hsd), Star and orphan nuclear receptors (nr2c1 and Ad4BP/SF-1). In contrast, the transcripts of *cfGnRH* and *tph2* were upregulated in the brain of all treated groups with maximum elevation in the endosulfan group. However, combination of endosulfan and flutamide (E + F) treatment showed minor antagonism in a few results of transcript quantification. Levels of T and 11-KT were elevated after flutamide and E + F treatments while no change was seen in the endosulfan group signifying the effect of flutamide as an androgen receptor antagonist. All the treatments modulated testis growth/function by decreasing the progression/differentiation of spermatogonia to spermatocytes. Based on these results, we suggest that the exposure to endosulfan and flutamide, even at low doses, impairs testicular development either directly or indirectly at the level of brain.

1. Introduction

Endosulfan is a cyclodiene broad spectrum pesticide, which is extensively used in many parts of the world on wide variety of crops (Mersie et al., 2003). Flutamide is a non-steroidal anti-androgen, which is widely used as a drug for prostate cancer and infertility (Pucci et al., 1995; Culig et al., 2004). The use of endosulfan has been prevalent in many countries including India since its inception, but, it has been banned recently. On the other hand, use of flutamide is increasing for the treatment of prostate cancer (Kojima et al., 2002). With an increase in the use of both endosulfan and flutamide, there is a possible risk of endocrine disruption if they end up reaching aquatic ecosystem. In this regard, non-target effects of endosulfan and flutamide on aquatic organisms like fish is a

good model to study. Technical grade endosulfan is a mixture of two biologically active stereoisomers endosulfan I (α) and endosulfan II (β) in the ratio of 70:30 (Mersie et al., 2003) with traces of its byproducts as alcohol and ether. In natural environmental conditions, the isomers transform into diol and sulfate forms in water and soil, which adhere well to clay particles and persist for several years (Naqvi and Vaishnavi, 1993). In the recent past, with increased number of studies on plethora of health hazards associated with endosulfan, many nations have come forward for phase-out/ban. As a result, it was added to annexure-A of the Stockholm convention on persistent organic pollutants (UNEP-POPS-COP5, 2011). Inclusion of endosulfan in annexure-A will impose a ban on the production and use. One of the leading producers and consumers of endosulfan, India, justified the use with its cost effectiveness and lack of good alternative product which leads to the requirement of prolonged time to phase-out endosulfan completely (Li and Macdonald, 2005; Lubick, 2010). Presence of endosulfan in the surface water (0.5-2.4 μg/l) and sediments (0.5-191 μg/l) of ponds/rivers/valleys had been reported in many parts of India (Ahmad et al., 1996; Rao and Pillala, 2001; Begum et al., 2009; KSCSTE, 2011). Like pesticides, many pharmaceuticals which have the potency to mimic sex steroids were found in sewage treatment plants and river/lake waters at higher concentrations (Hirsch et al., 1999; Fent et al., 2006). When a drug is administered to patients, some of its active ingredients may not be completely metabolized and may reach water bodies through sewage, and thus, water bodies may contain pharmaceuticals in metabolized and/or original bioactive forms (Lienert et al., 2007). The best example of a drug entering the aquatic environment and having effects on aquatic organisms is the synthetic hormone, EE_2 due to its use in contraceptive pills (Fent et al., 2006). The

presence of flutamide in water bodies has not been reported yet. We are assuming such a probability either directly or due to related pharmaceutical compounds, as it is widely used for the treatment of prostate cancer, acne vulgaris, hirsutism and polycystic ovary syndrome (Pucci et al., 1995; Culig et al., 2004). Earlier reports on flutamide used wide range of concentrations (320-651 μg/l; Jensen et al., 2004; Filby et al., 2007) which were higher than the one used in the present study. On this perspective, we have chosen to examine the effects of flutamide at a low level (33 μg/l) on catfish testicular development. A range of 20-300 ng/g of endosulfan has been reported as pesticide bioaccumulation in fishes living in northern part of Indian riverine systems (Singh and Singh, 2008). Endosulfan is normally more toxic to fish than to aquatic invertebrates (Naqvi and Vaishnavi, 1993). It can affect central nervous, immune and reproductive systems and can cause behavioral abnormalities in juveniles as well as adult fishes (Jonsson and Toledo, 1993; Narita et al., 2007; Stanley et al., 2009). The impact of effect varies with time span and concentration of exposure. The LC_{50} values reported for the different fish species vary from 0.4 to 60 μg/l or ppb (EFSA, 2011) and for *C. batrachus*, the LC_{50} value is 60 μg/l (Tripathi and Verma, 2004). The effects of endosulfan on reproductive system are largely mediated via hormonal effects rather than direct toxicity. In southern part of India, Kerala state, male children exposed to endosulfan had lowered serum T levels (Saiyed et al., 2003). Endosulfan imparts estrogenic effects in human breast cancer cells (Soto et al., 1994). It can compete with E_2 for estrogen receptor β and cause transactivation gene response (Lemaire et al., 2006). Flutamide inhibits the actions mediated by androgens such as testicular growth, spermatogenesis and development of secondary sexual characters in male guppyfish (Baatrup and Junge, 2001; Jensen et al.,

2004) by competing with T and 11-KT efficiently (Wilson et al., 2007). Flutamide modulates hypothalamic centers regulating GtH secretion and disrupts the negative feedback action of endogenous androgens and thus increasing the steroid production (Jensen et al., 2004). It is also known to modulate the expression of many steroidogenic enzyme genes in mammals (Ohsako et al., 2003). Exposure of flutamide, increased the expression of genes encoding for enzymes involved in sex steroid biosynthesis like *P450c17, 11β-hsd2, cyp19a1a, cyp19a1b* and decreased the expression of *growth hormone receptor, igf-1* in fathead minnow, *Pimephales promelas* (Filby et al., 2007). Thus both endosulfan and flutamide exert similar effects with different mechanism/mode of action. In the present study, endosulfan and flutamide were chosen based on their synergism in exerting their effects on reproductive system. Fishes are best bioindicators for studying endocrine disruption because of their sexual plasticity and sensitivity to sex steroids or xenobiotics (Nagahama et al., 2004; Swapna and Senthilkumaran, 2009). The teleost model, *C. batrachus* is an annually breeding gonochoristic fresh water fish, which are known for their resistance to high concentrations of xenobiotic compounds/steroids which might serve as a useful reference for the study on other less-tolerant and popularly consumed fish species (Tripathi and Verma, 2004; Swapna and Senthilkumaran, 2009). Endosulfan is known to cause drastic reduction in vitellogenin production in adult *C. batrachus* (Chakravorty et al., 1992). Earlier studies often used adult fish or embryos exposed to high levels of endosulfan (10-1000 μg/l; Pandey, 1988; Stanley et al., 2009) and flutamide (320-651 μg/l; Jensen et al., 2004; Filby et al., 2007). But, the impact of these compounds at low doses during testicular differentiation/development has not been analyzed at molecular level. In the present study, after exposing to endosulfan and/or

flutamide, we analyzed the expression pattern of certain steroidogenic enzyme genes such as *P450scc/cyp11a1*, *P450c17*, *11β-h*, *11β-hsd2*, *17β-hsd12* and testis related transcription factors such as *dmrt1*, *sox9a and wt1* which play a vital role in catfish testicular differentiation/development (Raghuveer et al., 2011a). In addition, expression patterns of orphan nuclear receptor (*nr2c1/TR2*), *Ad4BP/SF-1* and *star* genes were also analyzed because of their supportive roles in testicular development and steroidogenesis. Our study was also extended to brain as gonadal development and recrudescence is precisely entrained by H-H-G- axis in vertebrates (Peter et al., 1991; Senthilkumaran and Joy, 1996; Goos et al., 1999; Charlton, 2008). Similar to mammals, H-H-G- axis regulate gonadal development and function through negative and positive feed-back mechanisms (Senthilkumaran and Joy, 1996; Goos et al., 1999; Schulz and Goos, 1999; Charlton, 2008; Swapna and Senthilkumaran, 2009). Consequently, hormone mimics/steroid analogs and xenobiotic compounds can disrupt gonadal development by modulating H-H-G- axis (Swapna and Senthilkumaran, 2009). Recently, we have implicated tryptophan hydroxylase2 (tph2) as a male brain sex marker in teleosts (Sudhakumari et al., 2010; Raghuveer et al., 2011b). On these perspectives, we have analyzed the expression patterns of catfish gonadotropin-releasing hormone (cfGnRH) and tph2 to assess the impact of endosulfan and flutamide on H-H-G- axis regulation on testicular development and function.

