Role of *sycp3* and *c-kit* during gonadal development and recrudescence in catfish

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

Doctor of Philosophy

in

Animal Sciences

Supervisor:

Prof. B. Senthilkumaran

By:

Laldinsangi

Department of Animal Biology
School of Life Sciences
University of Hyderabad
P.O. Central University
Hyderabad 500 046, Telangana, India

May, 2017

Regd. No.: 11LAPH03

University of Hyderabad

(A Central University by an Act of Parliament)
Department of Animal Biology
School of Life Sciences
P.O. Central University, Gachibowli, Hyderabad-500046

CERTIFICATE

This is to certify that the thesis entitled **"*Role of sycp3 and c-kit during gonadal development and recrudescence in catfish*"** submitted by Ms. **Laldinsangi** bearing registration number **11LAPH03** in partial fulfillment of the requirements for award of **Doctor of Philosophy** in the School of Life Sciences is a bona fide work carried out by her under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Parts of this thesis have been:

A. Published in the following publications:

- Environmental Toxicology and Pharmacology 37, 1006-1014 (ISSN: 1382-6689)

B. Presented in the following conferences:

1. *International Symposium on Reproductive Biology and Comparative Endocrinology*, 25^{th}-27^{th} March, 2015, BHU, Varanasi (International)
2. *International symposium on Comparative Endocrinology and Integrative Physiology*, 4^{th}-7^{th} August, 2015, Thiruvananthapuram, Kerala (International)
3. *8^{th} Congress of the Asia and Oceania Society for Comparative Endocrinology*, 20^{th}-24^{th} June, 2016, Seoul, South Korea (International)

Further, the student has passed the following courses towards the fulfillment of course work required for the award of Ph.D

Course Code	Name	Credits	Pass/Fail
AS 801	Seminar 1	1	Pass
AS 802	Research Ethics & Management	2	Pass
AS 803	Biostatistics	2	Pass
AS 804	Analytical Techniques	3	Pass
AS 805	Lab Work	4	Pass

Supervisor **Head of the Department** **Dean of the School**

University of Hyderabad
(A Central University by an Act of Parliament)
Department of Animal Biology
School of Life Sciences
P.O. Central University, Gachibowli, Hyderabad-500046

DECLARATION

I, **Laldinsangi,** hereby declare that this thesis entitled **"Role of *sycp3* and *c-kit* during gonadal development and recrudescence in catfish"** submitted by me under the guidance and supervision of ***Prof. B. Senthilkumaran*** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other University or Institute for the award of any degree or diploma.

Date:

Name: Laldinsangi

Signature of the Student

Regd. No.: 11LAPH03

Dedicated to my loving family

Acknowledgements

- *At the outset, I express my heartfelt gratitude to my supervisor Prof. B. Senthilkumaran for his outstanding guidance and supervision. His passion for work has been a constant source of inspiration for me, and I will be eternally indebted to him for his undying support and patience.*

- *My sincere gratitude goes to Prof. Aparna Dutta-Gupta for her timely suggestions and invaluable support, as well as her generosity in lending her laboratory facilities whenever required.*

- *I would like to thank my Doctoral Committee members Dr. Anita Jagota and Dr. Y. Suresh for their encouragement, advice and direction throughout the course of my research work.*

- *I express my gratitude to the present Head of Department, Prof. Jagan Pongubala and former Heads Prof. B. Senthilkumaran, Prof. Manjula Sritharan and Prof. S. Dayananda, Department of Animal Biology, for providing good equipment facilities in the department.*

- *I thank the present Dean Prof. P. Reddanna and former Deans Prof. A.S. Raghavendra, Prof. R.P. Sharma, Prof. Aparna Dutta-Gupta and Prof. M. Ramanadham for access to the school facilities.*

- *I would like to thank all the faculty members of School of Life Sciences for their generosity in providing access to their laboratory facilities.*

- *I thank Dr. C.C. Sudhakumari for her help and support. Her contribution to the laboratory through funds from DST-Women Scientist grant is also greatly acknowledged.*

- *My sincere gratitude to UPE-II for providing me funding to attend the 8th AOSCE conference in Seoul, South Korea.*

- *I thank Department of Biotechnology, Govt. of India, for the much appreciated financial support through Junior and Senior Research Fellowships.*

- *I also thank DST, DBT, DBT-TATA, CSIR and UGC for funding our laboratory (Grants provided to Prof. BS) and WOS-(A) given to Dr. CCS and Dr. SKM.*

- *DST-FIST, DST-PURSE, DBT-CREBB, UGC-SAP/CAS, UPE and University of Hyderabad funding to the Department and the School are also acknowledged.*

❖ *I also express my sincere gratitude to my present and former lab-mates, Dr. A. Rajakumar, Dr. C. Swati, Dr. S.K. Mamta, Dr. Y. Prathibha, Dr. Murugananthkumar, Dr. S. Durai, Dr. J. Varshini and Dr. S. Anshu, Mr. Yugantak Raj Gupta, Ms. S. Deepa, Mrs. A. Anitha, for all their all invaluable support, both on the academic and personal fronts. All the experiences I encountered and life lessons that I have learned from them will always be cherished.*

❖ *I thank all my friends and research scholars of SLS for their timely help.*

❖ *I thank Mrs. Leena Bashyam, Mrs. Monica Kannan and Mrs. Nalini for their help in Genomics, Proteomics and Confocal microscope facilities, respectively.*

❖ *I thank all the non-teaching staff in the School, Department and Animal house for their help and all our lab attendants for the maintenance of the laboratory and aquaculture facilities.*

❖ *I thank the University of Hyderabad for providing access to extensive online resources in the form of journal subscriptions as well as internet facility provided via the CNF that has aided my research work every step of the way.*

❖ *I thank all my friends in campus especially the Mizo student community who have made this campus feel like a second home.*

❖ *My sincere gratitude to my best friends, who have lent their ears, shoulders and hearts without hesitation through all these years, and I am a better person because of them.*

❖ *Words are inadequate to describe the love and gratitude I have for my family, whose boundless support and encouragements have made everything possible for me to get to where I am now.*

❖ *Above all, I thank **God**, whose grace has led me thus far.*

~Laldinsangi~

Abbreviations

11-KT	–	11-ketotestosterone
11β-h	–	11β-hydroxylase
3β-hsd	–	3β-hydroxysteroid dehydrogenase
ACTH	–	Adrenocorticotropic hormone
AMH	–	Anti-Müllerian hormone
Cyp19a1a	–	Ovarian 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
DTT	–	Dithiothreitol
EDCs	–	Endocrine disrupting chemicals
ESCO2	–	Establishment of cohesion 1 homolog 2
esiRNA	–	Endonuclease prepared small interfering RNAs
FBS	–	Fetal bovine serum
FITC	–	Fluorescein isothiocyanate
FSH	–	Follicle stimulating hormone
Gata4	–	GATA binding protein 4
GnRH	–	Gonadotropin releasing hormone
hCG	–	Human chorionic gonadotropin
IF	–	Immunofluorescence
IHC	–	Immunohistochemistry
IU	–	International Units
IVF	–	*In vitro* fertilization
kDa	–	Kilodalton
L-15	–	Leibovitz -15
LC	–	Lethal concentration
LH	–	Luteinizing hormone
MALDI-TOF	–	Matrix-assisted laser desorption/ionization-time of flight

MS 222	–	Ethyl 3-aminobenzoate methanesulfonate
PBS	–	Phosphate buffered saline
PEI	–	Polyethylenimine
PGC	–	Primordial germ cells
qPCR	–	Quantitative real-time PCR
RACE	–	Rapid Amplification of cDNA ends
SC	–	Synaptonemal complex
SDS-PAGE	–	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sox3	–	SRY related HMG-box3
Sox9a	–	SRY-related high mobility group box 9a
Sry	–	Sex determining region Y
Sycp3	–	Synaptonemal complex protein 3
T	–	Testosterone
TBS	–	Tris buffer saline
Wt1	–	Wilms' tumor suppressor 1

Contents

Reproduction is a fundamental process in living organisms that is crucial for the perpetuation of a species and comprises of various stages including sex determination, differentiation and gonadal development that occur throughout ontogeny. The main purpose of these processes is the consequent development of required anatomy and physiology of reproductive organs for successful sexual reproduction to occur. The processes of sex determination and differentiation involve a cascade of molecular, genetic, and physiological mechanisms that control the development of a bi-potential gonad into either a testis or an ovary. Ultimately gonads produce viable gametes namely sperm and eggs, respectively, which are essential for vertebrate reproductive function. Sex determination has proven to be one of the most diverse pathways during development and various studies in both mammalian and non-mammalian species have shown that there is very little conservation in both mechanical and molecular aspects (Bachtrog *et al.*, 2014). The pathways involved in the regulation of sex determination have been extensively studied in eukaryotes, *Drosophila melanogaster* and *Caenorhabditis elegans* (Kuwabara and Kimble, 1992; Salz and Erickson, 2010). In non-mammalian vertebrates such as birds, sex determination depends on the inheritance of sex chromosomes ZZ in males and heterogametic ZW in females (Schartl, 2004; Chue and Smith, 2011). Reptiles also show a very diverse pattern of sex determination patterns (Ciofi and Swingland, 1997). However, in a majority of the species, there are no identifiable sex chromosomes, with temperature being the main determinant of sex, and this pattern is also observable in amphibians (Manolakou *et al.*, 2006). Several studies have indicated the role of melatonin produced by the pineal organ as a key factor in photoperiodic response and control of seasonal maturation of gonads in fish which is synchronised with changes in climate/temperature, day length and food availability (Falcón *et al.*, 2010). The role of melatonin in reproductive activity of avian species in

response to seasonal variability of hormone levels and photoperiodic conditions have also been suggested (Deviche and Small, 2001; Maitra *et al.*, 2002). Although these various environmental factors have been associated as possible cues, day length which is season-dependent has been considered the major factor for the cueing and timing of reproduction in most teleost species (Bromage *et al.*, 2001) and melatonin may have a substantial role in feedback control of gonadotropin signalling (Senthilkumaran and Joy, 1995; Migaud *et al.*, 2010).

In mammals, the genetic sex of the organism is established at the time of fertilization with the inheritance of a paternal X or Y chromosome, and sex-determination is initiated subsequently after the process of gonadal development (Swain and Lovell-Badge, 1999). The sex determining gene *Sry* found in the Y-chromosome is crucial for instigating male sex determination (Figure 1) and is conserved in a vast range of mammalian species (Sinclair *et al.*, 1990; Berta *et al.*, 1990).

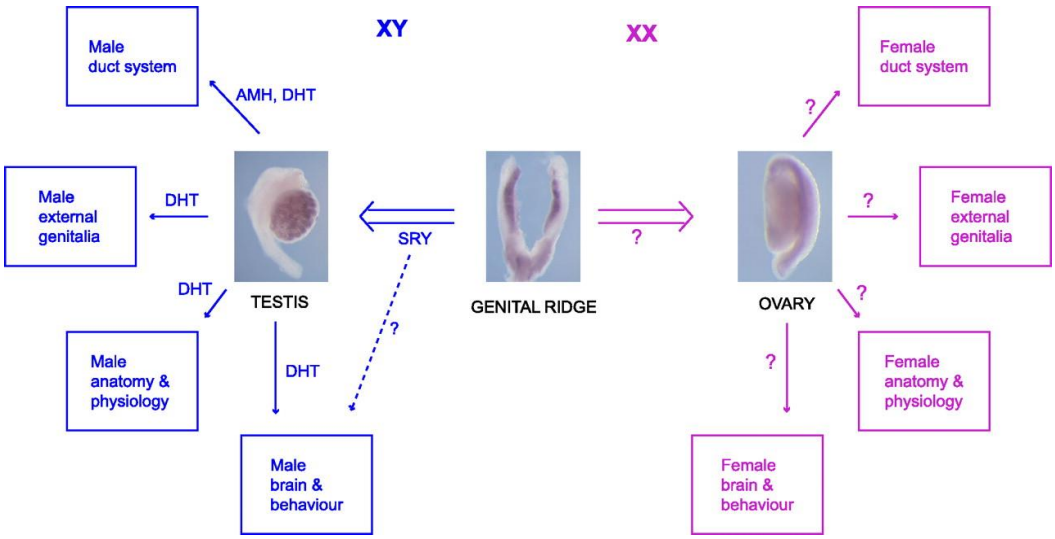


Fig. 1. Overview of the development of sexual phenotype in mammals

Adapted from Wilhelm *et al.*, 2007

The *Sry* gene, in coordination with hormones like androgens and anti-Müllerian hormone (AMH) produced by the developing testis, directs the progress of a bipotential gonad into secondary sexual structures including behaviour. If *Sry* is absent, this process proceeds towards the female pathway where the sex steroid estradiol-17β (E_2) seems to play a crucial role (Wilhelm *et al.*, 2007). Hence in mammals, the process of primary sex determination as well as gonadal development for both male and female is strictly gene-dependent.

Teleost fishes, the largest and most diverse group of vertebrates, exhibit an astounding variability in the mechanisms of sex determination that can be grouped into hermaphroditism and gonochorism, which are further influenced by environmental and genetic factors (Devlin and Nagahama, 2002; Kobayashi *et al.*, 2013). However, distinct and identifiable sex-chromosomes are rare, and the absence of strictly defined chromosomal sex may result in gonadal sex differentiation that is exceptionally plastic, and this very process is a result of the effects of numerous factors that act in concert with each other (Devlin and Nagahama, 2002; Nakamura *et al.*, 2003; Sudhakumari and Senthilkumaran, 2013). In a rare instance, a sex determining gene of teleosts, *DMY* was identified in the Japanese medaka *Oryzias latipes* (a well-studied fish model for genetics and genomics) a decade after the discovery of *Sry* in mammals and is a duplicate of an autosomal gene *dmrt1a* named as DMY found on LG 9 of the Y-chromosome (Matsuda *et al.*, 2002, Nanda *et al.*, 2002). This was followed by the identification a similar gene in the closely related Hainan ricefish or tiger medaka, *O. curvinotus* (Matsuda *et al.*, 2003).

In males, the undifferentiated gonads exclusively express *DMY*, and further induces a downstream gene *dmrt1*, which contributes to the differentiation and development of gonads in

fish, birds and mammals (Figure 2). In females, the precise genetic cascade elicited by the absence of *DMY* is still uncertain, but may involve sex-specific gene expression, such as *FIGa* and sex steroid/aromatase regulation (Manolakou *et al.*, 2006; Kinoshita *et al.*, 2009).

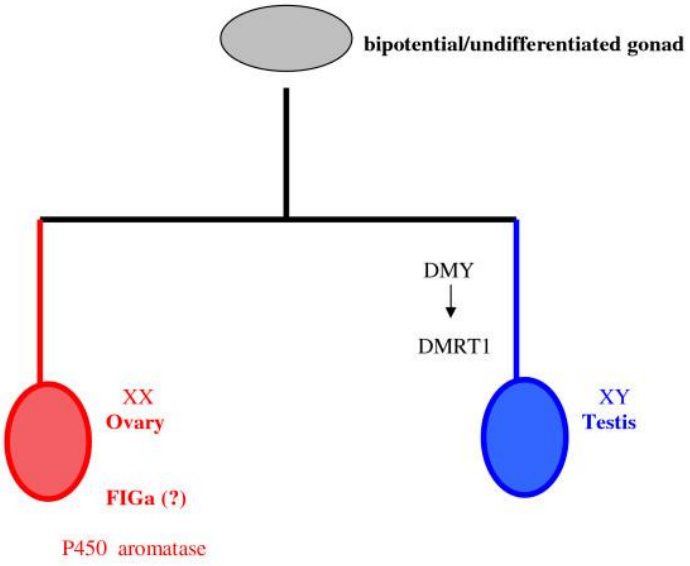


Fig. 2. Sex determination in Japanese medaka

Adapted from Manolakou *et al.*, 2006

Following the identification of DMY in medaka, four other sex determining candidate genes had been found in other fish species namely *amhy* in *Odontesthes hatchei*, *gsdf* in *O. luzonensis*, *amhr2* in *Takifugu rubripes* and *sdY* in *Oncorhynchus mykiss* (Kikuchi and Hamaguchi, 2013; Arezo *et al.*, 2014). Hence the diversity of such identified sex determining genes in fish implies various alternative processes that occur at the undifferentiated stage of gonads to alter and influence gonadal fate, with some genes having a more prominent role than others (Heule *et al.*, 2014; Martinez *et al.*, 2014).

Apart from the main sex determining genes, there are also numerous genes that play crucial roles in the process of sex determination and differentiation even though the exact hierarchy of function or gene cascade is still not yet fully elucidated. More widely studied genes that participate in sex determination/differentiation (Figure 3) include *wt1* (Wilm's tumor suppressor-1), *sox-9* (Sry-related HMG box-9), *dmrt1* (double sex and mab-3 related gene-1), *foxl2* (Forkhead transcription factor-2), *amh* and steroidogenic enzymes like aromatases (Cyp19a1a, oP450arom) and more recently sox-3 (Swain and Lovell-Badge, 1999; Hughes 2001; Yoshiura *et al.*, 2003; Kobayashi *et al.*, 2004; Baron *et al.*, 2005; Piferrer and Blázquez , 2005; Wang and Orban, 2007; Klüver *et al.*, 2009; Raghuveer and Senthilkumaran, 2009, 2010; Le Page *et al.*, 2010; Takehana *et al.*, 2014; Rajakumar and Senthilkumaran, 2016).

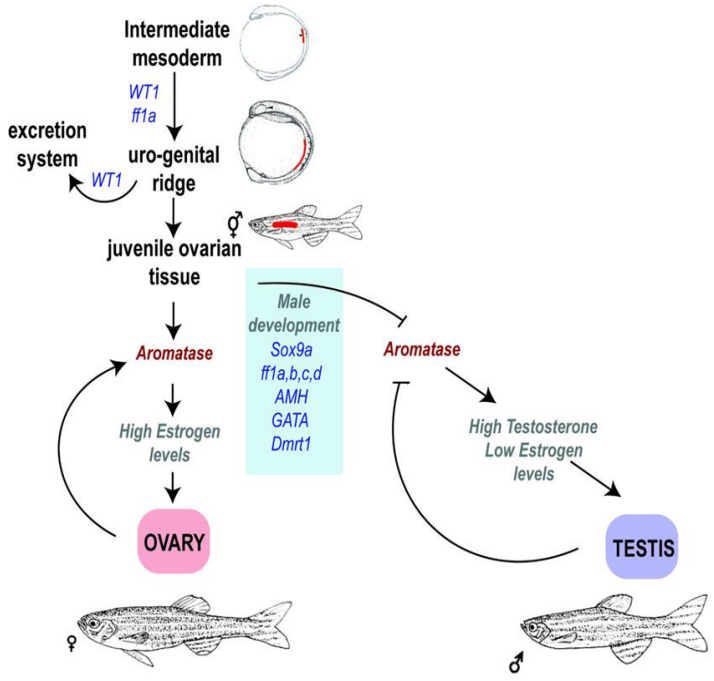


Fig. 3. Overview of factors involved in the sex determination and differentiation

Adapted from von Hofsten and Olsson, 2005

In the zebrafish, *Danio rerio*, an important vertebrate model where basic developmental mechanisms of sex determination have been studied, the above-mentioned group of genes determine the gonadal development pathway even though the interaction and regulatory mechanisms are still undefined (Figure 3). There is a possibility that any one of the genes may be responsible for sex determination/differentiation where the absence or alteration in these genes during critical stages of development or recrudescence, due to induced physiological or environmental insults, have resulted in sex reversal or impairment of gonadal function (Hofsten and Olsson, 2005, Swapna and Senthilkumaran, 2009; Rajakumar *et al.*, 2011, Baroiller and D'Cotta, 2016). In addition, the switch responsible for the male pathway may depend on the combined effects of allelic variants between the participant genes, while the regulation of *cyp19a1a* seems to play a crucial role for zebrafish sex determination/differentiation (von Hofsten and Olsson, 2005).

Gonadal development

The process of sex determination in most vertebrates triggers the cascade of genetic events downstream that lead to sexual differentiation, by activating the necessary signalling pathways and networks, and repressing the alternative or antagonistic pathway. This progression induces steroid hormone production and regulation, and subsequently leads to a gonad that corresponds to the sexual phenotype, i.e. testis or ovary, (Munger and Capel, 2012; Heule *et al.*, 2014). The development of gonads is essential for vertebrate reproduction, as they are the functional niche for gametogenesis and a wide spectrum of vertebrate species has a similar basic structure of mature testis and ovary (DeFalco and Capel, 2009). Although the structures of gonads in males and females are distinctly different, they have similarities in their composition of germ,

supporting and interstitial cells which develop from common cell lineages (Nishimura and Tanaka, 2014). After the establishment of sex in the gonads, morphogenetic changes ensue until gonadal maturation is completed.

In mammals, the mouse model has been used extensively to investigate sex determination/differentiation as well as gonad morphogenesis. The bipotential gonad or genital ridge start to develop as paired structures within the intermediate mesoderm epithelium and the gonads appear on the ventromedial surface of the mesonephros at ~10.5 days post coitum (dpc) after which cell divisions in this epithelium subsequently cause their development into the somatic component of gonads (Karl and Capel, 1998; DeFalco and Capel, 2009). As explained earlier, the gonadal differentiation is then initiated in a subclass of somatic cells in the XY gonad by the expression of *Sry* during 10.5—and 12.0 dpc (Gubbay *et al.* 1990; Hacker *et al.* 1995; Eggers *et al.*, 2014). The somatic cells expressing *Sry* develop into Sertoli cells (supportive cells that functions in close contact with germ cells) while a subgroup of interstitial cells start to differentiate into Leydig cells between 12.5 and 13.5 dpc, and have an important function of expressing steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase and cholesterol side chain cleavage enzyme (DeFalco and Capel, 2009). On the contrary, in the absence of *Sry* that prevents development of Sertoli and Leydig cells, the lack of AMH sanctions the development of Müllerian duct hat differentiates into female reproductive parts such as oviduct, uterus (Mackay, 2000; Wilhem *et al.*, 2007). Primordial germ cells (PGCs), which are the germ cells prior to gonadal sex differentiation originate from the epiblast of mesodermal layer and are first identifiable in the mouse at ~7.25 dpc (Ginsburg *et al.*, 1990). They then migrate and reach the genital ridges by 10–11 dpc via a well-defined path, while continuously expressing genes that are responsible for the preservation of their undifferentiated

pluripotent state (Kerr *et al.*, 2006; Wilhem *et al.*, 2007). In the presumptive gonad, the mitotic division of PGCs continue upto ~13 dpc after which male germ cells arrest at the G1/G0 stage as T-prospermatogonia, while an extra round of DNA replication occurs in female germ cells before entry into meiosis (Swain, 2006; Saitou and Yamaji, 2012; Sakashita *et al.*, 2015).

In teleosts, the process of gonadal development differs from higher mammals due to prolonged periods of undifferentiation in the presumptive gonads which can continue from weeks to years, and might enable the sexual fate to be influenced by environmental factors during this development period (Penman and Piferrer, 2008; Saito and Tanaka, 2009). In addition, due to their vast diversity, teleosts display variation in the morphogenetic events leading to gonad development depending on the species, which may include differences in the general sex differentiation patterns, somatic and germ cell interaction, the time period in which they occur and also the relative weight of various processes (Martinez *et al.*, 2014).

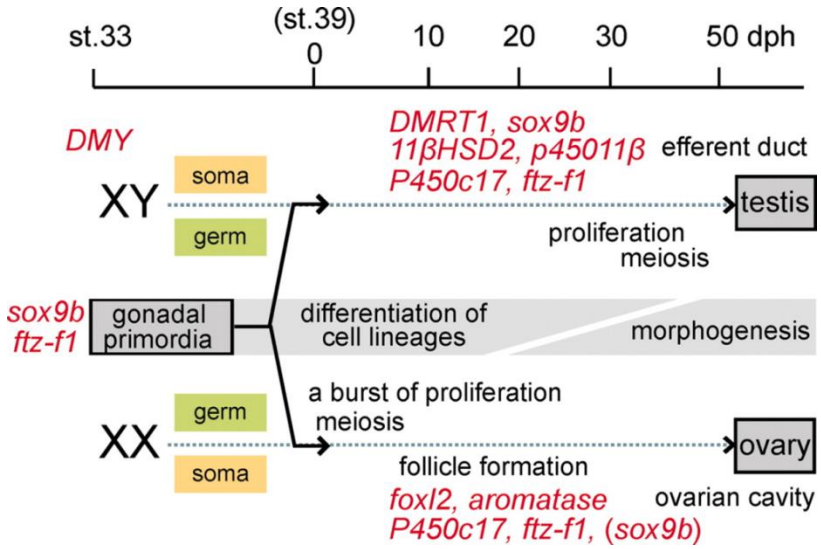


Fig. 4. Schematic representation of gonadal development in medaka

Adapted from Kurokawa *et al.*, 2006

In fishes like medaka (Figure 4), proliferation of germ cells that precedes somatic cell differentiation indicates initiation of sex differentiation (Kondo *et al.*, 2009). The gonadal primodia is formed by the PGCs that can be identified at the early gastrula stage in teleosts (Kurokawa *et al.*, 2006) and specified by maternally-inherited cytoplasmic determinants such as *nanos*, *vasa* and *tudor* (Aoki *et al.*, 2008). In zebrafish, *dead end* (*dnd*) was identified as crucial germ plasm determinant required for PGC migration and survival (Weidinger *et al.*, 2003) and stable expression of *vasa* has also been identified in the embryonic stages of catfish, *Clarias gariepinus* (Raghuveer and Senthilkumaran, 2010). The migration of PGCs to the presumptive gonads is directed by the influence of various molecules expressed by somatic cells on the defined pathway, such as Cxcr4, a chemokine receptor and its ligand, SDF1 (stromal cell-derived factor 1) in zebrafish (Knaut *et al.*, 2003; Blaser *et al.*, 2006). In medaka, the arrival of PGCs at the gonadal primordium is followed by proliferation which is sexually dimorphic where there is a significant increase in the number of female germ cells while the same pattern is not observed in male germ cells (Saito *et al.*, 2007; Nishimiura and Tanaka, 2014). This pattern of sexually dimorphic proliferation of germ cells appears to be a common feature across vertebrate species (Saito *et al.*, 2007). The interaction between somatic and germ cells is crucial for gonadal development since several genes responsible for initial sex differentiation such as *dmrt1*, *amh*, or *sox9* are expressed in Sertoli cells in males. On the other hand, *cyp19a1a* or *foxl2* are expressed in the granulosa/theca cells of females (Nakamura *et al.*, 2009; Wu *et al.*, 2010; Martinez *et al.*, 2014). However, a certain degree of overlapping expression of *dmrt1* and/or *sox9* in germ and supporting cells is seen in catfish (Raghuveer and Senthilkumaran 2009, 2010; Raghuveer *et al.*, 2011). The developing germ cells also significantly influence gonad formation and sex differentiation, since germ cell ablation

experiments have resulted in female-to-male secondary sex reversal phenotype or masculinization, with the supporting cell lineages expressing *dmrt1* and *sox9b* which are Sertoli cell markers (Kurokawa *et al.*, 2006).

Proliferation of the germ cells is also another important feature that leads to gametogenesis. The PGCs during the period of migration have been found to be mitotically inactive, and resume activity post migration (Hamaguchi, 1982). On reaching the gonadal anlage which subsequently develops into two bilateral primordia, germ cells start to proliferate with two distinct types of division: type I and type II (Figure 5), which is induced by various factors such as *amh* (Shiraishi *et al.*, 2008). Type I division involves maintenance of self-renewal, mirroring the characteristic feature of stem cells, where the germ cell division results in two daughter cells which are then bordered by supporting cells. Type II division involves the successive and exponential division of germ cells which consequently enter meiosis.

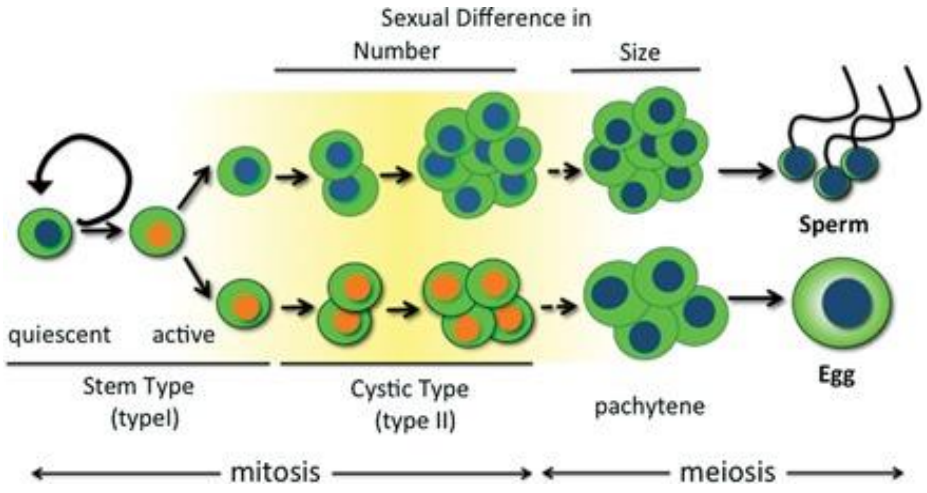


Fig. 5. Representative overview of two types of division in embryonic germ cells

Adapted from Tanaka, 2016

The germ cells that enter type II division are programmed to proceed towards gametogenesis, and female germ cells have an earlier entry into this type of division compared to male germ cells (Saito *et al.*, 2007; Tanaka *et al.*, 2008; Nishimura and Tanaka, 2014).

As mentioned earlier, the principal outcome of sex differentiation is the canalisation of the gonads into either ovaries or testes and functionally competent gametes are essential for proper fertilization as well as early embryonic development (MacLaughlin and Donahoe, 2004; Grier and Aranzábal, 2009, Grier *et al.*, 2009).

Germ cells then proceed into the subsequent stages of gametogenesis known as spermatogenesis in testis and oogenesis in ovary (Figure 6), and develop in close association with supporting cells that express different genes at various stages of development.

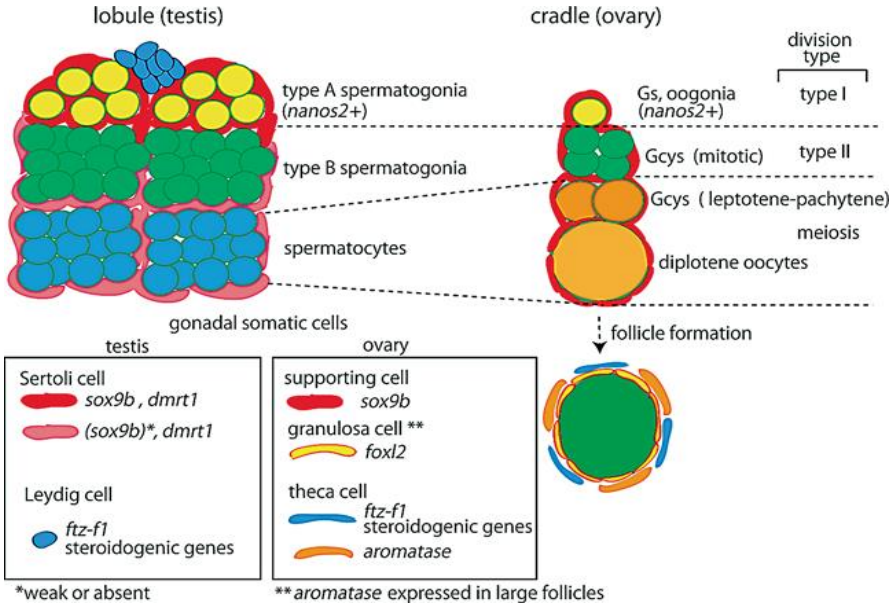


Fig. 6. Germ cells and somatic cells in the gonads

Adapted from Nishimura and Tanaka, 2014

This process of gametogenesis in sexually reproducing organisms is the central biological process that ultimately results in the reduction of the number of chromosomes in half to yield haploid germ cells (sperm and oocyte) via meiosis (Ward *et al.*, 2003). The rudimentary morphological structures of testes and ovaries have certain parallels as well as major differences. In males, Sertoli cells express *sox9b* and *dmrt1*, while Leydig cells express *ftz-f1* while in females, the granulosa cells express *sox9b* and *foxl2* and thecal cells express *ftz-f1* and *aromatase* (Nakamura *et al.*, 2011, 2012; Nishimura and Tanaka, 2014).

However, the process of spermatogenesis, from the initial germ cell differentiation, physiological regulation as well as the process of meiosis of spermatogenic cells is less understood (Orth *et al.*, 2000; Cavaco, 2005; Otaka *et al.*, 2015) when compared to oogenesis where there is availability of profound reports regarding oocyte growth to final maturation process especially in teleosts (Balamurugan and Haider, 1995; Chaube and Haider, 1997; Nath and Maitra, 2001; Bhattacharya *et al.*, 2007; Nagahama and Yamashita, 2008; Das *et al.*, 2016) This discrepancy between the two processes of gametogenesis has resulted in the disposition of this study towards a more focussed analysis on spermatogenesis.

Spermatogenesis

Spermatogenesis is an intricate process that involves the sequential progress of spermatogonial stem cells (SSCs) into differentiating spermatogonia, resulting in the production of functional sperm. The steps involved in spermatogenesis across all vertebrates have specific durations which are tightly regulated by pathways that are paracrine, autocrine, juxtacrine, and endocrine in nature, and influence various genes that have definitive roles in the development of germ cells (Chocu *et al.*, 2012). Sertoli cells are vital for spermagonial development as they harbour

the receptors for hormones (testosterone [T], follicular stimulating hormone [FSH] and luteinizing hormone [LH]) which are main regulators of the process (Sofikitas *et al.*, 2008). In mammals spermatogenesis is divided into three phases. Phase I involves mitotic division of primary spermatogonia, phase II where haploid spermatids are produced from spermatocytes via two consecutive divisions in meiosis, and phase III that produces spermatocytes, a process also known as spermiogenesis (Eddy, 2012; Chocu *et al.*, 2012).

This spermatogenic cycle takes place in the seminiferous tubules of testis (Figure 7), a convoluted tubular structure, composed of somatic Sertoli and peritubular myoid cells while Leydig cells (that produce Testosterone) occupy the interstitial space (Yoshida, 2008).