2. Materials and methods

2.1. Animals

Catfish hatchlings used in the present study were obtained by IVF as described earlier in the chapter 1. The juveniles were reared carefully with proper aeration and feeding under ambient (natural) photothermal conditions. Similarly sized juvenile males of 50 dph were sorted out to different tanks for the purpose of treatments for the present study.

2.2. Experimental design and sampling

Juvenile male catfish (50 dph), divided into four groups of 50 larva each were maintained in well-aerated aquarium tanks (50 L) containing filtered water with or without treatment compounds. Endosulfan group comprised of catfish larva maintained in filtered water where, 2.5 μg/l endosulfan was dissolved (Lot & Batch 11020104; Parrysulfan 35 EC, Coromandel fertilizers Ltd., Secunderabad, India). Flutamide group comprised of catfish larva maintained in filtered water where 33 μg/l flutamide was dissolved (Lot & Batch 048K1189; Sigma). Endosulfan and flutamide combined (E + F) group comprised of catfish larva maintained in filtered water where 2.5 μg/l endosulfan and 33 μg/l flutamide were dissolved. Age-matched control group was maintained without any treatment. Each group was maintained with a replicate. Pilot studies were conducted to finalize the use of one single low dose. Endosulfan was diluted from the commercial stock directly with milliQ water. Flutamide used in the study was first dissolved in absolute ethanol, air-dried and then reconstituted in milliQ water. Working stocks of endosulfan and flutamide were prepared freshly and used for treatments. After 50 days of exposure, juvenile (male) catfishes (100 dph) were sacrificed by anesthetizing with 100 mg/l of MS 222 (Sigma) in ice-cold water following general animal ethical guidelines. Body weight and length of all fishes were measured prior to dissection. Blood was collected by caudal puncture, using a heparinized syringe. Collected blood was centrifuged at 15,000 xg for 10 min at 4 °C to

obtain plasma for the estimation of T and 11-KT. Testes and brains were dissected out from male fishes under a stereo zoom dissection microscope (Leica, Wetzlar, Germany) using fine sterile scissors and forceps. Testes were measured for weight and length promptly. Then the testes and brain were snap frozen with liquid nitrogen and stored at -80 °C for the isolation of total RNA. The testis of 3-4 fishes have been pooled (as one biological sample) to get sufficient quantities of total RNA and subsequent cDNA synthesis. Five biological samples were used for each group. For histological studies, the body cavity was surgically exposed to divulge testicular tissue to ensure better fixation in Bouin's (saturated picric acid: formaldehyde: glacial acetic acid at 15:5:1) fixative for 2-3 h and processed further as described below.

2.3. Histological analysis

The Bouin's fixed testicular tissues (n = 5) were dehydrated with graded series of ethanol to finally be embedded in paraplast (Sigma). Sections of 5-6 μm thickness were cut serially using a rotatory microtome (Leitz, Wetzlar, Germany). Hematoxylin-eosin staining was carried out following deparaffinization in xylene, rehydration and dehydration with graded series of alcohol. The sections were finally mounted on the slides using DPX mountant (SRL, Mumbai, India). Microscopic examination was carried out using an Olympus CX41 bright field microscope to assess the frequency distribution of testicular germ cell types in percentage for each group.

2.4. Measurement of plasma T and 11-KT

Plasma levels of T and 11-KT were measured by EIA kit (Cayman) by following the manufacturer's protocol. Volume of plasma collected from juvenile catfishes was lower

than required for each assay as per the manufacturer's protocol, and hence plasma from five fish for each group was pooled to obtain one biological plasma sample (n = 1). Five biological samples were analyzed for all the groups (n = 5). The cross reactivity of T antisera for 11-KT was 2% while the opposite was ~ 0.01%. The sensitivity of EIA kit for T and 11-KT measurement is 6 and 1.3 pg/ml, respectively. Intra- and inter-assay variations were negligible and fell within the limits specified in the manufacturer's protocol.

2.5. qPCR

Total RNA of brain and testicular tissues were prepared using TRI reagent (Sigma) as per the manufacturer's protocol. Purity and quantity of total RNA was assessed by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The first strand cDNA was prepared using 500 ng of testis or brain total RNA using iscript® reverse transcriptase (Bio-Rad) according to the manufacturer's instructions. Successful reverse transcription was confirmed for all samples by performing PCR amplification of internal control, β-actin (*C. gariepinus*, GenBank accession No. JN806115). The relative expression was analyzed by qPCR using SYBR® Green detection method except for *dmrt1* and *tph2* where Taqman probes were used as per the method reported earlier (Raghuveer and Senthilkumaran, 2010; Raghuveer et al., 2011b). The primers for qPCR were designed for amplicon length of ~150-200 bp which was spanning two adjacent exons to exclude the amplification of genomic DNA, except for *18S rRNA*. The primers used for *sox9a*, *Ad4BP/SF-1*, *P450c17*, *11β-hsd2*, *star*, *cfGnRH* and *tph2* were designed initially for the closely related species *C. gariepinus*. Later, their suitability for *C. batrachus* was confirmed by sub-cloning and sequencing the cDNA fragments pertaining

to each gene. The partial cDNA sequence data were found to be identical to the respective sequences of the closely related species, *C. gariepinus*. Minor nucleotide variations in *dmrt1* and *wt1* lead to new GenBank submissions for *C. batrachus*. The primers for *cyp11a1*, *11β-h*, *17β-hsd12* and *nr2c1* were designed directly for *C. batrachus*. The list of primers used for qPCR analysis is given in Table 1. The GenBank accession numbers of the genes are as follows: *dmrt1* (FJ596554/FJ596557), *sox9a* (HM149258), *Ad4BP/SF-1* (HQ680985), *wt1* (JF510005/JN848589), *nr2c1* (JN848591), *cyp11a1* (KF739411), *P450c17* (FJ790422), *17β-hsd12* (JN848590), *11β-h* (KJ475435), *11β-hsd2* (GU220074), *star* (FJ793811), *cfGnRH* (X78049), *tph2* (GU290195) and *18S rRNA* (KM018296). The qPCR assays were carried out in triplicate for five different samples using power SYBR® Green PCR master mix (Applied Biosystems) in a 7500 fast thermal cycler (Applied Biosystems) according to the manufacturer's universal thermal cycling conditions. Melting-curve analysis was performed for each sample to check the specificity of PCR amplification and further analysis was done using sequence detection software (Applied Biosystems). All assays were carried out with no template controls which yielded no amplification. Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification and expression of genes was normalized against expression of 18S rRNA, generating a ΔCt value (Ct of target gene-Ct of reference gene). Relative expression was calculated by taking control as a calibrator using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak, 2008).