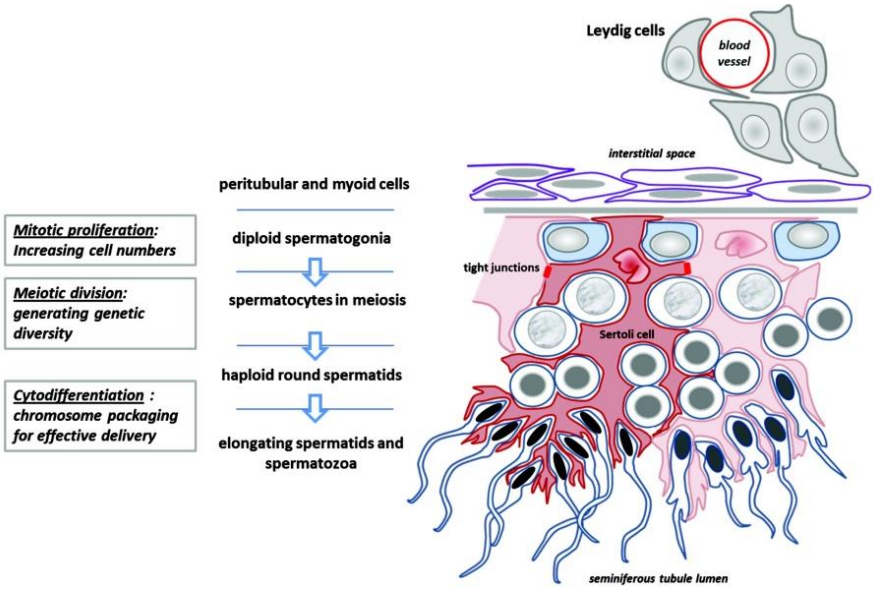


Fig. 7. Schematic diagram to illustrate spermatogenesis in mouse

Adapted from Hunter *et al.*, 2012

In teleosts, the basic structural and functional characteristics of this process bear strong similarities to that of higher vertebrates such as mammals, and are highly conserved within various fish species except for ductal system of origin (Miura and Miura, 2003; Schulz *et al.*, 2010). The testis, as in most vertebrates, is divided into two main compartments - the interstitial compartment (that constitutes Leydig cells, blood/lymphatic vessels, and connective tissue elements, peritubular myoid cells) and the germinative compartment that consists of the germinal epithelium, Sertoli cells and various stages of germ cells namely spermatogonia, spermatocytes and spermatids (Figure 8). These two compartments are essentially separated by a basement membrane (Schulz and Nóbrega, 2011).

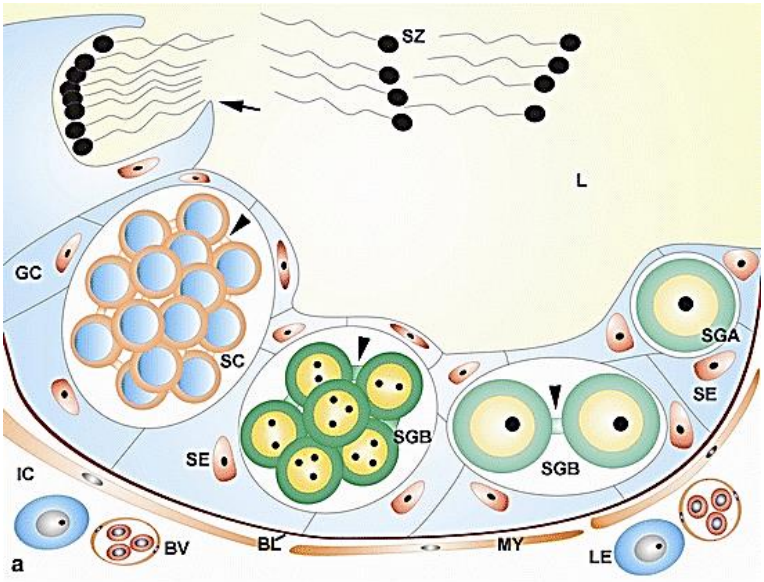


Fig. 8 : Schematic representation of fish testicular structure.

Adapted from Nóbrega *et al.*, 2009

The spermatogenic phases, namely the mitotic, meiotic and spermiogenic, that involve the propagation of spermatogonia to develop into spermatozoa also follow the same patterns as

that of mammals although distinct characteristics of spermiogenesis and spermatozoa may show variability among different teleost species (Nóbrega *et al.*, 2009).

However, unlike mammals, spermatogenesis in teleosts occurs in cysts found in the seminiferous tubules, which does not give a comparative spermatogenic cycle as in higher vertebrates (Figure 9) and these cysts arise from the communication between Sertoli cells and primary spermatogonia (Schulz *et al.*, 2010).

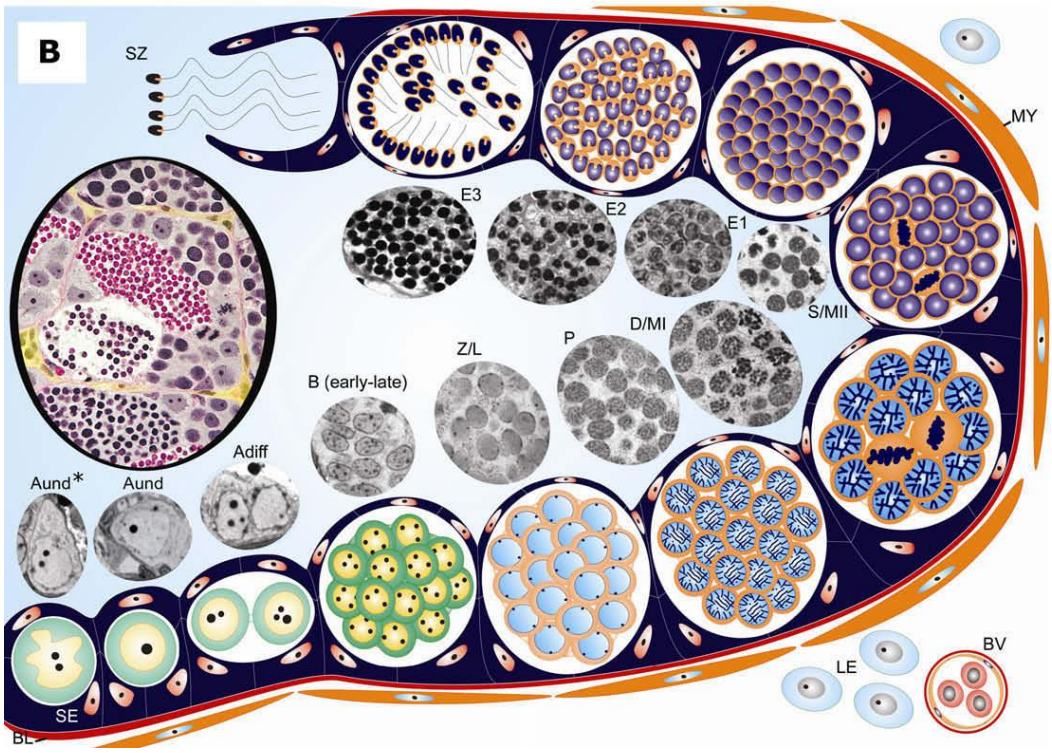


Fig. 9. Schematic representation of cystic spermatogenesis in fish.

Adapted from Schulz *et al.*, 2010

Sertoli cells play a vital role in testicular development and germ cell survival, and their proliferation even in sexually mature fish results in enlarged testicular lumen and increased

sperm production (Schulz *et al*., 2005). The completion of spermatogenesis is marked by spermiation which is essentially the cessation of close contact between Sertoli and germ cells leading to the presence of mature sperm (milt) in the spermatic duct system, and the whole process of spermatogenesis, depending on certain abiotic factors, may range from 1 to 3 weeks. In addition, spermatozoa of fishes lack acrosome, and entry into the oocyte occurs through the micropyle for fertilization to form a diploid zygote of the offspring generation. (Schulz *et al*, 2010).

The process of spermatogenesis at successive stages of differentiation and proliferation can be regulated by factors that originate from both somatic and germ cells. The genes that influence and regulate various stages of mitosis and meiosis are temporally expressed that result in the sequential development of germ cells. The Sertoli cells express extrinsic factors such as KIT ligand (KITL) and growth factors belonging to the tissue growth factor β superfamily that includes gonad-soma derived factor and AMH (Klüver *et al.*, 2007; Schulz *et al.*, 2010; Zhang *et al.*, 2011; Uribe *et al.*, 2014; Kaneko *et al.*, 2015). Other factors that are known to influence the proliferation of spermatogonia and spermatocytes include growth factor activin, insulin-like growth factors and growth hormone (Miura *et al.*, 1995; Ge, 2000; Reinecke *et al.*, 2005; Reinecke, 2010). This proliferation resulting in a large number spermatocytes and spermatids is marked by the increase in expression of meiotic and post-meiotic proteins such as dyneins and synaptonemal complex proteins Sycp1 and Sycp3 (Iwai *et al.*, 2006; Ozaki *et al.*, 2011; Dzyuba and Cosson, 2014). However, there is still insufficient information to decipher the complete underlying mechanisms and specific mode of actions, particularly in the teleost testis compared to ovary that necessitates the investigation of additional factors that are involved in germ cell development.

Spermatogenesis is significantly regulated by the pituitary-gonadal axis, and in most vertebrates, steroid hormone synthesis is induced by gonadotropins which in turn influences different spermatogenic stages (Figure 10). Principally the two types of gonadotropins, LH and FSH activate their receptors in Leydig and Sertoli cells respectively, to implement spermiogenesis as well as spermiation (Mylonas *et al.*, 2010). In species like catfish, only one form of gonadotropin could be purified that is homologous to LH (Koide *et al.*, 1992; Schulz *et al.*, 2001) while certain studies identified the occurrence of two gonadotropin receptors in salmon where the FSH receptor displays affinity to LH, although having a preference for FSH (Miwa *et al.*, 1994; Schulz and Miura, 2002), which may imply that there are distinct variations amongst species although LH in certain cases has been found to be capable of initiation and maintenance of gametogenesis (Gen *et al.*, 2000). Studies in tilapia and African catfish have also suggested that higher plasma levels of FSH during initial spermatogonial proliferation may be directly linked to Sertoli cells proliferation, since an increase in these cells is related to the mitotic expansion of spermatogonial cysts (Schulz *et al.*, 2005). However, both Leydig and Sertoli cells have been found to express the receptor for FSH in *C. gariepinus*, while receptors of LH were exclusive in Leydig cells (Garcia-Lopez *et al.*, 2008) which suggests that steroidogenesis in Leydig cells is under the direct influence of LH and FSH, while elevated levels of LH during spawning seasons might have the capacity to cross-activate FSH receptors (Schulz *et al.*, 2010). Incidentally, both LH and/or FSH levels show seasonal pattern correlating to the reproductive cycle (Joy *et al.*, 2000; Kirubagaran *et al.*, 2005; Levavi-Sivan *et al.*, 2010). The stimulation of somatic cells in the testis by gonadotropins results in the synthesis of potent androgens which are vital regulating factors of the entire spermatogenenic process.

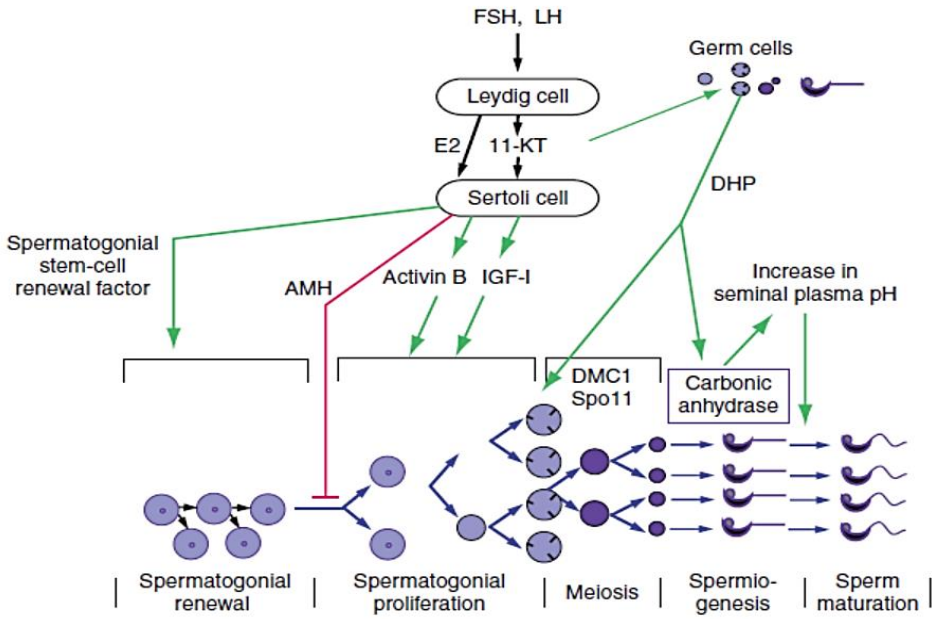


Fig. 10. Overview of endocrine mechanisms regulating spermatogenesis.

Adapted from Yaron and Levavi-Sivan, 2011

Spermatogonial renewal is regulated by E_2 and spermatogonial proliferation toward meiosis and spermiogenesis are regulated by 11-ketotestosterone (11-KT), which is the potent form of androgen in fish, via androgen receptors (Amer *et al.*, 2001; Schulz and Miura, 2002). The proliferative activity of 11-KT is also mirrored by another factor, activin B which is of Sertoli cell origin (Nagahama, 1994; Yaron and Levavi-Sivan, 2011). Finally, the process of sperm maturation is regulated by 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP), which is a progestin in teleosts synthesized by the enzyme, 20β-hydroxysteroid dehydrogenase under the influence of LH (Sakai *et al.*, 1996; Senthilkumaran *et al.*, 2002; Scott *et al.*, 2010; Sreenivasulu *et al.*, 2012; Liu *et al.*, 2014). Incidentally, 17α,20β-DP directly affects immature spermatozoa to active the carbonic anhydrase that escalates seminal plasma pH levels which in turn promotes motility of the spermatozoa (Miura and Miura, 2003). In spite of all these

findings, the process of germ cell development and spermatogenesis is still least understood. This warranted a detailed study using catfish as an animal model.

Catfish as a model of study

Catfish are a diverse group of ray-finned fish that are members of the order 'Siluriformes' and are excellent models for genomic studies, in particular, for agriculturally important issues due to their commercial significance. *C. batrachus*, also known as the Asian air-breathing catfish, is found widespread over Asia including India. *C. gariepinus*, native to north Africa has also been spread across Europe, the Middle East, and in parts of Asia including India.

These two catfish species undergo seasonal reproductive cycle or recrudescence and become mature within approximately a year. The scientific classification and different phases of catfish reproductive cycle are described below.

Clarias batrachus

Scientific classification

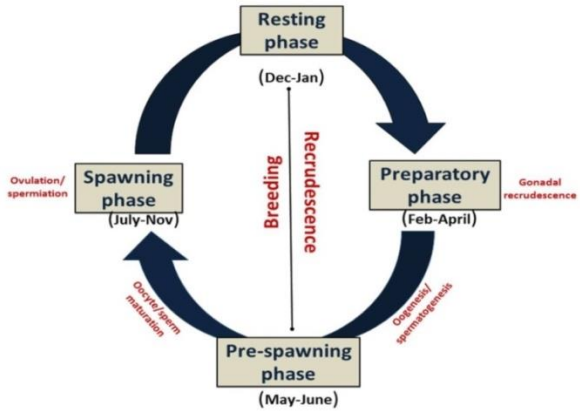
Kingdom : Animalia
Phylum : Chordata
Class : Actinopterygii
Order : Siluriformes
Family : Clariidae
Genus : *Clarias*
Species : *batrachus*

Clarias gariepinus

Scientific classification

Kingdom : Animalia
Phylum : Chordata
Class : Actinopterygii
Order : Siluriformes
Family : Clariidae
Genus : *Clarias*
Species : *gariepinus*

Seasonal reproductive phases	*C. batrachus*	*C. gariepinus*
Preparatory	February - April	February - April
Prespawning	May – June	May – June
Spawning	July – August	July – November
Post-spawning/regressed	September – January	December - January



The seasonal reproductive cycle for these two species is mostly similar, except for the spawning phase which can be up to three months longer in *C. gariepinus.*

The use of catfish as a model has various advantages-

- Its annual reproductive cycle enables the analysis of gonadal maturation events more than once
- Their sexual plasticity allows for manipulation and sex-reversal using sex steroid treatments during the critical window of sex determination/differentiation
- Absence of blood barrier renders it a simplified system for several experimental manipulations

- Catfish respond well to human chorionic gonadotropin (hCG) induction, with a high hatchability rate and this allows to carry out *in vitro* fertilization (IVF) easily
- Breeding and rearing of these two species have been well established in our laboratory conditions
- Morphological sex distinction is possible during adulthood (by observation of external papillae) that enables induced breeding

For breeding, adult male and female catfish were used for IVF. Gravid females and males were selected and injected 5000 and 3000 IU, respectively, of hCG intraperitoneally. Eggs were collected after 10-14 hours by applying gentle pressure to the abdomen of females and milt was collected from the testes by mincing on ice, after dissecting it out. Both were mixed carefully in a sterilized bowl with gentle shaking to allow fertilization. The clumping of fertilized oocytes was avoided by frequent washing steps. They were then transferred to tanks connected with circulating filtered tap water. Hatching generally takes 24-36 hours after fertilization after which the fingerlings were kept in the incubator with filtered water. Once the yolk sac of the fingerlings gets completely absorbed, live feed i.e. tubeworms (*Tubifex tubifex)* are fed *ad libitum* daily, and maintained in ambient photo-thermal conditions.

As mentioned before, teleosts show sexual plasticity and the mechanisms of gonadal sex determination and differentiation can be easily influenced by exogenous compounds or endocrine disruptors (EDs), which are usually of anthropogenic origin (Nagahama *et al.*, 2004; Diamanti-Kandarakis *et al.*, 2009, Senthilkumaran, 2015). Even though catfish have been known to have a high resilience to elevated concentrations of xenobiotic compounds/steroids than other fish species, various studies have shown that even sub-lethal doses of EDs have the

capacity to disrupt normal reproductive physiology in both adults and juveniles by altering the expression levels of various factors, steroidogenic enzyme genes and proteins related to gonad and germ cell development (Singh and Singh, 2007; Rajakumar *et al.*, 2012; Lal *et al.*, 2013; Prathibha *et al.*, 2014). The capacity of EDs to insult normal testicular and ovarian development as supported by prior reports may inadvertently serve as a means of further investigating the molecular mechanisms of germ cell and gonadal development and in identifying new and crucial factors that play significant roles in such important reproductive processes.

In view of this, the current study aimed to identify new factors which are involved in gonadal development by employing a proteomic approach via analysis of differential expression patterns of various proteins, in both altered (endocrine disruption) and normal physiological (juvenile and adult) conditions. This method enabled the identification of a germ-cell marker *sycp3* which was found to be upregulated during juvenile stages. This was followed with further analysis of the role of *sycp3* along with *c-kit* during germ cell development and spermatogenesis at juvenile and adult stages using localization and gene expression pattern analysis in various ontogenic stages, tissue-wise and during seasonal reproductive cycle. The possible interactions of these two genes, either directly or indirectly, were also investigated by transient silencing both genes using polyethylenimine mediated transfection of esiRNA, followed by analysis of the impact of their knockdown on various testis-related transcription factors and steroidogenic enzyme genes.

The results of these studies were compiled into three major chapters for the present Ph.D thesis as indicated below with a general introduction and a consolidated summary.

1. **Proteome analysis of catfish gonads: using endocrine disruption and comparing juvenile and adulthood**
    a. **Two-dimensional proteomic analysis of gonads of air-breathing catfish, *Clarias batrachus* after the exposure of endosulfan and malathion**
2. **Identification, cloning and expression profile of Sycp3 during gonadal cycle and after esiRNA silencing in catfish**
3. **Expression analysis of *c-kit* during germ cell development, recrudescence and after esiRNA silencing in catfish**

Each chapter has been provided with separate bibliography to correlate previous findings with the current study.

References

Amer M.A., Miura T., Miura C., Yamauchi K., 2001. Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). Biol. Reprod. 65, 1057-1066.

Aoki Y., Nagao I., Saito D., Ebe Y., Kinjo M., Tanaka M., 2008. Temporal and spatial localization of three germline-specific proteins in medaka. Dev. Dyn. 237, 800–807.

Arezo M.J., Papa N., Guttierrez V., García G., Berois N., 2014. Sex determination in annual fishes: Searching for the master sex-determining gene in *Austrolebias charrua* (Cyprinodontiformes, Rivulidae). Genet. Mol. Biol. 37, 364-74.

Bachtrog D., Mank J.E., Peichel C.L., Kirkpatrick M., Otto S.P., Ashman T.L., Hahn M.W., Kitano J., Mayrose I., Ming R., Perrin N., Ross L., Valenzuela N., Vamosi J.C. 2014. Sex determination: why so many ways of doing it? PLoS Biol. 12: e1001899

Balamurugan K., Haider S., 1995. Effects of actinomycin D and cycloheximide on gonadotropin- or 17α,20β-dihydroxy-4-pregnen-3-one-induced *in vitro* maturation in oocytes of the catfish, *Clarias batrachus*. J. Biosciences 20, 151-156.

Baroiller J.F., D'Cotta H., Sex. Dev., 2016. The reversible sex of gonochoristic fish: insights and consequences. 10, 242-266.

Baron D., Batista F., Chaffaux S., Cocquet J., Cotinot C., Cribiu E., De Baere E., Guiguen Y., Jaubert F., Pailhoux E., Pannetier M., Vaiman D., Vigier B., Veitia R., Fellous M., 2005. Foxl2 gene and the development of the ovary: a story about goat, mouse, fish and woman.

Berta P., Hawkins J.R., Sinclair A.H., Taylor A., Griffiths B.L., Goodfellow P.N., Fellous M., 1990. Genetic evidence equating SRY and the testis-determining factor. Nature 348, 448-450.

Bhattacharya S., Basu D., Ak N., Priyadarshini A., 2007. Molecular mechanism of oocyte maturation. Soc. Reprod. Fertil. Suppl. 63, 45-55.

Blaser H., Reichman-Fried M., Castanon I., Dumstrei K., Marlow F.L., Kawakami K., Solnica-Krezel L., Heisenberg C.P., Raz E., 2006. Migration of zebrafish primordial germ cells: a role for myosin contraction and cytoplasmic flow. Dev. Cell. 11, 613-627.

Bromage, N., Porter M., Randall C., 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197, 63-98.

Cavaco J.E.B., 2005. Sex steroids and spermatogenesis in the African catfish (*Clarias gariepinus*). Arch. Androl. 51, 99-107.

Chaube S.K., Haider S., 1997. Effects of cAMP forskolin and cyanoketone on *in vitro* oocyte maturation in the catfish, *Clarias batrachus*. J. Biosci. 22, 255-265.

Chocu S., Calvel P., Rolland A.D., Pineau C., 2012. Spermatogenesis in mammals: proteomic insights. Syst. Biol. Reprod. Med. 58, 179-190.

Chue J., Smith C.A., 2011. Sex determination and sexual differentiation in the avian model. FEBS J. 278, 1027-1034.

Ciofi C., Swingland I.R., 1997. Environmental sex determination in reptiles. Appl. Anim. Behav. Sci. 51, 251–265.

Das D., Pal S., Maitra S., 2016. Releasing prophase arrest in zebrafish oocyte: synergism between maturational steroid and Igf1. Reproduction. 151, 59-72.

DeFalco T., Capel B., 2009. Gonad morphogenesis in vertebrates: divergent means to a convergent end. Annu. Rev. Cell. Dev. Biol. 25, 457-482.

Deviche P., Small T., 2001. Photoperiodic control of seasonal reproduction: neuroendocrine mechanisms and adaptations. In: Dawson, A., Chaturvedi, C.M. (Eds.) Avian endocrinology. Narosa Publishing House, New Delhi, India, pp. 113-128.

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

Diamanti-Kandarakis E., Bourguignon J.P., Giudice L.C., Hauser R., Prins G.S., Soto A.M., Zoeller R.T., Gore A.C., 2009. Endocrine-disrupting chemicals: an endocrine Society scientific statement. Endocr. Rev. 30, 293-342.

Dzyuba V., Cosson J., 2014. Motility of fish spermatozoa: from external signalling to flagella response. Reprod. Biol. 14, 165–175.

Eddy E.M., 2002. Male germ cell gene expression. Recent Prog. Horm. Res. 57, 103–128.

Eggers S., Ohnesorg T., Sinclair A., 2014. Genetic regulation of mammalian gonad development. Nat. Rev. Endocrinol. 10, 673-83.

Falcón J., Migaud H., Muñoz-Cueto J.A., Carrillo M., 2010. Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469-82.

Garcia-Lopez A., Bogerd J., Granneman J.C., van Dijk W., Trant J.M., Taranger G.L., Schulz R.W., 2008. Leydig cells express FSH receptors in African catfish. Endocrinology 150, 357–365.

Ge W., 2000. Roles of the activin regulatory system in fish reproduction. Can. J. Physiol. Pharmacol. 78, 1077-1085.

Gen K., Okuzawa K., Senthilkumaran B., Tanaka H., Moriyama S., Kagawa H., 2000. Unique expression of gonadotropin-I and -II subunit genes in male and female red seabream (*Pagrus major*) during sexual maturation. Biol Reprod. 63, 308-319.

Ginsburg M., Snow M.H.L., McLaren A., 1990. Primordial germ cells in the mouse embryo during gastrulation. Development 110, 521–528.

Grier H.J., Aranzábal M.C.U., Patrio R., 2009. The ovary, folliculogenesis, and oogenesis in teleosts. In: Jamieson, B.G.M. (Ed.), Reproductive biology and phylogeny of fishes (Agnathans and Bony Fishes). Enfield, NH, USA, pp. 25–84.

Grier H.J., Aranzábal M.C.U. 2009. The testis and spermatogenesis in teleosts. In: Jamieson, B.G.M. (Ed.), Reproductive biology and phylogeny of fishes (Agnathans and Bony Fishes). Enfield, NH, USA, pp. 121–142.

Gubbay J., Collignon J., Koopman P., Capel B., Economou A., Münsterberg A., Vivian N., Goodfellow P., Lovell-Badge R., 1990. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 346, 245-250.

Hacker A., Capel B., Goodfellow P., Lovell-Badge R., 1995. Expression of *Sry*, the mouse sex determining gene. Development 121, 1603-1614.

Hamaguchi S., 1982. Light- and electron-microscopic study of the migration of primordial germ cells in the teleost, *Oryzias latipes*. Cell Tissue Res. 227, 139-151.

Heule C., Salzburger W., Böhne A., 2014. Genetics of sexual development: an evolutionary playground for fish. Genetics 196, 579-591.

von Hofsten J., Olsson P.E., 2005. Zebra-fish sex determination and differentiation: Involvement of FTZ-F1 genes. Reprod. Biol. Endocrinol. 3: 63.

Hughes I.A., 2001. Minireview: sex differentiation. Endocrinology 142, 3281–3287.

Hunter D., Anand-Ivell R., Danner S., Ivell R., 2012. Models of *in vitro* spermatogenesis. Spermatogenesis 2, 32-43.

Iwai T., Yoshii A., Yokota T., Sakai C., Hori H., Kanamori A., Yamashita M., 2006. Structural components of the synaptonemal complex, SYCP1 and SYCP3, in the medaka fish *Oryzias latipes.* Exp. Cell Res. 312, 2528-2537.

Joy K.P., Singh M.S., Senthilkumaran B., Goos H.J., 2000. Pituitary gonadal relationship in the catfish *Clarias batrachus* (L): a study correlating gonadotrophin-II and sex steroid dynamics. Zool. Sci. 17, 395-404.

Kaneko H., Ijiri S., Kobayashi T., Izumi H., Kuramochi Y., Wang D.S., Mizuno S., Nagahama Y., 2015. Gonadal soma-derived factor (*gsdf*), a TGF-beta superfamily gene, induces testis differentiation in the teleost fish *Oreochromis niloticus*. Mol. Cell Endocrinol. 415, 87-99.

Karl J., Capel B., 1998. Sertoli cells of the mouse testis originate from the coelomic epithelium. Dev. Biol. 203, 323–333.

Kerr C.L., Shamblott M.J., Gearhart J.D., 2006. Pluripotent stem cells from germ cells. Methods Enzymol. 419, 400-426.

Kikuchi K., Hamaguchi S., 2013. Novel sex-determining genes in fish and sex chromosome evolution. Dev. Dyn. 242, 339–353.

Kinoshita M., Okamoto G., Hirata T., Shinomiya A., Kobayashi T., Kubo Y., Hori H., Kanamori A., 2009. Transgenic medaka enables easy oocytes detection in live fish. Mol. Reprod. Dev. 76, 202-207.

Kirubagaran R., Senthilkumaran, B., Sudhakumari C.C., Joy K.P., 2005. Seasonal dynamics in gonadotropin secretion and E(2)-binding in the catfish *Heteropneustes fossilis*. Fish Physiol. Biochem. 31, 183-188.

Klüver N., Herpin A., Braasch I., Driessle J., Schartl M., 2009. Regulatory back-up circuit of medaka Wt1 co-orthologs ensures PGC maintenance. Dev. Biol. 325, 179-188.

Klüver N., Pfennig F., Pala I., Storch K., Schlieder M., Froschauer A., Gutzeit H.O., Schartl M., 2007. Differential expression of anti-Müllerian hormone (*amh*) and anti-Müllerian hormone receptor type II (*amhrII*) in the teleost medaka. Dev. Dyn. 236, 271-281.

Knaut H., Werz C., Geisler R., Nusslein-Volhard C., 2003. A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. Nature 421, 279–282.

Kobayashi T., Matsuda M., Kajiura-Kobayashi H., Suzuki A., Saito N., Nakamoto M., Shibata N., Nagahama Y., 2004. Two DM domain genes, DMY and DMRT1, involved in

testicular differentiation and development in the medaka, *Oryzias latipes*. Dev. Dyn. 231, 518–526.

Kobayashi Y., Nagahama Y., Nakamura M., 2013. Diversity and plasticity of sex determination and differentiation in fishes. Sex. Dev. 7, 115-125.

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

Kondo M., Nanda I., Schmid M., Schartl M., 2009. Sex determination and sex chromosome evolution: insights from medaka. Sex. Dev. 3, 88-98.

Kurokawa H., Aoki Y., Nakamura S., Ebe Y., Kobayashi D., Tanaka M., 2006. Time-lapse analysis reveals different modes of primordial germ cell migration in the medaka *Oryzias latipes*. Dev. Growth Differ. 48, 209–221.

Kuwabara P.E., Kimble J. 1992. Molecular genetics of sex determination in *C. elegans.* Trends Genet. ,8, 164-168.

Lal B., Sarang M.K., Kumar P., 2013. Malathion exposure induces the endocrine disruption and growth retardation in the catfish, *Clarias batrachus* (Linn.). Gen. Comp. Endocrinol. 181, 139-145.

Le Page Y., Diotel N., Vaillant C., Pellegrini E., Anglade I., Mérot Y., Kah O., 2010. Aromatase, brain sexualization and plasticity: the fish paradigm. Eur. J. Neurosci. 32, 2105-2115.

Levavi-Sivan B., Bogerd J., Mañanós E.L., Gómez A., Lareyre J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412-37.

Liu G., Luo F., Song Q., Wu L., Qiu Y., Shi H., Wang D., Zhou L., 2014. Blocking of progestin action disrupts spermatogenesis in Nile tilapia (*Oreochromis niloticus*). J. Mol. Endocrinol. 53, 57-70.

Mackay S., 2000. Gonadal development in mammals at the cellular and molecular levels. Int. Rev. Cytol. 200, 47-99.

MacLaughlin D.T., Donahoe P.K., 2004. Sex determination and differentiation. N. Engl. J. Med. 350, 367-378.

Maitra S.K., Dey M., Sengupta A., Haldar C., 2002. Pineal in regulation of avian reproduction. In: Haldar, C., Singaravel, M., Maitra, S.K. (Eds.), Treatise on pineal gland and melatonin. Science Publisher Inc., Delhi, pp. 299-321.

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

Martínez P., Viñas A.M., Sánchez L., Díaz N., Ribas L., Piferrer F., 2014. Genetic architecture of sex determination in fish: applications to sex ratio control in aquaculture. Front. Genet. 5: 340.

Matsuda M., Nagahama Y., Shinomiya A., Sato T., Matsuda C., Kobayashi T., Morrey C. E., Shibata N., Asakawa S., Shimizu N., Hori H., Hamaguchi S., Sakaizumi M., 2002. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417, 559-563.

Matsuda M., Sato T., Toyazaki Y., Nagahama Y., Hamaguchi S., Sakaizumi, M., 2003. *Oryzias curvinotus* has DMY, a gene that is required for male development in the medaka, *O. latipes*. Zoolog. Sci. 20, 159-161.

Migaud H., Davie A., Taylor J.J., 2010. Current knowledge on the photoendocrine regulation in temperate fish species. J. Fish Biol. 76, 27–68.

Miura T., Miura C., Yamauchi K., Nagahama Y., 1995. Human recombinant activin induces proliferation of spermatogonia in vitro in the Japanese eel *Anguilla japonica*. Fisheries Sci. 63, 434–437.

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

Miwa S., Yan L., Swanson, P. 1994. Localization of two gonadotropin receptors in the salmon gonad by in vitro ligand autoradiography. Biol. Reprod. 59, 629–642.

Munger S.C., Capel B., 2012. Sex and the circuitry: progress toward a systems-level understanding of vertebrate sex determination. Wiley Interdiscip. Rev. Syst. Biol. Med. 4, 401-412.

Mylonas C.C., Fostier A., Zanuy S., 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165, 516-534.

Nagahama Y, Yamashita M., 2008. Regulation of oocyte maturation in fish. Dev. Growth Differ. 50, S195-219.

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

Nagahama Y., Nakamura M., Kitano T., Tokumoto T., 2004. Sexual plasticity in fish: a possible target of endocrine disruptor action. Environ. Sci. 11, 73-82.

Nakamura M., Bhandari R.K., Higa M., 2003. The role estrogens play in sex differentiation and sex changes of fish. Fish Physiol. Biochem. 28, 113.

Nakamura S., Kurokawa H., Asakawa S., Shimizu N., Tanaka M., 2009. Two distinct types of theca cells in the medaka gonad: germ cell-dependent maintenance of *cyp19a1*-expressing theca cells. Dev. Dyn. 238, 2652-2657.

Nakamura S., Kobayashi K., Nishimura T., Tanaka M., 2011. Ovarian germline stem cells in the teleost fish, medaka (*Oryzias latipes*). Int. J. Biol. Sci. 7, 403–409.

Nakamura S., Watakabe I., Nishimura T., Toyoda A., Taniguchi Y., Tanaka M., 2012. Analysis of medaka *sox9* orthologue reveals a conserved role in germ cell maintenance. PLoS One 7: e29982.

Nanda I., Kondo M., Hornung U., Asakawa S., Winkler C., Shimizu A., Shan Z., Haaf T., Shimizu N., Shima A., Schmid M., Schartl M., 2002. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. Proc. Natl. Acad. Sci. USA 99, 11778-11783.

Nath P., Maitra S., 2001. Role of two plasma vitellogenins from Indian major carp (*Cirrhinus mrigala*) in catfish (*Clarias batrachus*) vitellogenesis. Gen. Comp. Endocrinol. 124, 30-44.