Table 1. List of primers used for qPCR analysis

S. no.	Primer Name	Primer Sequence 5′ to 3′
1.	**dmrt1F**	**GCAGAGCTCAGCAAAACCCGG**

2.	dmrt1R	GCGGCTCCCAGAGGCAGCAGGAGA
3.	sox9aF	TCTGGCGGCTGCTGAATGAAGG
4.	sox9aR	CTCGGTATCCTCGGTTTCACC
5.	wt1F	ACGCGCACAGGGTGTTCGA
6.	wt1R	GGTACGGTTTCTCTCCTTGTG
7.	Ad4BP/SF-1F	TCACTATGCACCTGCCT
8.	Ad4BP/SF-1R	CGCTTGTACATGGGGCCGAAC
9.	nr2c1F	GAGTCCTGATGCCTATGAGTACG
10.	nr2c1R	GACACTTATCGTCTGTCGAAGTTG
11.	cyp11a1F	TCAACCAAGCGGACCACTGT
12.	cyp11a1F	TCAGGATGCCGTGCCAACTC
13.	P450c17F	CCATGGCTCCAGCTCTTTCC
14.	P450c17R	CAGTAAGACCAACATCCTGAGTGC
15.	17β-hsd12F	AGCCATCGAGAGCAAGTACCATGT
16.	17β-hsd12R	AAGCCGAGTCATCTGACAAACCGA
17.	11β-hF	GGTTCCCCTGCAGTTTGC
18.	11β-hR	TGCACCAGCGTCCCAGCTG
19.	11β-hsd2F	ATCACAGGGTGCGACTCGGGTTTC GGG
20.	11β-hsd2R	CGGCTGAGTGATGTCCACCTGA
21.	starF	TCGTCCGAGCCGAGAACGG
22.	starR	TGCCTCCTCCACTCCACTG
23.	cfGnRHF	AGCGTGCCGTGATGCAGGAG
24.	cfGnRHR	TCTCTCCCAGCGACAGGCGT
25.	tph2F	CAGTTCTCACAGGAAATTGG
26.	tph2R	TGACTTTCTCTTTGGCATCTTC
27.	18S rRNA F	GCTACCACATCCAAGGAAGGCAGC
28.	18S rRNA R	CGGCTGCTGGCACCAGACTTG

2.6. Statistical analysis

All data (n = 5) were expressed as mean ± SEM. All statistical analyses were performed using SigmaPlot 11.0 software (Systat Software Inc.). All data passed homogeneity and

normality tests and were compared by one-way ANOVA followed by SNK post hoc test. A probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Changes in the expression of *dmrt1, sox9a, wt1, Ad4BP/SF-1, nr2c1* and *star*

The exposure of fish to endosulfan and flutamide, alone and in combination, significantly down regulated ($P < 0.05$) the expression (Fig. 1A-F) of *sox9a*, *dmrt1*, *wt1*, *Ad4BP/SF-1*, *nr2c1* and *star* compared to control. Reduction in expression of genes in the treatment groups in comparison to control are indicated below. The expression of *dmrt1* (Fig. 1A) was down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P = 0.001$) to 22, 8 and 69% respectively. Similarly, the expression of *sox9a* (Fig. 1B) was down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 55, 15 and 46% respectively. The expression of *wt1* (Fig. 1C) was also down regulated by endosulfan ($P < 0.003$), flutamide ($P < 0.002$) and E + F ($P < 0.002$) to 25, 13 and 30% respectively. The expression of *Ad4BP/SF-1* (Fig. 1D) was down regulated by endosulfan ($P < 0.035$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 85, 58 and 48% respectively. Similarly, the expression of *nr2c1* (Fig. 1E) was down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 63, 59 and 53% respectively. The expression of *star* (Fig. 1F) was down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 3, 4 and 16% respectively. The expressions of *dmrt1* and *sox9a* were significantly different ($P < 0.002$) between endosulfan, flutamide and E + F groups, while those of *wt1* and *nr2c1* were not (Fig. 1A-C and E). The expression of *Ad4BP/SF-1* was significantly higher ($P < 0.004$) in the

endosulfan group when compared to other groups. (Fig. 1D) The expression of *star* (Fig. 1F) was significantly higher ($P < 0.001$) in the E + F group when compared to other groups.

3.2. Changes in the expression of *cyp11a1, P450c17, 17β-hsd12, 11β-h* and *11β-hsd2*

The exposure of fish to endosulfan and flutamide, alone and in combination, significantly down regulated ($P < 0.05$) the expression of *cyp11a1*, *P450c17*, *17β-hsd12*, *11β-h* and *11β-hsd2* compared to control (Fig. 2A-E). The expression of *cyp11a1* (Fig. 2A) was down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 26, 41 and 60% respectively. The expression of *P450c17* (Fig. 2B) was also down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 19, 30 and 10% respectively. Expression of *17β-hsd12* (Fig. 2C) was down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 11, 16 and 30% respectively. Expression of *11β-h* (Fig. 2D) was down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 22, 48 and 47% respectively. Similarly, the expression of *11β-hsd2* (Fig. 2E) was down regulated by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) to 17, 9 and 41% respectively.

3.3. Changes in the expression of *cfGnRH* and *tph2*

The exposure of fish to endosulfan and flutamide, alone and in combination, significantly elevated ($P < 0.05$) the expression of *cfGnRH* and *tph2* (Fig. 3A & B). The expression of *cfGnRH* (Fig. 3A) was increased by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P < 0.001$) groups to 55-, 19- and 9-folds respectively. The expression of *tph2* (Fig. 3B) was also increased by endosulfan ($P < 0.001$), flutamide ($P < 0.001$) and E + F ($P <$

0.001) groups to 23-, 10- and 5-folds respectively. Individual comparison among treatment groups revealed that the expressions of *cfGnRH* and *tph2* were significantly different ($P < 0.001$) between groups.

3.4. Testicular somatic index (TSI)

TSI of control fish (Table 2) revealed normal testicular development while it was significantly lowered in the endosulfan, flutamide and E + F treated groups (Table 2). Individual comparison among treatment groups revealed that the TSI of endosulfan and flutamide groups were significantly different from the E + F group. However, body and testicular lengths of all the groups were not significantly different between each other.

3.5. Histological analysis

Exposure of fish to endosulfan and flutamide, alone and in combination, affected the germ cell differentiation and thus modulated the progression from primary spermatogonia to differentiating/secondary spermatogonia and further to spermatocytes (Table 3). In control group, there was a uniform development of primary and differentiating/secondary spermatogonia along with the spermatocytes. Though the number of primary spermatogonia was not significantly different between control, endosulfan and E + F groups, the number was decreased significantly in the flutamide (decreased to 18%; $P = 0.044$) group. The exposure of fish to flutamide speeded up the progression from primary spermatogonia to differentiating spermatogonia, but not further to spermatocytes. All the treatments slowed down the progression from differentiating spermatogonia to spermatocytes, which caused a significant increase in the number of differentiating spermatogonia in endosulfan (to 43.3%; $P = 0.01$), flutamide (to 59.33%; $P < 0.001$) and

E + F (to 58.67%; $P < 0.001$) groups when compared to control (31.3%; Table 3). Consequently, significant decrease in the number of spermatocytes was observed in endosulfan (to 25.3%; $P = 0.044$), flutamide (to 22.67%; $P = 0.047$) and E + F (to 14.67%; $P = 0.010$) groups when compared to control (38%; Table 3).

3.6. Plasma T and 11-KT levels

Plasma T levels in endosulfan treated fishes showed no significant change compared to control (Fig. 4A). On the other hand, the levels of T were significantly increased by flutamide (to 6 ng/ml; $P < 0.001$) and E + F (to 6.64 ng/ml; $P < 0.001$). The levels of 11-KT were also significantly increased by flutamide (to 0.68 ng/ml; $P < 0.001$) and E + F (to 0.89 ng/ml; $P < 0.001$) treatments but not by endosulfan treatment (Fig. 4B).

Table 2. TSI, body and testicular lengths of juvenile catfishes at end of the treatments (100 dph).

	TSI	Body length (cm)	Testis length (mm)
Group I Control	0.059 ±0.003	6.10 ±0.32	5.30 ±0.70
Group II Endosulfan	0.037^{ab} ±0.004	5.68 ±0.51	5.37 ±0.58
Group III Flutamide	0.036^{ab} ±0.004	5.57 ±0.42	4.82 ±0.09
Group IV E + F	0.049^{c} ±0.002	5.63 ±0.35	5.10 ±0.29

The data (n=5) represented as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

Table 3. Effects of endosulfan and flutamide, alone and in combination, on the frequency distribution of testicular germ cell types in juvenile male catfish.

	Stages of Germ cells		
	Primary Spermatogonia	Differentiating Spermatogonia	Spermatocytes
Group I Control	30.67 ±1.33	31.33 ±1.33	38.0 ±2.31
Group II Endosulfan	31.33 ±3.53	43.33[a] ±2.9	25.34[a] ±5.46
Group III Flutamide	18.0[a] ±4.16	59.33[b] ±1.76	22.67[a] ±2.9
Group IV E + F	26.67 ±3.53	58.67[a] ±3.5	14.66[b] ±3.53

The data (n=5) represented as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

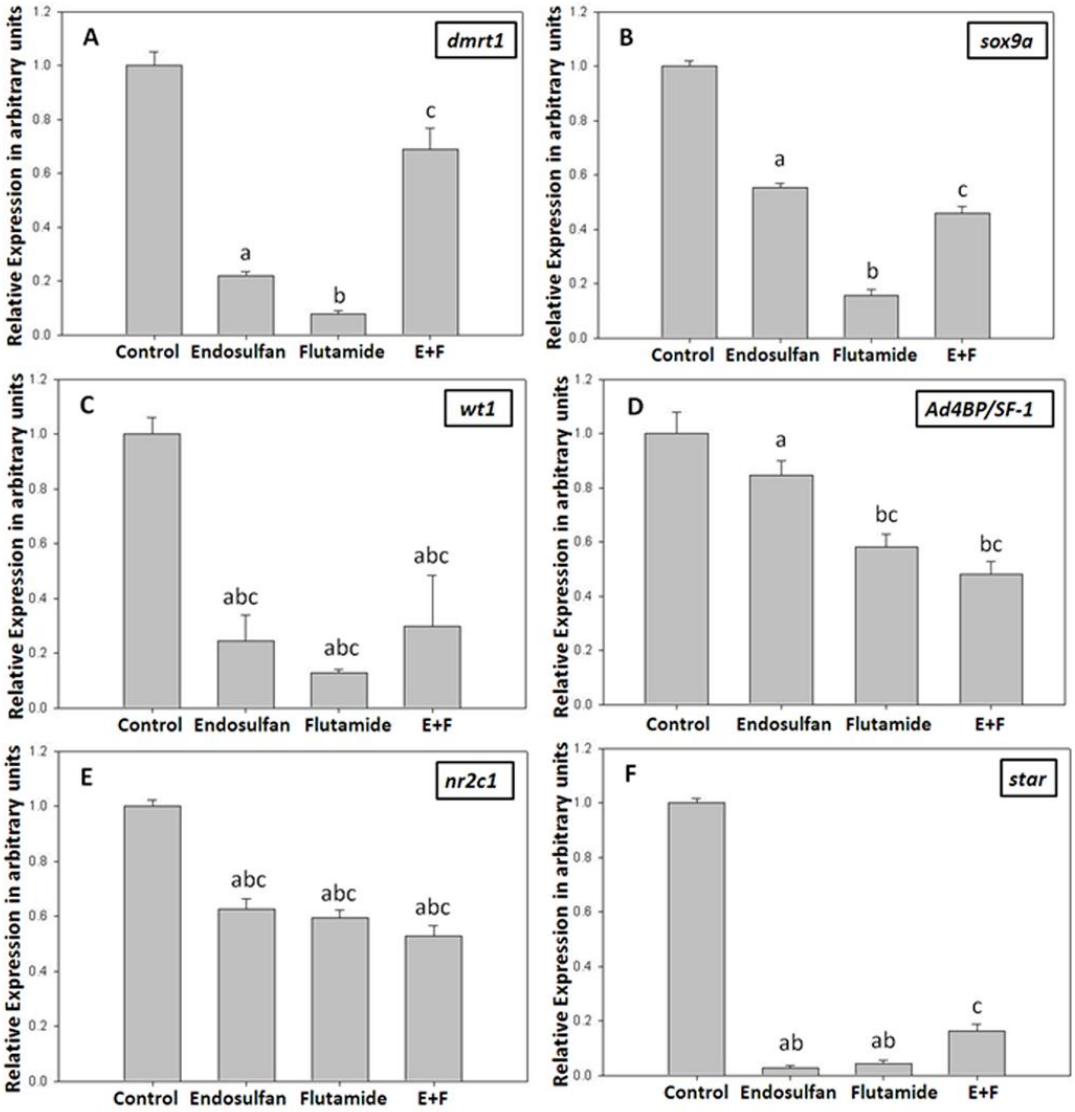

Fig. 1. Relative expression of (A) *dmrt1*, (B) *sox9a*, (C) *wt1*, (D) *Ad4BP/SF-1*, (E) *nr2c1* and (F) *star* in the control and treated testis of juvenile catfish. The relative expression was normalized with 18S rRNA and the values were calculated by using comparative Ct method. Data (n=5) were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post hoc test).