Nishimura T., Tanaka M., 2014. Gonadal Development in Fish. Sex. Dev. 8, 252-261.

Nóbrega R.H., Batlouni S.R., França L.R., 2009. An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. Fish Physiol. Biochem. 5, 197-206.

Orth J.M., Jester W.F., Li L.H., Laslett A.L., 2000. Gonocyte-Sertoli cell interactions during development of the neonatal rodent testis. Curr. Top. Dev. Biol. 50, 103-124.

Otaka K., Hiradate Y., Kobayashi N., Shirakata Y., Tanemura K., 2015. Distribution of the sex chromosome during mouse spermatogenesis in testis tissue sections. J. Reprod. Dev. 61, 375-381.

Ozaki Y., Saito K., Shinya M., Kawasaki T., Sakai N., 2011. Evaluation of *Sycp3*, *Plzf* and *Cyclin B3* expression and suitability as spermatogonia and spermatocyte markers in zebrafish. Gene Expr. Patterns 11, 309-315.

Penman D.J., Piferrer F., 2008. Fish gonadogenesis. Part I: genetic and environmental mechanisms of sex determination. Rev. Fish. Sci. 16, 16–34.

Piferrer F., Blázquez M., 2005. Aromatase distribution and regulation in fish. Fish Physiol. Biochem. 31, 215-226.

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.

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

Raghuveer K., Senthilkumaran B., 2010. Cloning and differential expression pattern of *vasa* in the developing and recrudescing gonads of catfish, *Clarias gariepinus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 79-85.

Raghuveer K., Senthilkumaran B., 2010. Isolation of *sox9* duplicates in catfish: localization, differential expression pattern during gonadal development and recrudescence, and hCG-induced up-regulation of *sox9* in testicular slices. Reproduction 140, 477–487.

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.

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.

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.

Rajakumar A., Senthilkumaran B., 2016. Sox3 binds to 11β-hydroxysteroid dehydrogenase gene promoter suggesting transcriptional interaction in catfish. J. Steroid Biochem. Mol. Biol. 158, 90-103.

Reinecke M., 2010. Insulin-like growth factors and fish reproduction. Biol. Reprod. 82, 656-661.

Reinecke M., Björnsson B.T., Dickhoff W.W., McCormick S.D., Navarro I., Power D.M., Gutiérrez J., 2005. Growth hormone and insulin-like growth factors in fish: where we are and where to go. Gen. Comp. Endocrinol. 142, 20-24.

Saito D., Morinaga C., Aoki Y., Nakamura S., Mitani H., Furutani-Seiki M., Kondoh H., Tanaka M., 2007. Proliferation of germ cells during gonadal sex differentiation in medaka: insights from germ cell-depleted mutant zenzai. Dev. Biol. 310, 280–290.

Saito D., Tanaka M., 2009. Comparative aspects of gonadal sex differentiation in medaka: a conserved role of developing oocytes in sexual canalization. Sex. Dev. 3, 99-107.

Saitou M., Yamaji M., 2012. Primordial germ cells in mice. Cold Spring Harb. Perspect. Biol. 4: a008375.

Sakai N., Suzuki N., Nagahama Y., 1996. Distribution of the site of 17α, 20β-dihydroxy-4-pregnen-3-one production in the spermiating amago salmon (*Oncorhynchus rhodurus*) testis. Biomed. Res. 17, 473-477.

Sakashita A., Kawabata Y., Jincho Y., Tajima S., Kumamoto S., Kobayashi H., Matsui Y., Kono T., 2015. Sex specification and heterogeneity of primordial germ cells in mice. PLoS One. 10: e0144836.

Salz H.K., Erickson J.W., 2010. Sex determination in Drosophila: The view from the top. Fly (Austin). 4, 60-70.

Schartl M., 2004. Sex chromosome evolution in non-mammalian vertebrates. Curr. Opin. Genet. Dev. 14, 634-641.

Schulz R.W., de França L.R., Lareyre J.J., Le Gac F., Chiarini-Garcia H., Nobrega R.H., Miura T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

Schulz R.W., Menting S., Bogerd J., Franca L.R., Vilela D.A.R., Godinho H.P., 2005. Sertoli cell proliferation in the adult testis: evidence from two fish species belonging to different orders. Biol. Reprod. 73, 891–898.

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

Schulz R.W., Nóbrega R.H., 2011. Anatomy and histology of fish testis. In: Farrell A.P. (Ed.). Encyclopedia of fish physiology: from genome to environment, San Diego: Academic Press, pp. 616–626.

Schulz R.W., Vischer H.F., Cavaco J.E., Santos E.M., Tyler C.R., Goos H.J., Bogerd J., 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 407-417.

Scott A.P, Sumpter J.P., Stacey N., 2010. The role of the maturation-inducing steroid, 17, 20β-dihydroxypregn-4-en-3-one, in male fishes: a review. J. Fish Biol. 76, 183–224.

Senthilkumaran B., Joy K.P., 1995. Effects of melatonin and p- cholorophenylalanine and α-methylparatyrosine on plasma gonadotropin level and ovarian activity in the catfish, *Heteropneustes fossilis*: a study correlating hypothalamic monoamines. Fish Physiol. Biochem. 14, 471-480.

Senthilkumaran B., Sudhakumari C.C., Chang X.T., Kobayashi T., Oba Y., Guan G., Yoshiura Y., Yoshikuni M., Nagahama, Y. 2002. Ovarian carbonyl reductase-like 20β-hydroxysteroid dehydrogenase shows distinct surge in messenger RNA expression during

natural and gonadotropin-induced meiotic maturation in nile tilapia. Biol. Reprod. 67, 1080-1086.

Senthilkumaran B., 2015. Pesticide- and sex steroid analogue-induced endocrine disruption differentially targets hypothalamo-hypophyseal-gonadal system during gametogenesis in teleosts - A review. Gen. Comp. Endocrinol. 219, 136-142.

Shiraishi E., Yoshinaga N., Miura T., Yokoi H., Wakamatsu Y., Abe S., Kitano T., 2008. Müllerian Inhibiting Substance is required for germ cell proliferation during early gonadal differentiation in Medaka (*Oryzias latipes*). Endocrinology 149, 1813-1819.

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

Singh P.B., Singh V., 2007. Endosulfan induced changes in phospholipids in the freshwater female catfish, *Heteropneustes fossilis* (Bloch). J. Env. Biol. 28, 605-610.

Sofikitis N., Giotitsas N., Tsounapi P., Baltogiannis D., Giannakis D., Pardalidis N.J., 2008. Hormonal regulation of spermatogenesis and spermiogenesis. Steroid Biochem. Mol. Biol. 109, 323-330.

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.

Sudhakumari C.C., Senthilkumaran B., 2013. Expression profiling of various marker genes involved in gonadal differentiation of teleosts: molecular understanding of sexual plasticity. In: Senthilkumaran, B. (Ed.), Sexual plasticity and gametogenesis in fishes. Nova Biomedical, USA, pp. 401–421.

Swain A., 2006. Sex determination: time for meiosis? The gonad decides. Curr. Biol. 16, R507–509.

Swain A., Lovell-Badge R., 1999. Mammalian sex determination: a molecular drama. Genes Dev. 13, 755-767.

Swapna I., Senthilkumaran B., 2009. Influence of ethynylestradiol and methyltestosterone on the hypothalamo–hypophyseal–gonadal axis of adult air-breathing catfish, *Clarias gariepinus*. Aquat. Toxicol. 95, 222–229.

Takehana Y., Matsuda M., Myosho T., Suster M.L., Kawakami K., Shin-I T., Kohara Y., Kuroki Y., Toyoda A., Fujiyama A., Hamaguchi S., Sakaizumi M., Naruse K., 2014. Co-option of Sox3 as the male-determining factor on the Y chromosome in the fish *Oryzias dancena*. Nat Commun. 5: 4157.

Tanaka M., 2016. Germline stem cells are critical for sexual fate decision of germ cells Bioessays. 38, 1227–1233.

Tanaka M., Saito D., Morinaga C., Kurokawa H., 2008. Cross talk between germ cells and gonadal somatic cells is critical for sex differentiation of the gonads in the teleost fish, medaka (*Oryzias latipes*). Dev. Growth Differ. 50, 273–278.

Uribe M.C., Grier H.J., Mejía-Roa V., 2015. Comparative testicular structure and spermatogenesis in bony fishes. Spermatogenesis 4: e983400.

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

Ward J.O., Reinholdt L.G., Hartford S.A., Wilson L.A., Munroe R.J., Schimenti K.J., Libby B.J., O'Brien M., Pendola J.K., Eppig J., Schimenti J.C., 2003. Toward the genetics of mammalian reproduction: induction and mapping of gametogenesis mutants in mice. Biol. Reprod. 69, 1615-1625.

Weidinger G., Stebler J., Slanchev K., Dumstrei K., Wise C., Lovell-Badge R., Thisse C., Thisse B., Raz E., 2003. *dead end*, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr. Biol. 13, 1429-1434.

Wilhelm D., Palmer S., Koopman P., 2007. Sex determination and gonadal development in mammals. Physiol. Rev. 87, 1-28.

Wu G.C., Tomy S., Lee M.F., Lee Y.H., Yueh W.S., Lin C.J., Lau E.L., Chang C.F., 2010. Sex differentiation and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol. 167, 417-421.

Yaron Z., Levavi-Sivan B. 2011. Endocrine Regulation of Fish Reproduction. In: Farrell, A.P. (Ed.) Encyclopedia of Fish Physiology: From Genome to Environment. Academic Press, San Diego, USA, pp 1500-1508.

Yoshida S., 2008. Spermatogenic stem cell system in the mouse testis. Cold Spring Harb. Symp. Quant. Biol. 73, 25-32.

Yoshiura Y., Senthilkumaran B., Watanabe M., Oba Y., Kobayashi T., Nagahama Y., 2003. Synergistic expression of Ad4BP/SF-1 and cytochrome P-450 aromatase (ovarian type) in the ovary of Nile tilapia, *Oreochromis niloticus*, during vitellogenesis suggests transcriptional interaction. Biol. Reprod. 68, 1545-53.

Zhang L., Tang J., Haines C.J., Feng H.L., Lai L., Teng X., Han Y., 2011. C-kit and its related genes in spermatogonial differentiation. Spermatogenesis 3, 186-194.

Abstract

Proteomic profile analysis of factors that are involved in important physiological processes such as gonadal and germ cell development can contribute greatly to identify novel factors. In the present study, the differential expression patterns of various proteins were analyzed in both altered (endocrine disruption) and normal physiological (juvenile and adult) conditions. The first approach utilized endocrine disruption as a model to alter the normal physiological conditions in adult (recrudescing) gonads in order to perceive any changes in the expression patterns of proteins that may play critical roles in reproductive competency. Endocrine disruption using pesticides such as endosulfan and malathion, have been well established in their capacity to insult normal testicular and ovarian development as supported by previous reports. In the second approach, differential protein expressions were analyzed in the gonads of juvenile and adults (in both males and females) to identify any factors that are significantly expressed during critical stages of development (in juvenile) and gonadal recrudescence (In adults). Through this approach, the present study identified several proteins that were differentially expressed in the pesticide-treated gonad when compared to the control, as well as in the normal physiological conditions (juvenile and adult).

1. Introduction

The processes of sex determination and differentiation that results in the development of a functional gonad are influenced by an array of molecular, genetic, and physiological mechanisms that are not yet completely elucidated especially in lower vertebrates like teleosts. Although there have been extensive genome-wide analyses at the mRNA level which have provided insights into these processes, there is comparatively less data available

on the corresponding protein profiles, despite proteins being established to be more significant markers of gene function (Pandey and Mann, 2000; Groh *et al*., 2011; Spaink *et al*., 2014). In addition, since there is generally a poor correlation between the levels of mRNA and protein due to reasons such as post-translational modifications, it is essential to supplement mRNA profiles with the information on protein expression (Greenbaum *et al*., 2003).

In the present study, a proteomic approach with two dimensional gel electrophoresis and MALDI/TOF was utilized prior to genomic techniques to identify proteins and study changes in their patterns of expressions. This proteomic data can potentially provide the essential functional and mechanistic information on gene expressions, since only a restricted amount of mRNA essentially gets translated into protein, and such details may provide a more comprehensive insight into the crucial factors that are involved during decisive stages of gonadal development and recrudescence in catfish. The first approach employed endocrine disruption as a model to alter the normal physiological conditions in adult (recrudescing) gonads of catfish, *Clarias batrachus*, in order to perceive any changes in the expression patterns of proteins that may play critical roles in reproductive competency. Endocrine disruption using pesticides such as endosulfan and malathion, have been well established in their capacity to insult normal testicular and ovarian development as evidenced by previous reports in catfish as well as other fish species (Rajakumar *et al*., 2012; Söffker and Tyler, 2012; Prathibha *et al*., 2014; Senthilkumaran, 2015). The second approach was the use of normal physiological conditions to examine the differential expression of proteins, comparing adult gonads with juvenile stages of *Clarias gariepinus* to identify any factors that are significantly expressed during critical stages of development (in juvenile) and gonadal

recrudescence (in adults). Through this approach, the present study identified several proteins which showed differential expression in the pesticide-treated gonad when compared to the control, which is explained in detail in Chapter 1a. In addition, due to the advantages of *C. gariepinus* in terms of fecundity, hatchability and growth process compared to *C. batrachus* in the laboratory conditions, the former catfish model has been used for testing proteome changes in the normal physiological status, juvenile and adulthood. This resulted in the identification of a germ-cell marker Synaptonemal complex protein-3 (Sycp3) which was upregulated in juvenile catfish testis when compared to adult, which is explained in detail in Chapter 2.

Abstract

The effects of endocrine disrupting chemicals have raised widespread concern, as numerous studies have proven their capacity to impede normal physiological functions of numerous organisms, particularly during critical development and reproductive stages. Commonly used agricultural pesticides, endosulfan and malathion, have been identified as disruptors of normal physiological function in organisms of aquatic systems. The present study examines the results of exposure of catfish (*Clarias batrachus*) to endosulfan (2.5 parts per billion [ppb]) and malathion (10 ppb) on the reproductive physiology by assessment of protein expression profiles after 21 days. After exposure of catfish to endosulfan, the proteomic profiles of gonads revealed downregulation of proteins such as ubiquitin and ESCO2, and upregulation in melanocortin-receptor-2, respectively. Ovary of fish exposed to malathion showed upregulated levels of prolactin. Identifying proteins in gonads that show differential expression as a result of exposure to these chemicals may provide critical indications of the aberrant effects of pesticides at the protein level.

1. Introduction

Endocrine disruptors and their toxicological effects on the environment have gained substantial attention in recent times due their possibly detrimental consequences on the normal physiological systems of humans as well as organisms of both terrestrial and aquatic ecosystems. Endocrine disrupting chemicals (EDCs) consist of a diverse group of chemicals, commonly of anthropogenic origin, that have the capacity to mimic natural hormones in the physiological systems of both humans and animals. They have been associated with altered physiological and reproductive functions in various ways, and numerous studies have highlighted their particular ability to bind and/or block estrogen and androgen receptors

(Tabb and Blumberg, 2006; Dutta-Gupta, 2013). These EDCs constitute many man-made chemicals such as industrial effluents, pesticides, pharmaceutical products, food additives, metals, etc., (Diamanti-Kandarakis *et al.*, 2009). Out of these numerous examples, the negative consequences of endosulfan, an organochloride (Embrandiri *et al.*, 2012) and malathion, an organophosphate pesticide (Prathibha *et al.*, 2014) are notable due to their extensive agricultural use on a global scale, including India, which has caused unchecked run-off into water bodies, directly threatening the aquatic ecosystem.

Numerous research on endosulfan exposure has highlighted the adverse consequences on biological systems such as deterioration of plasma vitellogenin levels (Chakravorty *et al.*, 1992), damaged metabolism (Tripathi and Verma, 2004), hyperactivity and altered behavioral patterns (Jonsson and Toledo, 1993), neurotoxicity (Carlson *et al.*, 1998), impairment of reproductive capability and spermatogenesis (Dutta *et al.*, 2006) in fish. Studies on endosulfan have shown that it can competitively bind to androgen receptors in humans, cause congenital defects, mental retardation, cancers and also manifest estrogenic effects in breast cancer cell lines (Embrandiri *et al.*, 2012). Zebrafish exposed to endosulfan concentrations as minimal as 10 ng/L, have shown a significant decrease in hatching rates, decreased gonadosomatic index, and increased vitellogenin levels in males, which indicates its antagonistic effect on fish reproduction (Han *et al.*, 2011). Sublethal concentrations of this pesticide on the catfish, *Heteropneustes fossilis* have also resulted in a reduction of total phospholipids levels at critical phases of reproduction like pre-spawning and spawning phases, which may consequently distress fertility and the annual reproductive cycle, since these lipids have significant roles in the production of nutrition and energy (Singh and Singh, 2007). Analysis of key genes associated with testicular development using quantitative real-time PCR and histological examination in juvenile catfish, *Clarias batrachus* from our

laboratory have revealed significant decrease in transcript levels of testis-related transcription factors and steroidogenic enzyme genes, which may impair spermatogonial proliferation as a result (Rajakumar *et al.*, 2012). Despite the ban of this pesticide in the Stockholm Convention 2011 (Rajakumar *et al.*, 2012), numerous studies have established its persistence in the environment via bioaccumulation. Therefore, a study on this pesticide at sub-lethal doses comparable to its environment concentration levels is still very much relevant.