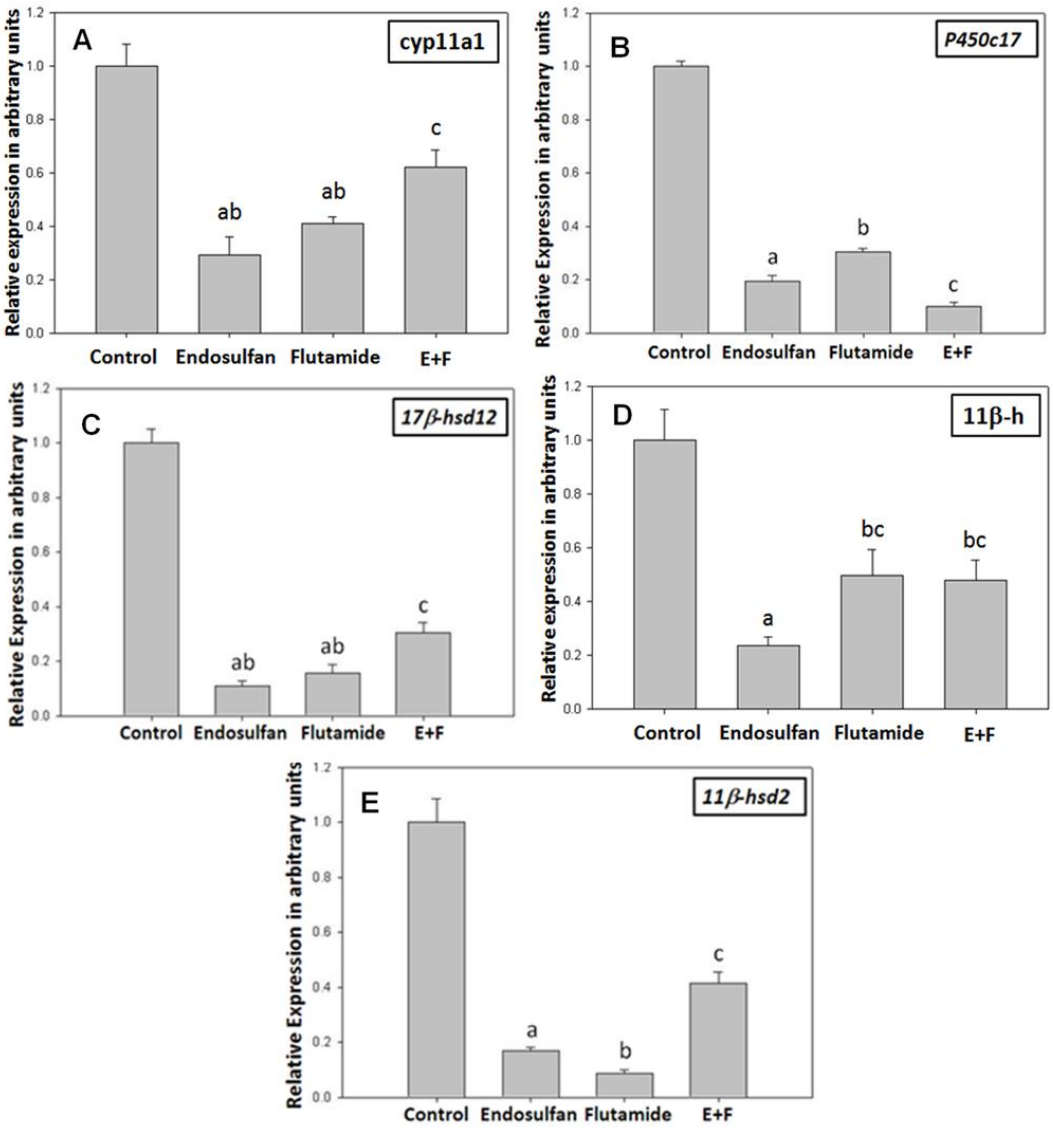

Fig. 2. Relative expression of (A) *cyp11a1*, (B) *P450c17*, (C) *17β-hsd12*, (D) *11β-h* and (E) *11β-hsd2* in the control and treated testis of juvenile catfish. Other details are as in Fig.1.

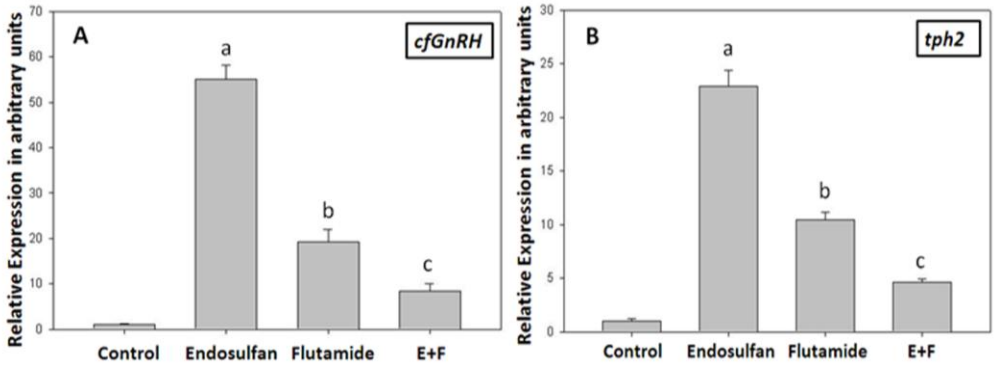

Fig. 3. Relative expression of (A) *cfGnRH* and (B) *tph2* in the control and treated brain of juvenile catfish (100 dph). Other details are as in Fig.1.

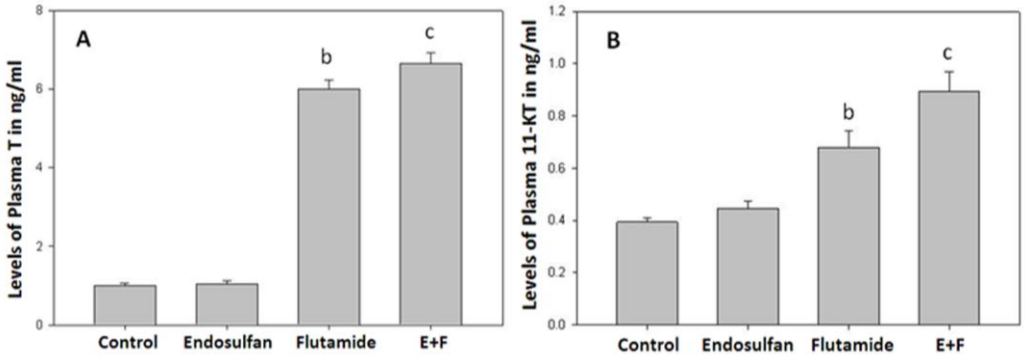

Fig. 4. Levels of plasma (A) T and (B) 11-KT of control and treated juvenile catfish. Data (n=5) were expressed as mean ± SEM. Means with different letters differ significantly ($P < 0.05$; ANOVA followed by SNK post-hoc test).

4. Discussion

The present study demonstrated profound impact of endosulfan and/or flutamide on the transcript levels of various genes, encoding testis related transcription factors, orphan nuclear receptors, steroidogenic enzymes and star, which are involved in multiple aspects of testicular development/differentiation and steroidogenesis of catfish (Raghuveer et al., 2011a). In addition, the compounds modulated transcript levels of brain *cfGnRH* and *tph2*

suggesting an indirect effect in addition to the direct action. The results were substantially supported with the data of testicular germ cell count and endogenous androgen (T and 11-KT) levels. Histological analysis followed by testicular germ cell count in the endosulfan, flutamide and E + F groups revealed slow progression of spermatogonia to spermatocytes which was evident from the increased number of differentiating spermatogonia (secondary spermatogonia). It is also known that elevated levels of androgens in the flutamide and E + F groups might impair testicular function/development by inhibiting spermatogonial differentiation into spermatocytes and spermatids (Meistrich and Shetty, 2003). In the case of endosulfan, it might have interfered with the mode of action of endogenous hormones. Significant histological changes together with no change in the levels of T and 11-KT in endosulfan treatment may be due to endosulfan mimicking estrogen action (Lemaire et al., 2006). An earlier report from our laboratory demonstrated that E_2 analog depletes sperm number in adult male catfish without affecting the levels of sex steroids (Swapna and Senthilkumaran, 2009). Endosulfan exposure caused down regulation of proteins such as ubiquitin and Esco2 in catfish testes, and upregulation of melanocortin receptor 2 in ovaries (Laldinsangi et al., 2014). Related studies on this line showed that the exposure of fish to 1-50 μg/l endosulfan caused enlargement of seminiferous tubules, reduction in the number of spermatogonia and Sertoli cells with an increase in the number of interstitial cells in teleosts (Balasubramani and Pandian, 2008; Holdway et al., 2008). Thus, either way, the treatment of fish with endosulfan or flutamide, alone and in combination, affected testicular architecture. Presence of endosulfan in surface waters (0.5-2.4 μg/l) and sediments (0.5-191 μg/l) of ponds/rivers/valleys in many parts of India (Ahmad et al., 1996; Rao and Pillala, 2001;

Begum et al., 2009; KSCSTE, 2011) supports the selection of our low dose for endosulfan treatment. This is also true for flutamide, as earlier studies used over 100 μg/l of flutamide (Jensen et al., 2004; Filby et al., 2007). The vehicle and stabilizing chemicals added to endosulfan during its technical formulation may have some biological effects. But such an effect is negligible, considering the low concentration used in the present study.