Studies on malathion exposure at sublethal levels have resulted in genotoxicity (Amer *et al.*, 2002), impairment of sperm function and mobility (Akbarsha *et al.*, 2000) in mice, alteration of normal physiology and behavior (Beauvais *et al.*, 2009), inhibition of enzyme acetylcholinesterase (Brewer *et al.*, 2001), teratogenic effects during development (Cook *et al.*, 2005), diminished metabolic function (Venkataramana *et al.*, 2006), and impaired immune responses (Plumb and Areechon, 1990; Harford *et al.*, 2005) in fish. Assessment of the effects of malathion in a protogynous teleost, *Monopterus albus* showed that thyroid hormone levels were also affected, indicating disruption of the endocrine systems, subsequently resulting in potential diminished reproductive function in fishes (Singh, 1989), and these findings also corroborate with those in the catfish, *C. batrachus* where malathion exposure caused impediments in growth process (Lal *et al.*, 2013), and altered levels of gonad and brain specific gene expressions (Prathibha *et al.*, 2014).

The toxicological evaluation of these and other commonly used pesticides on fish species has gained significant momentum and attention due to their ecological consequence and economic impact. Since previous studies have established the capacity of these compounds to impair reproductive function in both adult and juvenile fishes (Arcand-Hoy and Benson, 1998; Rajakumar *et al.*, 2012), it is essential to elucidate the various aspects of their exerted

effects, and classify various standards, both at the transcriptomic and proteomic levels, which can specify critical levels of exposure. Since the normal physiology of fishes are easily susceptible to even minimal levels of exposure to external contaminants that result in alterations of genes and proteins expression patterns, analysis of the changes at the level of proteome would prove advantageous due to its dynamic nature. Further, detailed evaluation of the proteomic profiles would serve as valuable bridges between the effects at the molecular and organismal levels (Lemos *et al*., 2009). Several studies have defined parameters that have been considered for assessing the negative consequences of these chemicals, one being reproductive function which has been shown to be susceptible to chemicals that disturb endocrine functions in the hypothalamo-hypophyseal-gonadal axis. Evaluation of the phenotypic outcomes of such exposures should be aided by detailed information that account for disruption in functional aspects like proteins, which may support establishment of 'expression signatures' for different classes of chemicals, as well as elucidating their modes of action (Biales *et al*., 2011).

The present study aims to analyze the effects of two widely used pesticides, endosulfan and malathion, on the reproductive function and physiology of catfish after exposure at sub-lethal concentrations, by assessment of protein expression profiles. The differential expression of proteins in the gonads were evaluated, both ovary and testis, after separate exposures as an effort to highlight the effects of these compounds on crucial reproductive functions at the level of proteins, and to identify any affected factors that may play key roles in processes such as gametogenesis, by analysis of their altered expression levels.

2. Materials and Methods

2.1 Animals

Rearing of catfish of various age groups was done in fresh water tanks (25 ± 2°C) under ambient conditions. *In vitro* fertilization was carried out with mature males and gravid female catfishes to obtain larva as per the method described by Raghuveer *et al*. (2011) and also in the General Introduction. Maturity is usually attained approximately in a year. The *in vitro* fertilization was carried out in accordance with the seasonal reproductive cycle culminating in spawning phase. Catfish, *C. batrachus*, exhibits an annual seasonal pattern of reproductive cycle which can be divided in four different phases, preparatory (February-April), pre-spawning (May-June), spawning (July-August) and regressing or post spawning phase (September-January). This pattern of gonadal attenuation and recrudescence which is characteristic of annual breeders like catfish compared to continuously mature species is crucial in highlighting the roles of genes during development as well as recrudescence.

Gravid females were selected and injected with 2500 IU of human chorionic gonadotropin (hCG) intramuscularly and intraperitoneally. After ~10 hours, mature eggs were extracted from the female by applying gentle pressure on the abdomen. Milt was collected from the testes by dissecting it out from the male followed by thorough mincing on ice. Both were mixed carefully in a sterilized bowl with gentle shaking to allow fertilization. The embryos were then transferred to tanks connected with circulating filtered tap water. Hatching generally takes 24-36 hours after fertilization. After hatching, the fingerlings were kept in the incubator with filtered water. Once the yolk sac of the fingerlings gets completely absorbed, live tubeworms *Tubifex tubifex* were fed *ad libitum* daily. The water tanks were properly aerated and filtered tap water was circulated under normal photoperiod and ambient

temperature (25 ± 2°C). The catfish in these stages are comparable to fishes that undergo recrudescence or mid-preparatory phase, where females principally develop pre-vitellogenic follicles while males develop overwhelmingly higher amounts of primary to secondary spermatocytes.

Both male and female catfish of similar age were divided into three groups: one group was kept as control, and the other two groups were separately treated either with 2.5 parts per billion (2.5 ppb or µg/L) endosulfan (Lot and Batch : E0344, Nagarjuna Agrichem Limited, Hyderabad India) or 10 ppb malathion (Hyderabad Chemicals Limited, Hyderabad, India), for 21 days. The chemicals were administered to the fish by dissolution of the pesticides into the water in the tanks. In one treatment group, 8 month old catfish were maintained in filtered water and treated with 2.5 ppb of endosulfan dissolved in the water. The other group consisted of 8 month old catfish maintained in filtered water treated with 10 ppb of malathion dissolved into the water. Replicates were maintained for each group. The doses used for treatment were established from our preliminary studies which have indicated that the used doses are lower than the LC_{50} values reported for the two pesticides. Working stocks for both endosulfan and malathion were carefully prepared and maintained daily for treatment. All the treatment groups were maintained in similar conditions, and all the tanks were replaced daily with fresh filtered water (control) or endosulfan or malathion added filtered water for a period of 21 days. The fishes were fed with live tube worms *ad libitum* throughout the period of treatment. After the experiment period, tissues were dissected from all three groups by sacrificing the fish as per the guidelines given by the Institutional Animal Ethics Committee, University of Hyderabad. Testis and ovary were dissected out, snap frozen with liquid nitrogen, and stored in at -80 ^{o}C for proteomic analysis. Pooling of gonads (~3-4 for each gonad taken as one biological sample) was also done to get adequate amounts of tissue for

higher protein yield. Analysis with 2D was performed a minimum of 3 times with separate gonad samples each time for consistent results.

2.2 Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF-MS) analysis

2.2.1 Sample preparation and tissue extraction

Protein was isolated from the gonads by homogenization using buffer containing 6 M Urea, 4% CHAPS, 50 mM DTT and 100 mM PMSF on ice. After homogenization, centrifugation was done at 12,000 xg for 30 minutes at 4 ^{o}C. The supernatant was collected with a pipette, precipitated, and used for 2D gel electrophoresis. Protein concentration in the sample was assessed using Bradford method.

2.2.2 Two-dimensional gel electrophoresis

Clean-up with 2-D Clean-up Kit (GE Healthcare Life Sciences, USA) was done for the supernatant collected after homogenization of the tissue samples, as per the manufacturer's protocol. After the clean-up, the separated protein precipitate was dissolved in rehydration buffer containing 7 M Urea, 2 M Thiourea, 4% CHAPS, 2% Ampholytes of pH 3-10, 70 mM DTT and 3.5% Bromophenol blue. About 350 µl of the protein solution was then used for rehydration of IPG strips (Immobiline Drystrip, 3-10 pH Linear 18 cm, GE Healthcare) for ~20 hours with low viscosity mineral oil (Merck Genei, Bangaluru, India). Isoelectric Focusing in Ettan IPGphor3 gel apparatus (GE Healthcare) was then performed for the rehydrated IPG strips loaded with 300 µg protein at 20 ^{o}C for 70,000 Volt hours (Vh)-50 V-1 hour, 500 V-5 hours, 500 V-5 hours, 10000 V-8 hours, 500 V-10 hours (a total of 70,000 volt hours). The IPG strips, after IEF, were equilibrated using buffer (75 mM Tris HCl pH 8.8,

30% glycerol, 2% SDS and a trace of bromophenol blue) added with 10 mg/ml of DTT followed by another round of equilibration for 20 minutes in buffer added with 25 mg/ml of iodoacetamide (an alkylating agent that can reduce cysteine residues via inhibition of disulfide bonds). The IPG strips were then subjected to SDS-PAGE and the gels were stained using coomassie brilliant blue R-250.

2.2.3 Image analysis

The gels were then analyzed with Image Master 2-D Platinum (GE Healthcare) software. Three different gels were examined for each sample and the protein spots that were observed to display significant differential expressions were selected for further analysis.

2.2.4 In-gel digestion, Protein identification and Database search

MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Bremen, Germany) was used to perform in-gel digestion and MALDI-TOF-MS analysis as per the method described by Shevchenko *et al.* (1996) with slight modifications. After manual excision of the protein spots from the gels, destaining was done for 5 times using 100 µL of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3). After this, treatment of protein-containing gel fragments was done with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56 °C for 1 h. This sample was then treated for 45 min with 55 mM iodoacetamide in 25 mM NH_4HCO_3 at 25 °C, washed with 25 mM NH_4HCO_3 and ACN, dried with speed vac (Labconco, San Diego, USA) and rehydrated in ~30 µL of 25 mM NH_4HCO_3 added with 12.5 ng/µL trypsin (Promega, Wisconsin, USA). This sample was incubated for 10 min on ice and digested overnight at 37 °C. Post digestion, centrifugation was done and the supernatant was pipetted out into a different eppendorf tube. The gel pieces were treated with 50 µL of 1% trifluoroacetic acid (TFA) and ACN (1:1) for 15 min and vortexed intermittently. The supernatants were collected, pooled and dried with speed vac

and reconstituted in 5 µL of 1:1 ACN and 1% TFA. From this sample, about 2 µL was added with 2 µL of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 1 µL was spotted on target plate. Database searches were performed for protein identification (Peptide Mass Fingerprinting and MS/MS) with MASCOT program (http://www.matrixscience.com) using Biotools software (Bruker Daltonics). The similarity examination for mass values was performed with existing digests and sequence information from NCBInr and Swiss Prot database. The search parameters used were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^+ and monoisotropic. According to the MASCOT program, the probability of $P < 0.05$ was considered statistically significant and only significant hits were selected for protein identification.

3. Results

3.1 Changes in the protein profile of catfish gonads after the exposure of endosulfan

The proteomic profile of gonads, obtained using 2D gel electrophoresis, of fish exposed to endosulfan displayed several differentially expressed protein spots between control and treated fishes. Differential expression of proteins was measured in fold changes, and only the spots with fold changes greater than 2 were selected for MALDI analysis. Protein spots with lower molecular weight were also preferred, since most hormones/factors essential for gonadal and reproductive functions may have low molecular weights, which is an important condition in our experiment.

In the control and endosulfan treated testis samples, comparison of gels after quantitative image analysis using Image Master 2-D Platinum (GE Healthcare) system (Figure 1) showed that out of 76 spots analyzed, 15 spots in treated group had significant changes in their

expressions compared to control, with fold changes greater than a factor of 2 (either upregulation or downregulation). Out of these 15 spots, 6 spots were downregulated (DR) in the treated group relative to control and protein spots were selected for MALDI analysis based on their low molecular weights. Spots that newly appeared (NE) or completely disappeared (DP1) after treatment were also selected because of the drastic changes in their expression profile. Table 1 shows the list of proteins identified using MALDI-TOF analysis.

In the ovary samples of catfish group treated with endosulfan, quantitative image (Figure 2) analysis revealed that out of 50 protein spots analyzed, 5 protein spots had a significant upregulated in expression (UR), with a fold change greater than a factor of 2 when compared to the control samples (Table 2). Out of these 5 upregulated spots, 3 protein spots having maximum fold increase were selected for MALDI-TOF analysis. One newly expressed (NE) spot from the gel in endosulfan treated group was also examined.

3.2 Changes in the protein profile of catfish ovary after the exposure of malathion

The ovary samples of catfish treated with malathion revealed several differentially expressed protein spots (Figure 3) when compared to control groups. The various differential spots identified and analyzed from the gels are listed in detail in Table 3. From a total of 123 spots analyzed, there were 9 spots that displayed significant change in expression, i.e., fold change greater than 2. Out of these 9 spots that showed significant differential expression, 2 spots that were downregulated (DR1 and DR2) and 1 upregulated spot (UR) were selected for MALDI-TOF analysis. The similar study could not be performed for testis due to limited sample amounts.

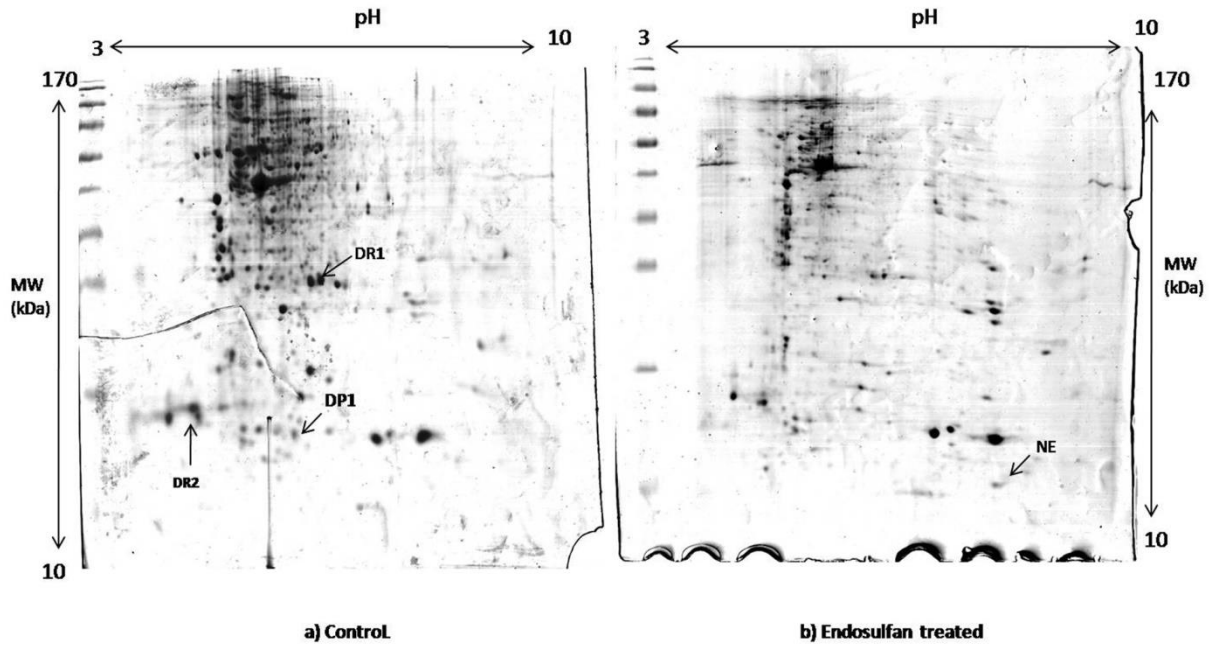


Fig. 1. Representative two-dimensional electrophoresis gels of catfish testis after endosulfan exposure and control: (a) control and (b) endosulfan treated. Spots indicated by arrows were downregulated (DR1, DR2) and disappeared (DP1) and newly expressed (NE) in the endosulfan exposed fish (n = 3 [pooled]). Spots were identified as Esco2 (DR1), LYR motif containing protein 4 (NE), Ubiquitin (DP1) and gamma-crystallin M1-1 (DR2).

Name of Spot	**Expression pattern**	***Molecular weight in kDa***	***pI***	**Protein name**	**Accession number**	**Species Identified**	**Score**
NE	Newly expressed	10.515	10.07	LYR motif-containing protein 4	gi|256000753	*Salmo salar*	21
DR1	Down regulated	59.076	10.01	ESCO2 (establishment of cohesion 1 homolog 2)	gi|325652170	*Oryzias latipes*	51
DP1	Disappeared	8.446	6.56	ubiquitin	gi|229532	*Homo sapiens*	103

DR2	Disappeared	22.15	8.05	gamma-crystallin M1-1	gi|632009	*Petenia splendida*	17

Table 1. List of identified protein spots from testis of catfish after the exposure of endosulfan with name of spot, expression pattern, molecular weight, pI, protein name, accession number, score and species of ray-finned fish to which the proteins were identified: protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (http://www.matrixscience.com) employing Biotools software (Bruker Daltonics). The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss Prot database. According to the MASCOT program, the probability of $P < 0.05$ was considered statistically significant and only significant hits were accepted for protein identification.