Considerable reduction in the progression of spermatogonia resulting in decreased number of spermatocytes in all the treated groups signifies that both endosulfan and flutamide likely impair testicular development by targeting steroidogenic enzyme and other testis related genes. The reduction in the transcript levels of genes encoding testis related transcription factors like dmrt1, sox9a, wt1 and Ad4BP/SF-1 indicate defective testicular development and function after the treatments of fish to endosulfan and/or flutamide considering the prominent role of these transcription factors in the testicular development (Gao et al., 2006; Raghuveer et al., 2011a). In catfish, dmrt1 plays a key role in early testicular development, recrudescence, as its expression is high during spermatogenesis and decreases gradually thereafter during spawning/spermiation and post spawning phases (Raghuveer and Senthilkumaran, 2009). The second important transcription factor, which is highly expressed during the period of spermatogenesis is sox9a (Raghuveer and Senthilkumaran, 2010). The expression of sox9 can be modulated by Ad4BP/SF-1 which may bind to the enhancer region of the gene (Sekido et al., 2004; Sekido and Lovell-Badge, 2008). Ad4BP/SF-1 belongs to NR5A1 nuclear receptor super family which regulates the transcription of many steroidogenic enzyme genes including *cyp11a1*, *cyp19a1a* and *P450c17* genes and the effect occurs through interactions with a

number of co-regulators to delineate cell-specific expression (Hu et al., 2001; Gurates et al., 2003; Yoshiura et al., 2003; Zhou et al., 2007; Schimmer and White, 2010). In contrast to this, the product of dmrt1/DMY represses Ad4BP/SF- 1 mediated activation of *P450c17* transcription in HEK 293 cells (Zhou et al., 2007). *Ad4BP/SF-1* knockouts lack expression of many of the steroidogenic enzymes and factors related to steroidogenesis including star, which is a mitochondrial phosphor protein that delivers cholesterol to inner mitochondrial membrane, the rate-limiting step in steroidogenesis (Caron et al., 1997; Stocco, 2000). Decreased expression of *star* in the present study might be due to decreased expression of *Ad4BP/SF-1*, which can stimulate the transcription of former at the promoter level (Yang and Gutierrez, 2009). The orphan nuclear receptor, nr2c1 is essential for testicular differentiation and it functions as a modulator for androgen receptor (AR) and it can suppress estrogen mediated transcription (Mu and Chang, 2003). The decrease in the expression of *nr2c1* would have modulated the transcript expression of genes related to testis indirectly as nr2c1 can interact with regulator proteins of gene expression like histone deacetylases (Franco et al., 2003). The other transcription factor gene analyzed was *wt1*. The product of this gene is a zinc finger transcription factor, which plays critical role in gonadal sex determination. Wt1 regulates Sox9 either directly or indirectly, after *sry* ceases, which thereby maintains Sertoli cells and seminiferous tubules in the developing testes (Gao et al., 2006). Further, such a possibility exists in teleosts like catfish were *sry* is absent except for the existence of *sox3*, an ancestor of *sry* (Chapter 5). Decreased expression of wt1 might have triggered reduction in the expression of *sox9a* along with *Ad4BP/SF-1* and changes in the tubular architecture of testicular lumen. The results correlated well

with a previous report, in which the loss of *wt1* caused disruption of developing seminiferous tubules and subsequent progressive loss of Sertoli and germ cells (Gao et al., 2006). In accordance to our findings, flutamide decreased *dmrt1* expression (Kobayashi et al., 2004) and modulated the expression of many steroidogenic enzyme genes (Filby et al., 2007). The enzyme P450c17 is essential for precursor steroid formation and any possible changes in its expression along with its positive regulator, Ad4BP/SF-1 can decrease gonadal androgens, estrogens and adrenal cortisol in vertebrates (Payne, 1990; Zhou et al., 2007). Reduction in the expression of *cyp11a1*, *P450c17*, *17β-hsd12*, *11β-h* and *11β-hsd2* after the treatments might compound this effect further as the products of these genes are the principal enzymes for T, 11-KT and E_2 production in teleosts (Zhou et al., 2005; Rasheeda et al., 2010; Chapter 1; 2; 3; 4). Any decrease in the androgen production may cause down regulation of the testis related transcription factors responsible for germ cell proliferation and testicular development in catfish (Raghuveer and Senthilkumaran, 2010; Rasheeda et al., 2010; Raghuveer et al., 2011a). In our studies, there is a considerable increase in plasma T and 11-KT levels in flutamide and E + F groups. The increase may be due to flutamide, which competes with androgen and irreversibly block AR thereby increasing the plasma levels of T and 11-KT (Wilson et al., 2007). We are hypothesizing that an increase in the expression of cfGnRH and tph2 may facilitate LH driven increase of plasma T and 11-KT in the treated fish to compensate the actions mediated by the anti-androgens. This was not the case in the endosulfan group wherein drastic increase in the expression of cfGnRH could not affect the enhanced androgen production by the testis via LH. Differential impact of these compounds indicates varied properties of anti-androgens and estrogens in male

reproductive system. Previous reports on this line supported our results that cfGnRH and tph2 can regulate the release of GtH with controlled feedback action on pituitary (Tsai et al., 2000; Senthilkumaran et al., 2001; Raghuveer et al., 2011b). The release of cfGnRH and GtHs can be further modulated by serotonin (5-HT) in teleosts (Senthilkumaran and Joy, 1996; Senthilkumaran et al., 2001) and hence any change in 5-HT affects the GtH release. The levels of 5-HT are modulated by tph2, which is the rate-limiting enzyme for 5-HT production (Sudhakumari et al., 2010; Raghuveer et al., 2011b). In addition, the increase in T and 11-KT might also be due to the loss of negative feedback action of T and 11-KT itself on H-H-G- axis. These data are in accordance with studies conducted in mammals where flutamide blocks the negative feedback action of T at H-H-G- axis to stimulate LH secretion and increased androgen production (Yamada et al., 2000; Ohsako et al., 2003). The decrease in transcripts of *11β-hsd2* in the present study, might contradict the observation made by Ohsako et al. (2003) in mice and Filby et al. (2007) in fathead minnow that the exposure of anti-androgens caused over expression of steroidogenic enzyme genes along with increased LH, which caused the increase in the testicular T production. On the contrary, endosulfan is not able to modulate androgen production, in spite of drastic increase in the expression of cfGnRH along with tph2, indicating no negative feedback blockade. Further studies are needed to give more molecular details and mechanism of action of these compounds. As of now, these discrepancies may be attributed to different development patterns, stages, species and differences in treatment doses.

5. Conclusions

In summary, exposure of juvenile catfish to endosulfan and flutamide, alone and in combination, impaired testicular differentiation or development in catfish by modulating the expression of several transcription factors, *star* and steroidogenic enzyme genes. Treatment induced changes in *cfGnRH* and *tph2* expression implied an effect at the level of brain in addition to testis. The observed changes in catfish convey potential impact on other less tolerant teleost species which may be exposed to similar compounds in nature. Our study will evoke an awareness to curtail the increased use of pharmaceuticals/pesticides to minimize pollutants for the betterment of aquatic ecosystem.

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Consolidated Summary

Present study examines the expression changes of *cyp11a1*, *17β-hsd1* and *12*, *11β-h* and *11β-hsd* together with *sox3* in gonads during sex differentiation, development, maturation and recrudescence (reproductive cycle) together with GtH induction in catfish. In addition, promoter motif analysis of *11β-hsd* was also carried out to understand its importance in steroidogenesis and spermatogenesis.

Chapter 1: Expression analysis of *cyp11a1* during gonadal development, recrudescence and after hCG induction and sex-steroid analog treatment in catfish, *Clarias batrachus*

Gonadal steroidogenesis is regulated primarily by the delivery of substrate, cholesterol into the inner mitochondrial membrane and its subsequent conversion to pregnenolone by Cyp11a1. In this study, full length cDNA of *cyp11a1* (2581 bp) was cloned from catfish testis to investigate the importance of Cyp11a1 by analyzing the expression of *cyp11a1* during gonadal development, seasonal reproductive cycle, after hCG induction and sex-steroid analog treatment. Phylogenetic analysis revealed that the Cyp11a1 is more conserved across teleosts. Tissue distribution analysis revealed that the *cyp11a1* expression was higher in the testis followed by the brain, head kidney, muscle and ovary compared to other tissues analyzed. High expression of *cyp11a1* in the head kidney and muscle revealed that Cyp11a1 could potentially regulate the extra-gonadal and/or circulating steroid levels in teleosts. Developing and mature testes showed higher expression of *cyp11a1* than the ovary of corresponding age group. Further, *cyp11a1* expression was higher during pre-spawning and spawning phases of testicular cycle and was found to be upregulated by hCG, *in vivo* and *in vitro*, which indicates the possible

regulation by GtH. Exposure of MT (1 µg/l) and EE_2 (1 µg/l) for 21 days during catfish testicular development showed lower *cyp11a1* expression levels in the testis and brain indicating a certain feedback intervention. These results suggest possible role for Cyp11a1 in the testicular development and recrudescence.

Chapter 2: Molecular cloning and expression analysis of *17β-hydroxysteroid dehydrogenase 1* and *12* during gonadal development, recrudescence and after *in vivo* hCG induction

17β-hsds are important steroidogenic enzymes as they are involved in both T and E_2 biosynthesis. Full length cDNAs of *17β-hsd 1* (1791 bp) and *12* (1073 bp) were cloned from catfish gonad which encode protein of 295 and 317 amino acid residues, respectively. To understand the importance of these enzymes in teleost reproduction, mRNA expression was analyzed during gonadal development, seasonal reproductive cycle and after hCG induction. Phylogenetic analysis revealed that the 17β-hsd 1 and 12 share high homology with their respective 17β-hsd forms from other teleosts and both the forms belong to short chain dehydrogenase/reductase family. Tissue distribution analysis showed that the *17β-hsd 1* expression was higher in the ovary and gills, while *17β-hsd 12* was higher in the testis, ovary, brain, intestine and head kidney compared to other tissues analyzed. Developing and mature ovary showed higher expression of *17β-hsd 1*, while *17β-hsd 12* was higher in the testis than the ovary of corresponding stages. Further, *17β-hsd 1* and *12* transcripts together with E_2 and T levels were found to be modulated during different phases of the seasonal reproductive cycle. Expression of *17β-hsd 1* and *12* was upregulated after hCG induction, suggesting possible regulation by GtH. Our findings

suggest that 17β-hsd 1 and 12 might play important role in regulating gonadal development and gametogenesis through modulation of sex-steroid levels.

Chapter 3: Dynamic expression of *11β-hydroxylase* during testicular development, recrudescence and after *in vivo* and *in vitro* hCG induction

11β-h is involved in the production of 11-hydroxytestosterone (11-OHT), an immediate precursor for 11-KT, a potent androgen in teleosts. To understand the role of 11β-h in gonadal development, maturation, function and recrudescence in an annually reproducing teleost, the present study was conducted using *C. batrachus*. Four forms of *11β-h* cDNA, regular type (2253 bp), variant 1 (1290 bp), variant 2 (1223 bp) and variant 3 (1978 bp) were identified from the testis of catfish which expressed ubiquitously with high levels in testis. *11β-h* transcripts were detected as early as 0 dph further, stage- and sex-dependent increase in the *11β-h* transcripts were seen during gonadal differentiation/development. In addition, high expression of *11β-h* (regular type) in pre-spawning phase was detected. Corroboratively, levels of 11-KT in serum and testicular tissue were high during pre-spawning and spawning phases, which might facilitate initiation and normal progression of spermatogenesis. The expression of *11β-h* was upregulated after hCG induction both *in vivo* (all forms) and *in vitro* (regular type). Immunohistochemical and immunofluorescence localization showed the presence of 11β-h in Sertoli and interstitial/Leydig cells of the testis. These results suggest the involvement of 11β-h in late stages of testicular development, together with the regulation of seasonal reproductive cycle in catfish.

Chapter 4: Isolation of *11β-hsd* in catfish: localization, expression changes, promoter motif analysis and regulation by transcription factor Sox3

In teleosts, the expression of steroidogenic enzyme genes and their related transcription factors (TFs) are critical for the regulation of steroidogenesis and gonadal development. In fishes, 11-KT is the potent androgen and hence, 11β-hsd, enzyme involved in the conversion of 11-OHT to 11-KT is important. Expression of *11β-hsd* together with its promoter level regulation is critical for sex-, stage- and tissue-specific expression which was never studied in any teleosts. Hence to understand the importance of *11β-hsd* and its promoter level regulation, the present study was conducted using catfish as a teleost model. At first the ORF of *11β-hsd* was cloned from catfish testis and expression changes were analyzed during gonadal development and maturation together with the regulation of seasonal reproductive cycle in catfish. *11β-hsd* was expressed ubiquitously with high levels in testis and liver. *11β-hsd* expression starts very early during development in addition, stage- and sex-dependent increase were seen in the ontogenic studies. Further, *11β-hsd* expression was higher during spawning phase of reproductive cycle and was found to be GtH inducible both *in vivo* and *in vitro*. IHC and IF analysis revealed the presence of 11β-hsd immunoreactivity in the interstitial/Leydig cells and Sertoli of testis. To clone the 5′ upstream region of *11β-hsd*, genomic DNA library was prepared from catfish testis and used for two rounds of PCR reactions. In total, ~2kb of 5′ upstream region was cloned and *in silico* promoter and potential TF binding site prediction were done using various promoter prediction and TF binding site algorithms. Sox3, Wt1, Pax2, Dmrt1 and Ad4BP/SF-1 binding sites were found to exist in the cloned *11β-hsd* promoter

region. Luciferase reporter assay using the sequential deletion constructs of the promoter in Human embryonic kidney and Chinese hamster ovary cells revealed the high promoter activity of the constructs containing Sox3 sites, but not with other sites. Site-directed mutagenesis, Sox3 over expression and electrophoretic mobility shift assay with Sox3 binding sites further substantiated the importance of Sox3. These results suggest that Sox3 binds to the *11β-hsd* promoter and activates its transcription and thus it appears to be a critical factor that regulates 11β-hsd expression during gonadal development, maturation and seasonal cycle in *C. batrachus*.

Chapter 5: Expression Analysis of *sox3* during testicular development, recrudescence and after hCG induction

In teleosts, the expression of steroidogenic enzymes and related transcription factor genes occurs in a stage- and tissue specific manner causing sexual development. However the role of *sox3*, an evolutionary ancestor of *Sry*, has not been studied in detail. Therefore, the full length cDNA of *sox3* (1197 bp) was cloned from catfish testis, and mRNA expression was analyzed during gonadal development, during the seasonal reproductive cycle, and after hCG induction. Tissue distribution analysis showed that *sox3* expression was higher in testis, ovary, and brain compared to other tissues analyzed. Developing and mature testis showed higher *sox3* expression than the ovary of corresponding stages. *Sox3* transcripts were found to be higher during the spawning phase of the seasonal reproductive cycle. Furthermore, the expression of *sox3* was upregulated by hCG after *in vivo* and *in vitro* induction, suggesting that GtHs might stimulate its expression. *In situ* hybridization and immunohistochemistry showed the presence of *sox3* mRNA and

protein in somatic and interstitial cell layers of the testis. Sox3 could also be localized in the zona radiata of developing and mature oocytes. Exposure of MT (1 µg/l) and EE_2 (1 µg/l) for 21 days during testicular development showed lower *sox3* expression levels in the testis and brain, indicating a certain feedback intervention. These results suggest a possible role for Sox3 in the regulation of testicular development and function.