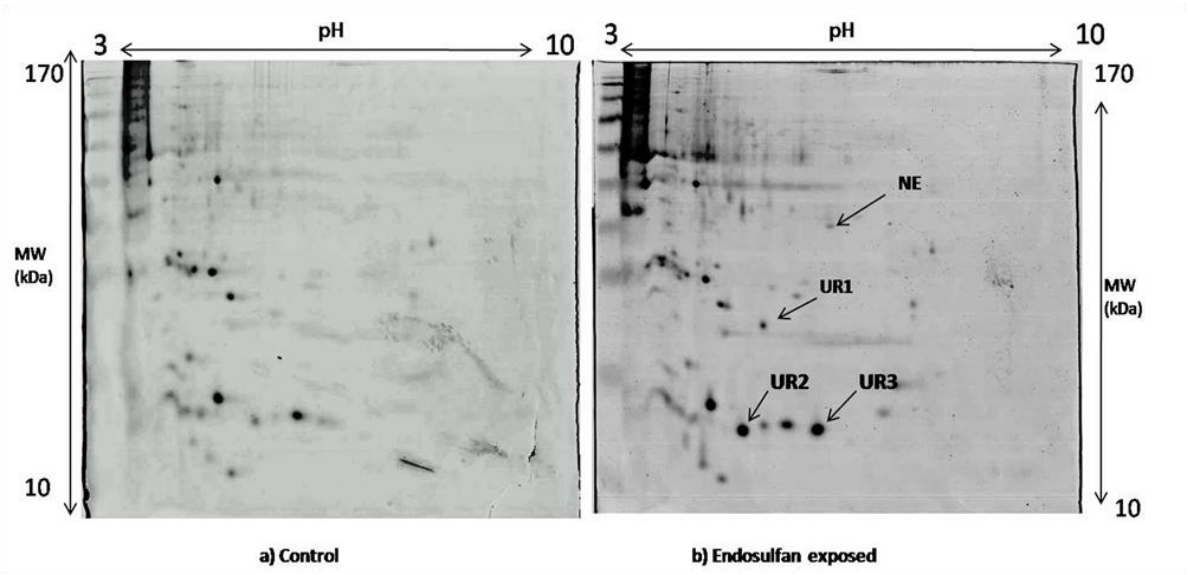


Fig. 2. Representative two-dimensional electrophoresis gels of catfish ovary after endosulfan exposure and control: (a) control and (b) endosulfan treated. Spots indicated by arrows were upregulated (UR1, UR2, UR3) and newly expressed (NE) in the endosulfan exposed fish (n = 3 [pooled]). Spots identified are disulfide-isomerase A3 precursor (UR1), melanocortin-2

receptor/ACTH receptor (UR2), myelin expression factor 2-like (UR3) and poly (rC)-binding protein 2 (NE).

Name of Spot	Expression pattern	Molecular weight in KDa	pI	Protein name	Accession number
UR1	Up regulated	55.337	5.46	Disulfide-isomerase A3 precursor	gi|209153384
NE	Newly expressed	33.595	8.18	Poly (rC)-binding protein 2	gi|41055221
UR2	Up regulated	34.701	8.92	Melanocortin-2 receptor/ACTH receptor	gi|343887257
UR3	Up regulated	53.290	6.49	Myelin expression factor 2-like	gi|348538711

Table 2. List of identified protein spots from ovary of catfish after the exposure of endosulfan (all other details are as in Table 1).

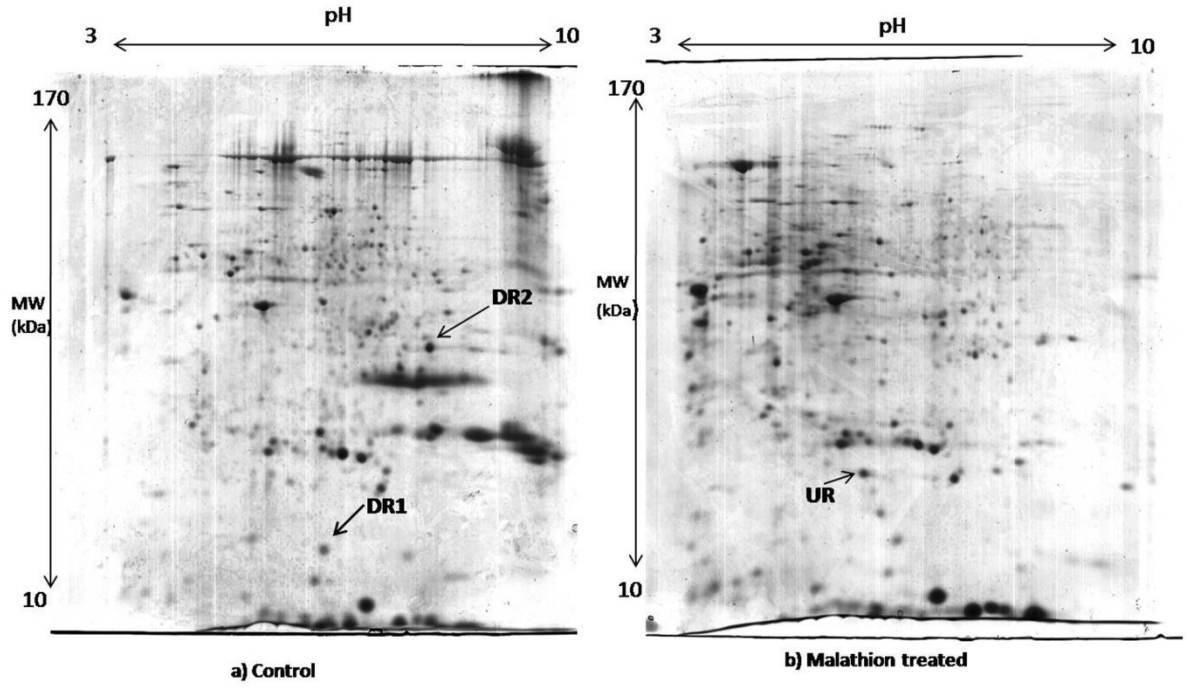


Fig. 3. Representative two-dimensional electrophoresis gels of catfish ovary after malathion exposure and control: (a) control and (b) malathion treated. Spots indicated by arrows were upregulated (UR) and downregulated (DR1, DR2) in the malathion exposed fish (n = 3 [pooled]). Spots were identified as prolactin (UR), progonadoliberin-2 (DR1) and cytochrome P450 1A1 (DR2).

Name of Spot	Expression pattern	Molecular weight in kDa	pI	Protein name	Accession number	Species Identified	Score
UR	Upregulated	23733	9.24	Prolactin	gi|464464|P34181.1	*Coregonus autumnalis*	16
DR1	Downregulated	9419	9.13	Progonadoliberin-2	gi|157278082	*Oryzias latipes*	17

DR2	Downregulated	59837	6.58	Cytochrome p450 1A1	gi|3913314	*Opsanus tau*	25

Table 3. List of identified protein spots from ovary of catfish after the exposure of malathion (all other details are as in Table 1).

4. Discussion

The current work revealed that both endosulfan and malathion have the potential to provoke responses independently at the proteomic level in developing gonads of catfish after exposure even at sub-lethal (minimal) doses of the pesticides. The 2D proteomic analyses together with MALDI-TOF showed aberrant effects of these pesticides on catfish gonads.

The treatment of endosulfan on male fishes resulted in the disappearance of protein ubiquitin, which may suggest the negative effects of this pesticide on reproductive function, due to the critical role of this protein in spermatogenesis as well as sperm maturation, and is established as a "marker for semen quality and fertility" in humans, and other mammals such as mice and bulls (Bebington *et al.*, 2001; Sutovsky *et al.*, 2004; Baska *et al.*, 2008; Lui and Lee, 2008). Various studies on the role of ubiquitin in reproduction have proposed that it may be crucial for protein degradation during gametogenesis which entails ubiquitin-dependent proteolysis (Baarends *et al.*, 1999). As such, the negative impact of endosulfan on this protein which consequently results in diminished reproductive and physiological processes may impede puberty. Despite the lack of studies in fish that associate ubiquitin in sperm maturation, our earlier finding on endosulfan impeding spermatogonial proliferation (Rajakumar *et al.*, 2012) might corroborate the above-mentioned claim. Significant downregulation of the "establishment of cohesion 1 homolog 2" (ESCO2) protein, which is a critical component of

chromosome segregation during mitosis (Bose *et al.*, 2012), may also indirectly link the adverse impact of endosulfan on reproductive functions, since reduction of ESCO2 in zebrafish models have caused elevated amounts of cell death and apoptosis (Mönnich *et al.*, 2011). In addition, ESCO2 has been established to have crucial functions in meiosis and renewal of germ cell due to its localization in both murine meiotic cells (spermatocyte) and embryonic ovary (Hogarth *et al.*, 2011; Evans *et al.*, 2012). This may infer that disturbances in this gene function may consequently hinder reproduction even in lower vertebrates such as teleosts.

In female fish treated with endosulfan, the protein profile displayed a significant increase in the expression levels of melanocortin-2-receptor, which is an essential component to receive ligands such as melanocorticoids, influenced by adrenocorticotropic hormone (ACTH). However, the molecular mechanisms have not been well elucidated in lower vertebrates such as teleosts. In rainbow trout, tissue distribution analysis of this receptor revealed increased expression levels in ovaries, suggesting the role of this ligand-receptor complex in the suppressing sex steroid levels triggered by external stressors (Aluru and Vijayan, 2008). Due to the suggested dynamic role of melanocortin receptors in ovulation and steroidogenic functions of bovine ovary (Amweg *et al.*, 2011), a disruption elicited by external antagonistic factors might negatively influence normal reproductive functions, which corroborates findings that have recognized the adverse impact of stress on reproduction in numerous vertebrates (Alsop *et al.*, 2009).

Catfish treated with sub-lethal doses of malathion showed significant increase in prolactin levels in ovary. Prolactin is an essential peptide hormone that plays a key role in a myriad of biological functions like metabolism, regulation of immune responses, behavior, lactation, reproduction, etc. (Cooke *et al.*, 2004). The significantly elevated levels of prolactin

expression corroborates results which validated that the elevated plasma levels of prolactin in freshwater teleosts may imply a hydromineral effect as a reaction to external stressors (Thangavel *et al.*, 2005). Persistent elevated levels of prolactin in mice have shown reduced GnRH secretion from the hypothalamus, which can potentially impede gonadotropins and gonadal steroidogenesis, and result in extensive impairment of reproductive functions (Freeman *et al.*, 2000). The disruption in expression levels of prolactin in the extra-pituitary tissues like liver, kidney and gonads where it has also been found, may also implicate aberrant sexual development and maturity in fishes (Whittington and Wilson, 2013). Such roles of prolactin in fishes remain to be investigated further. Nevertheless, the present study may suggest such a phenomenon.

The proteomic analysis of catfish treated with pesticides, endosulfan and malathion, at environmentally relevant, sub-lethal doses resulted in disruptions in the expression profiles of several proteins in gonads which seem to play critical roles in the normal physiology and reproductive function of catfish. Further detailed analyses are essential to confirm that pesticides have the potential to negatively impact and disrupt the endocrine systems. In addition, studies are required to investigate the mechanisms in impairment of hormone levels, either directly or indirectly, and also to explicate the identified protein spots as potential biomarkers to monitor critical pesticide levels in the environment. The present study reveals first evidence for alterations in proteomic profile of catfish gonads after limited period low level exposure with endosulfan and malathion. Despite these interesting findings, this approach did not yield any factors that were directly related to germ cell or gonadal development except for ubiquitin which is already a well-known protein related to apoptosis that partially determines progression of spermatogenesis.

References

Akbarsha M.A., Latha P.N., Murugaian P., 2000. Retention of cytoplasmic droplet by rat cauda epididymal spermatozoa after treatment with cytotoxic and xenobiotic agents. J. Reprod. Fertil. 120, 385-390.

Alsop D**.,** Ings J.S**.,** Vijayan M.M., 2009. Adrenocorticotropic hormone suppresses gonadotropin-stimulated estradiol release from zebrafish ovarian follicles. PLoS ONE 4: e6463.

Aluru N., Vijayan M.M., 2008. Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout. Endocrinology 149, 4577-4588.

Amer S.M., Fahmy M.A., Aly F.A.E., Farghaly A.A., 2002. Cytogenetic studies on the effect of feeding mice with stored wheat grains treated with malathion. Mutat. Res. 513, 1-10.

Amweg A.N., Paredes A., Salvettia N.R., Larab H.E., Ortegaa H.H., 2011. Expression of melanocortin receptors *mRNA*, and direct effects of ACTH on steroid secretion in the bovine ovary. Theriogenology 75, 628-637.

Arcand-Hoy L.D., Benson W.H., 1998. Fish reproduction: An ecologically relevant indicator of endocrine disruption. Environ. Toxicol. Chem. 17, 49-57.

Baarends W.M., Roest H.P., Grootegoed J.A., 1999. The ubiquitin system in gametogenesis. Mol. Cell. Endocrinol. 151, 5-16.

Baska K.M., Manandhar G., Feng D., Agca Y., Tengowski M.W., Sutovsky M., Yi Y.J., Sutovsky P., 2008. Mechanism of extracellular ubiquitination in the mammalian epididymis. J. Cell. Physiol. 215, 684-696.

Beauvais S.L., Jones S.B., Brewer S.K., Little E.E., 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout *Oncorhynchus mykiss* and their correlation with behavioral measures. Environ. Toxicol. Chem. 19, 1875-1880.

Bebington C., Doherty F.J., Fleming S.D., 2001. The possible biological and reproductive functions of ubiquitin. Hum. Reprod. Update 7, 102-111.

Biales A.D., Bencic D.C., Flick R.L., Blocksom K.A., Lazorchak J.M., Lattier D.L., 2011. Proteomic analysis of a model fish species exposed to individual pesticides and a binary mixture. Aquat. Toxicol. 101, 196-206.

Bose T., Lee K.K., Lu S., Xu B., Harris B., Slaughter B., Unruh J., Garrett A., McDowell, W., Box, A., Li, H., Peak, A., Ramachandran, S., Seidel, C., Gerton, J.L., 2012. Cohesin proteins promote ribosomal RNA production and protein translation in yeast and human cells. PLoS Genet. 8: e1002749.

Brewer S.K., Little E.E., DeLonay A.J., Beauvais S.L., Jones S.B., Ellersieck M.R., 2001. Behavioral dysfunctions correlate to altered physiology in rainbow trout (*Oncorynchus mykiss*) exposed to cholinesterase-inhibiting chemicals. Arch. Environ. Contam. Toxicol. 40, 70-76.

Carlson R.W., Bradbury S.P., Drummond R.A., Hammermeister D.E., 1998. Neurological effects on startle response and escape from predation by medaka exposed to organic chemicals. Aquat. Toxicol. 43, 51-68.

Chakravorty S., Lal B., Singh T.P., 1992. Effect of endosulfan (thiodan) on vitellogenesis and its modulation by different hormones in the vitellogenic catfish *Clarias batrachus*. Toxicology 75, 191-198.

Cook L.W., Paradise C.J., Lom B., 2005. The pesticide malathion reduces survival and growth in developing zebrafish. *Environ. Toxicol. Chem.* 24, 1745-1750.

Cooke, P.S., Holsberger D.R., Witorsch R.J., Sylvester P.W., Meredith J.M., Treinen K.A., Chapin, R.E., 2004. Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. Toxicol. Appl. Pharmacol. 194, 309-335.

Coppage D.L., Matthews E., 1974. Short-term effects of organophosphate pesticides on cholinesterases of estuarine fishes and pink shrimp. Bull. Environ. Contam. Toxicol. 11, 483-488.

Diamanti-Kandarakis E., Bourguignon J.P., Giudice L.C., Hauser R., Prins G.S., Soto A.M., Zoeller R.T., Gore A.C., 2009. Endocrine-disrupting chemicals: an endocrine Society scientific statement. Endocr. Rev. 30, 293-342.

Dutta H.M., Misquitta D., Khan S., 2006. The effects of endosulfan on the testes of bluegill fish, *Lepomis macrochirus*: a histopathological study. Arch. Environ. Contam. Toxicol. 51, 149-156.

Dutta-Gupta A. 2013. Teleosts as model for environmental risk assessment. In: Senthilkumaran, B., (Ed.), "Sexual plasticity and gametogenesis in fishes", Nova Medical, New York, pp. 49-56.

Embrandiri A., Singh R.P., Ibrahim H.M., Khan A.B., 2012. An epidemiological study on the health effects of endosulfan spraying on cashew plantations in kasaragod district, Kerala, India. Asian J. Epidemiol. 5, 22-31.

Evans E.B., Hogarth C., Evanoff R.M., Mitchell D., Small C., Griswold M.D., 2012. Localization and regulation of murine ESCO2 during male and female meiosis. Biol. Reprod. 87, 61, 1-8.

Freeman M.E., Kanyicska B., Lerant A., Nagy G., 2000. Prolactin: structure, function, and regulation of secretion. Physiol. Rev. 80, 1523-1631.

Greenbaum D., Colangelo C., Williams K., Gerstein M., 2003. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol. 4: 117.

Groh K.J., Nesatyy V.J., Segner H., Eggen R.I., Suter M.J., 2011. Global proteomics analysis of testis and ovary in adult zebrafish (*Danio rerio*). Fish Physiol. Biochem. 37, 619-647.

Han Z., Jiao S., Kong D., Shan Z., Zhang X., 2011. Effects of β-endosulfan on the growth and reproduction of zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 30, 2525-2531.

Harford A.J., O'Halloran K., Wright P.F., 2005. The effects of *in vitro* pesticide exposures on the phagocytic function of four native Australian freshwater fish. Aquat. Toxicol. 75, 330-342.

Hogarth C.A., Mitchell D., Evanoff R., Small, C., Griswold M., 2011. Identification and expression of potential regulators of the mammalian mitotic-to-meiotic transition. Biol. Reprod. 84, 34-42.

Jonsson C.M., Toledo M.C.F., 1993. Acute toxicity of endosulfan to the fish *Hyphessobrycon bifasciatus* and *Brachydanio rerio*. Arch. Environ. Contam. Toxicol. 24, 151-155.

Lal B., Sarang M.K., Kumar P., 2013. Malathion exposure induces the endocrine disruption and growth retardation in the catfish, *Clarias batrachus* (Linn.). Gen. Comp. Endocrinol. 181, 139-145.

Lemos M.F.L., Soares A.M.V.M., Correia A.C., Esteves A.C., 2009. Proteins in ecotoxicology - how, why, and why not? Proteomics 10, 873-887.

Lui W.Y., Lee W.M., 2008. Ubiquitin system in male reproduction and its relevance to contraception. *Immunol. Endocr. Metab. Agents. Med. Chem.* 8, 14-19.