Chapter 6: Endosulfan and flutamide impair testicular development in juvenile catfish

Sex steroid production and expression/regulation of transcription factors are highly dynamic and sensitive. Therefore analyzing the impact of hormone analogs and endocrine disruptors on gonadal development of fishes can provide new insights on regulatory aspects. On this perspective, the impact of few endocrine disruptors like endosulfan and flutamide on catfish gonadal development were analyzed. Endosulfan and flutamide, a widely used pesticide and a prostate cancer/infertility drug, respectively, have an increased risk of causing endocrine disruption if they reach water bodies. Though many studies are available on neurotoxicity/bioaccumulation of endosulfan and receptor antagonism of flutamide, very little is known about their impact on testicular steroidogenesis at molecular level. As sex steroids play an important role in sex differentiation of lower vertebrates including fishes, a small change in their levels caused by endocrine disruptors can affect the gonadal development of fishes significantly. The aim of this study was to evaluate the effects of endosulfan and flutamide on testis related transcription factor and steroidogenic enzyme genes with a comparison on the levels of androgens during critical period of catfish testicular development. We also analyzed the correlation between the above mentioned genes and catfish gonadotropin-releasing

hormone (cfGnRH)-tryptophan hydroxylase2 (tph2). *C. batrachus* males of 50 dph were exposed to very low dose of endosulfan (2.5 μg/l) and flutamide (33 μg/l), alone and in combination for 50 days. The doses used in this study were far less than those used in the previous studies of flutamide and reported levels of endosulfan in surface water and sediments. Sampling was done at end of the treatments (100 dph) to perform testicular germ cell count (histology), measurements of T and 11-KT by EIA and transcript quantification by qPCR. In general, treatments down regulated the expression of several genes including testis-related transcription factors (Dmrt1, Sox9a and Wt1]), steroidogenic enzymes (Cyp11a1, P450c17, 17β-hsd12, 11β-h and 11β-hsd), Star and orphan nuclear receptors (nr2c1 and Ad4BP/SF-1). In contrast, the transcripts of *cfGnRH* and *tph2* were upregulated in the brain of all treated groups with maximum elevation in the endosulfan group. However, combination of endosulfan and flutamide (E + F) treatment showed minor antagonism in a few results of transcript quantification. Levels of T and 11-KT were elevated after flutamide and E + F treatments while no change was seen in the endosulfan group signifying the effect of flutamide as an androgen receptor antagonist. All the treatments modulated testis growth/function by decreasing the progression/differentiation of spermatogonia to spermatocytes. Based on these results, we suggest that the exposure to endosulfan and flutamide, even at low doses, impairs testicular development either directly or indirectly at the level of brain.

Final Conclusions

Differential and dimorphic expression of specific steroidogenic enzyme genes during different stages of gonadal development, maturation and seasonal cycle were found. The final conclusions from the studies are

- **Steroidogenic enzyme gene expression is stage- tissue- and sex- specific.**
- **Sox3 binds to 11β-hsd promoter and transactivates its transcription.**
- **Steroidogenic enzyme genes and Sox3 might play more important role during testicular development and maturation.**
- **11β-h, 11β-hsd and Sox3 were localized in both interstitial/Leydig and/or Sertoli cells (Sertoli Steroidogenesis).**
- **Endocrine disruptors down regulate steroidogenic enzyme gene expression which thereby retards the testicular development.**

Research Publications

Research Publications from thesis work:

1. **Rajakumar, A.,** Senthilkumaran, B., **(2015).** Dynamic Expression of *11β-hydroxylase* During testicular Development, Recrudescence and after *in vivo* and *in vitro* hCG Induction in the Catfish, *Clarias batrachus.* **Gen. Comp. Endocrinol. 211: 69-80** (Elsevier Science, UK).

2. **Rajakumar, A.,** Senthilkumaran, B., **(2014).** Expression Analysis of *sox3* During Testicular Development, Recrudescence and after hCG Induction in Catfish, *Clarias batrachus.* **Sex. Dev. 8: 376-386** (Karger International, Germany).

3. **Rajakumar, A.,** Senthilkumaran, B., **(2014).** Molecular Cloning and Expression Analysis of *17β-hydroxysteroid dehydrogenase 1* and *12* During Gonadal Development, Recrudescence and after *in vivo* hCG Induction in Catfish, *Clarias batrachus.* **Steroids 92: 81-89** (Elsevier Science, UK).

4. **Rajakumar, A.,** Senthilkumaran, B., **(2014).** Expression Analysis of *cyp11a1* during Gonadal Development, Recrudescence and after hCG induction and Sex steroid analog Treatment in the Catfish, *Clarias batrachus.* **Comp. Biochem. Physiol. B 176: 42-47** (Elsevier Science, UK).

5. **Rajakumar, A.,** Singh, R., Chakrabarty, S., Murugananthkumar, R., Laldinsangi, C., Prathibha, Y., Sudhakumari, C.C., Dutta-Gupta, A., Senthilkumaran B., **(2012).** Endosulfan and Flutamide Impair Testicular Development in the Juvenile Asian Catfish, *Clarias batrachus*, **Aquat. Toxicol. 110-111: 123-132** (Elsevier Science, UK).

Review Chapter

1. **Rajakumar, A.,** Senthilkumaran, B., **(2013).** Sperm Maturation in Teleosts: Role of Androgens and Progestins in our Present Understanding to Emerging New Concepts *In*: **"Sexual Plasticity and Gametogenesis in Fishes" (**B.

Senthilkumaran, Ed.), Nova Biomedical, USA **(A Review) Chapter XXIII, 377-400.**

Research publications with members of Prof. B. Senthilkumaran's laboratory

1. Laldinsangi, C., Vijayaprasadarao, K., **Rajakumar, A.,** Murugananthkumar, R., Prathibha, Y., Sudhakumari, C.C., Mamta, S.K., Dutta-Gupta, A., Senthilkumaran B., **(2014).** Two-Dimensional Proteomic Analysis of Gonads of Air-Breathing Catfish, *Clarias batrachus* after the Exposure of Endosulfan and Malathion. **Environ. Toxicol. Pharmacol. 37: 1006-1014** (Elsevier Science, UK).

2. Prathibha, Y., Murugananthkumar, R., **Rajakumar, A.,** Laldinsangi, C., Sudhakumari, C.C., Mamta, S.K., Dutta-Gupta, A., Senthilkumaran B., **(2014).** Gene Expression Analysis in Gonads and Brain of Catfish, *Clarias batrachus* after the Exposure of Malathion. **Ecotoxicol. Environ. Saf. 102: 210-219** (Elsevier Science, UK).

3. Mamta, S.K., Raghuveer, K., Sudhakumari, C.C., **Rajakumar, A.,** Basavaraju, Y., Senthilkumaran, B., **(2014).** Cloning and Expression Analysis of Tyrosine Hydroxylase and Changes in Catecholamine Levels in Brain during Ontogeny and after Sex Steroid Analogues Exposure in the Catfish, *Clarias batrachus.* **Gen. Comp. Endocrinol. 197: 18-25** (Elsevier Science, UK).

4. Chakrabarty, S., **Rajakumar, A.,** Raghuveer, K., Sridevi, P., Mohanachary, A., Prathibha, Y., Bashyam, L., Dutta-Gupta, A., Senthilkumaran, B., **(2012).** Endosulfan and Flutamide, Alone and in Combination, Target Ovarian Growth in the Juvenile Catfish, *Clarias batrachus*. **Comp. Biochem. Physiol. C 155: 491-497** (Elsevier Science, UK).

5. Sreenivasulu, G., Senthilkumaran, B., Sridevi, P., **Rajakumar, A.,** Rasheeda, M.K., **(2012).** Expression and Immunolocalization of 20β-Hydroxysteroid Dehydrogenase During Testicular Cycle and After hCG Induction, *In vivo* in the Catfish, *Clarias gariepinus*. **Gen. Comp. Endocrinol. 175: 48-54** (Elsevier Science, UK).

6. Raghuveer, K., Senthilkumaran, B., Sudhakumari, C.C., Sridevi, P., **Rajakumar, A.,** Singh, R., Murugananthkumar, R., Majumdar, K.C., **(2011).** Dimorphic Expression of Various Transcription Factor and Steroidogenic Enzyme Genes During Gonadal Ontogeny in the Air-Breathing Catfish, *Clarias gariepinus*. **Sex. Dev. 5: 213-223** (Karger International, Germany).