Mönnich M., Kuriger Z., Print C.G., Horsfield J.A., 2011. A zebrafish model of roberts syndrome reveals that ESCO2 depletion interferes with development by disrupting the cell cycle. *PLoS ONE* 6: e20051.

Pandey A., Mann M., 2000. Proteomics to study genes and genomes. Nature 405, 837-846.

Plumb J. A., Areechon N., 1990. Effect of malathion on the humoral immune response of channel catfish. Dev. Comp. lmmunol. 14, 355-358.

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.

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.

Senthilkumaran B., 2015. Pesticide and sex steroid analogue-induced endocrine disruption differentially targets hypothalamo-hypophyseal-gonadal system during gametogenesis in teleosts. Gen. Comp. Endocrinol. 219, 136-142.

Shevchenko A., Wilm M., Vorm O., Mann M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850-858.

Singh H., 1989. Interaction of xenobiotics with reproductive endocrine functions in a protogynous teleost, *Monopterus albus*. Mar. Environ. Res. 28, 285-289.

Singh P.B., Singh V., 2007. Endosulfan induced changes in phospholipids in the freshwater female catfish, *Heteropneustes fossilis* (Bloch). J. Environ. Biol. 28, 605-610.

Söffker M., Tyler C.R., 2012. Endocrine disrupting chemicals and sexual behaviors in fish--a critical review on effects and possible consequences. Crit. Rev. Toxicol. 42, 653-668.

Spaink H.P., Jansen H.J., Dirks R.P., 2014. Advances in genomics of bony fish. Brief Funct. Genomics 13, 144–156.

Sutovsky P., Hauser R., Sutovsky M., 2004. Increased levels of sperm ubiquitin correlate with semen quality in men from an andrology laboratory clinic population. Hum. Reprod. 19, 628-638.

Tabb M.M., Blumberg B., 2006**.** New modes of action for endocrine-disrupting chemicals. Mol. Endocrinol. 20, 475-482.

Thangavel P., Sumathiral K., Karthikeyan S., Ramaswamy M., 2005. Endocrine response of the freshwater teleost, *Sarotherodon mossambicus* (Peters) to dimecron exposure. Chemosphere. 61, 1083-1092.

Tripathi G., Verma P., 2004. Endosulfan-mediated biochemical changes in the freshwater fish *Clarias batrachus*. Biomed. Environ. Sci. 17, 47-56.

Venkataramana G.V., Sandhya Rani P.N., Murthy P.S., 2006. Impact of malathion on the biochemical parameters of gobiid fish, *Glossogobius giuris* (Ham). J. Environ. Biol. 27, 119-122.

Whittington C.M., Wilson A.B., 2013. The role of prolactin in fish reproduction. Gen. Comp. Endocrinol. 191, 123-136.

Abstract:

Molecular mechanisms underlying germ cell development, proliferation and maturation in teleosts revealed the importance of transcription factors through functional genomic approaches which is often comparable to mammals. The present study employed a proteomic approach to find novel molecules with reference to germ cell development in teleosts. Based on this, in our study we have utilized two-dimensional gel electrophoresis combined with matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry in an attempt to identify and compare the proteins which are expressed differentially in the male gonads of catfish during juvenile and adult stages. Our 2D gel analysis identified the Sycp3 protein which was found to be up regulated in juvenile testis when compared to adult stages, and then the full length cDNA was cloned using the peptide sequence data from catfish testis. We explored the spatio-temporal expression pattern of *sycp3* during the critical time period of gonadal development as well as different phases of recrudescence in catfish using real-time PCR, followed by localization analysis by *in situ* hybridization and immunohistochemistry. In addition, *in vivo* transient knockdown using PEI mediated *sycp3*-esiRNA in adult catfish during recrudescence showed a decrease in *sycp3* expression, which also affected the expression level of various testis related genes. These findings imply that Sycp3 might have a potential role in the development and maintenance of testicular function in catfish.

1. Introduction

The processes of germ cell and gonadal development are the basis of sexual reproduction in vertebrates. Particularly in teleosts, these processes are under the influence of a cascade of molecular, genetic and physiological mechanisms. A decisive step in the attainment of sexual

maturity is the entry of germ cells into meiosis, followed by progression into viable gametes to produce either eggs or sperm. Although various transcription factors and genes which are implicated in gonadal development have been identified, there is still inadequate information to decipher underlying mechanisms and specific mode of actions. Earlier reports in teleost species in this context are mainly from daily or fortnight breeders, and there is a lack of similar data in annual breeders. To gain more insight, we aimed to identify genes/factors regulating meiosis in order to understand germ cell development. Taking this into account, we employed a proteomic approach involving a two dimensional (2D) electrophoretic separation of proteins from adult and juvenile male gonads of the catfish *Clarias gariepinus*, excision of differentially expressed spots, in-gel digestion and identification of resulting peptides by matrix assisted laser desorption/ionization time-of-flight (MALDI TOF/TOF) mass spectrometry. We then identified the synaptonemal complex protein-3 (Sycp3) which was found to be up regulated in juvenile testis when compared to adult stages.

SYCP3 is a meiosis-specific component of the synaptonemal complex, essential for the synapsis of homologous chromosomes, and is a marker of meiosis in mammals (Page *et al.*, 2006). The *Sycp3* gene encodes the SYCP3 protein which is a major component of the synaptonemal complex (SC), a structure specific to meiosis which comprises of two parallel lateral regions and a central element (Botelho *et al.*, 2001). Many studies, mostly in mammalian gametogenesis, have shown its crucial role in chromosome pairing, recombination, synapsis, and also in relation with their expression during sex differentiation of gonads and the stages of meiotic prophase in mouse oocytes (Di Carlo *et al.*, 2000). Its importance in gametogenesis and fertility has been studied in mammals as well as in a few teleost species such as medaka and zebrafish (Iwai *et al.*, 2006; Ozaki *et al.*, 2011). Male

mice that carry a null-mutation in the *Sycp3* gene have been found to be sterile with complete loss of spermatocytes (Yuan *et al.*, 2000). Male specific genes such *Dmrt1* knockdowns have also been observed to have an effect on expression and localization patterns of SYCP3 (Agbor *et al.*, 2013). Expression profile analysis in rats revealed its detection from day 15 of post natal period and its increased expression thereon might give a possibility of correlation with critical stages of fish gonadal development (Liu *et al.*, 2012) where its expression was found to be significant during early spermatogenesis. In black porgy, *Sycp3* along with *dazl* and *dmc1* have been found to be essential for meiotic competency, and was downregulated with estrogen treatment which affects meiotic arrest in the germ cells (Lau *et al.*, 2013). Although prior studies have established its role in meiosis and gametogenesis mostly in mammals, further information about the involvement of this testis-critical gene would be essential to elucidate its role in the process of gonadal germ cell development during critical stages, in teleost species where very limited reports are available in this context. Such studies, hence, may contribute to the pool of numerous genes that are responsible for the complex process of germ cell development, proliferation and maturation in teleosts. This assumes more importance where certain annually breeding teleosts undergo recrudescence to complete gonadal cycle repeatedly.

To enable our view, at first, based on the identified Sycp3 protein sequence using MALDI-TOF, we designed degenerate primers and cloned the full length cDNA of *sycp3* from the testis of catfish. The spatio-temporal expression pattern of *sycp3* was also studied during the critical stage of gonadal development and recrudescence in catfish using real-time PCR. Polyclonal antibodies were produced based on deduced amino acid sequences to localize the protein using immunohistochemistry (IHC) while transcripts in testis were identified using *in*

situ hybridization (ISH). We also transfected Endonuclease prepared small interfering RNAs (esiRNA) targeted against *sycp3* gene in catfish testis *in vivo* and analyzed its transient knockdown effects as well as the impact of the silencing on other genes crucial for germ cell development and testicular function.

2. Materials and Methods

2.1 Breeding of catfish and sample collections

Breeding with IVF and rearing of *C. gariepinus* at different age groups has already been described in detail in the Chapter 1a. Breeding was performed during spawning phase and hatched embryos were maintained in glass tanks with proper aeration. After the required number of days post hatch, samples were collected. Prior to dissection, catfish were anesthetized on ice using 100 mg/L of MS-222 (Sigma, St. Louis, MO, USA). Gonads of male and female catfish (adult, 400 dph and juvenile, 100 dph) were then dissected out and snap frozen in liquid nitrogen, stored at -80 ^{o}C for proteomic analysis/total RNA isolation. Sampling for fish was done as per the general guidelines and with approval of the Institutional Animal Ethics Committee, University of Hyderabad.

2.2 Two-dimensional gel electrophoresis and MALDI-TOF-MS analysis:

2.2.1 Sample preparation and tissue extraction

Adult and juvenile (n=20 for each pooled sample) catfish gonads were collected and homogenized for protein extraction in a buffer containing 6 M Urea, 4% CHAPS, 50 mM DTT and 100 mM PMSF on ice. Centrifugation was done at 12,000xg at 4^{o}C for 30 minutes.

The supernatant was then carefully extracted, precipitated, and used for 2D gel electrophoresis. Protein concentration was measured using the method of Bradford (1976).

2.2.2 Two-dimensional electrophoresis

The prepared protein samples from both adult and juvenile gonads were then subjected to clean-up process, and two-dimensional gel electrophoresis was performed as per the protocol described in detail in the Chapter 1a. The gels were then stained using coomassie brilliant blue R-250, and computer analysis were carried out using Image Master 2-D Platinum (GE Healthcare) system. Three different gels were analyzed for both juvenile and adult samples, for both male and female gonads. The spots that were found to display significant changes in their expression were chosen, and this was then followed by In-gel digestion, Protein identification and Database search, also described in Chapter 1a. In juvenile testis, the peptide obtained from MALDI-TOF from the protein sample was highly homologous to the peptide KVMMDTQQQEMATVR identified in *Oncorhynchus mykiss*, and the translated nucleotide sequence from this peptide was used to design the degenerate reverse primer for cDNA cloning.

2.3 Molecular cloning of *sycp3* from testis of catfish

Isolation of total RNA was done using TRI-reagent (Sigma) from testes of both adult and juvenile catfish. The concentration was measured by NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). First strand cDNA synthesis was performed using the isolated total RNA by Primescript RT-PCR (TaKaRa) Reverse transcriptase with 1 µl oligo d(T) primer, 1 µl dNTPs, 4 µl reaction buffer, 0.25 µl RNase inhibitor and 5 µg of total RNA in a 20 µl reaction at 42°C. Different sets of degenerate

primers for *sycp3* were designed (Table 1) by aligning the existing sequences of siluriformes in NCBI data base using Lasergene software (release 3.05; DNASTAR, Madison, WI, USA). The resulting amplicon was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced and then analyzed for confirmation as catfish *sycp3* partial cDNA. The full length sequence of *sycp3* cDNA was obtained by RACE strategy. Gene specific primers (GSPs) were designed using sequence information from the partial cDNA sequence of *sycp3*. The 5` and 3` cDNA templates for RACE were prepared using SMARTer™ RACE cDNA amplification kit (Clontech, Mountainview, CA, USA) as per the manufacturer's protocol. Then, touchdown PCR reactions were carried out with the primers (5P, 5N, 3P and 3N) designed for RACE (Table 1) along with universal primer A mix, nested universal primer and Advantage® 2 PCR kit (Clontech) to obtain the 5` and 3` ends. All thermal cycling conditions were done as per the protocols given by the manufacturer. The products obtained from RACE were gel purified, cloned into pGEM®-T easy vector (Promega), followed by sequencing and analysis by NCBI-BLAST.

2.4 Quantitative analysis of *sycp3* for tissue distribution, reproductive cycle and ontogeny studies using RT-PCR (qPCR)

Adult catfish (n=5), both male and female undergoing late preparatory phase were sacrificed and different tissues were dissected out (brain, heart, muscle, liver, kidney, testis, ovary). Testis samples were collected at different phases of the reproductive cycle for phase expression analysis. Samples of developing gonads from both male and female were also collected. In catfish, gonadal differentiation, resulting in development of either a testis or ovary, occurs from 35 to 50 days post hatch (dph). Therefore, mesonephric-gonadal complex were collected from 0 to 40 dph fish, and testes and ovaries were dissected from 50, 100, 200

dph and adult fishes. Total RNA was extracted from these tissues using TRI-reagent® (Sigma) as per the manufacturer's protocol. Reverse transcription was carried out with Verso® reverse transcriptase enzyme (Thermo Fisher Scientific Inc, Waltham, MA, USA), random hexamers and 1µg of total RNA as per the manufacturer's instructions. β-actin (internal control) amplification was done to confirm the quality of the cDNA templates. qPCR primers for *sycp3* with amplicon length of ~200 bp were designed. The expression pattern of *sycp3* was analyzed by qPCR in gonads and different somatic tissues, during various stages of gonadal development as well as different phases of the annual reproductive cycle. SYBR® Green Master mix (Clontech) was used to perform real-time PCRs in an ABI Prism® 7500 fast thermal cycler (Applied Biosystems, Foster City, CA, USA) at 95^{o}C (15 s), 60^{o}C (1 min) for 40 cycles. The resulting amplicons were then sequenced and verified to confirm the specificity of the PCR amplification followed by dissociation curve analysis with sequence detection software (Applied Biosystems). *18s rRNA* was used as a reference gene due to its stable expression based on our previous studies. For each sample, triplicate qPCR assays were performed using the *sycp3* specific primers and no template controls were also done which did not generate any amplification. Cycle threshold (C_t) values were acquired from exponential phase of PCR amplification and normalization of *sycp3* expression was done against expression of the internal control gene, *18S rRNA* to obtain a ΔC_t value (C_t of target gene - C_t of control). Differential gene expressions were then calculated using $2^{-\Delta Ct}$ method. All primers used are listed in Table 1.

2.5 hCG induction *in vivo* and *in vitro*:

The *in vivo* and *in vitro* induction using hCG on adult catfish at early prespawning phase were done as per the protocols described by Murugananthkumar *et al*. (2016). In brief, for *in vivo* induction, 5000 IU of hCG (Pubergen; Sanzyme Ltd., Shameerpet TS, India) dissolved in 100 µl of saline was loaded into an osmotic pump (ALZET® osmotic pumps, Cupertino, 116 CA, USA) and saline was used for control groups. Fishes were anesthetized with 100 mg/L of MS-222 (Sigma). A small incision of about 8mm was cut in the intraperitoneal region and saline or hCG loaded osmotic pump were then implanted into the peritoneal cavity close to the gonads. The incision was sutured using sterile 30 mm catgut and the fishes were carefully monitored for 3 days and then maintained in glass tanks. After the experimental period of 21 days, the fishes were sacrificed and samples were taken for analysis.

For *in vitro*, an adult male catfish was sacrificed during preparatory phase and the testis was dissected out under sterile conditions and kept it in ice-cold Leibovitz (L-15) culture medium (Sigma). Tissue slices of about 50 µm thickness were then cut from the testis using a McIIwain tissue chopper (Vibratome, Ted Pella Inc., Redding, CA, USA). The testicular slices were cultured in tissue culture plates to which ~2ml of L-15 medium were added. This medium was supplemented with sterile 10 mM HEPES buffer and an antibiotic mixture comprising of penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). The testicular slices were carefully maintained for ~24 h at ~20 °C. To each well, 100 IU/ml of hCG was added. For control groups, physiological saline was added. After incubation, the testicular slices were collected from control and hCG treated groups. Tissue collection was done at different time intervals of 2, 4, 6, 12, 20, and 24 h. The tissue samples were briefly washed in 1X PBS,

pH 7.4. Total RNA isolation was followed by cDNA synthesis and relative expression of *sycp3* was evaluated as previously described.

2.6 *In situ* hybridization (ISH)

ISH for localization of *sycp3* mRNA transcripts in testis of adult catfish was carried out based on the protocol described by Rajakumar and Senthilkumaran (2014) to. In brief, fixation of testis and sectioning was done in freezing medium as described for IF analysis. Sense and antisense 'cRNA' probes were synthesized using digoxigenin (DIG) RNA labelling mix (Roche) as per the instructions of the manufacturer. UFC3LKT column (Millipore) were used for purification of the cRNA probes and stored at -80 °C. Testis sections were prepared and washed with PBS-T (PBS with Tween 20-DEPC) and treated with proteinase K followed by 4 % PFA. About 1 µl each of purified probes (both sense and antisense) were diluted with 200 µl of hybridization buffer followed by denaturation for 5 min at 80 °C. The diluted probes were then pipetted on the slides and incubated at 50 °C in a sterile RNase free incubator for ~12 h. After incubation, blocking buffer solution (Roche) was used to wash the slides. Anti-DIG-ALP antibody was diluted 1:1000 in maleic acid buffer (Roche) and added to the slides and incubated for ~12 h at 4°C. The slides were washed repeatedly with DIG washing buffer (Roche) followed by incubation in detection buffer (Roche). BCIP-NBT (Roche) as substrates was used for colour development and counter stained with nuclear red. After colour development and washing, the slides were then dehydrated using a gradient series of alcohol. DPX mountant was used for fixed mounting. Images were obtained using Leica DM6 B digital research microscope (Leica Microsystems Gmbh, Wetzlar, Germany).

2.7 Polyclonal antibody generation for Sycp3

The deduced amino acid sequence of Sycp3 was used for commercial synthesis of an antigenic peptide, CLQQWETDVKKSEDQ, conjugated to keyhole limpet hemocyanin carrier protein (Biotech Desk Pvt. Ltd., Secunderabad, India). The peptide was dissolved in phosphate buffer saline (PBS; pH 7.4) and injected into three-month old New Zealand white rabbits. The rabbits were housed and handled with the specific approval and as per the guidelines of Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA No.151/1999 dt. 22.07.1999). Before peptide injection, blood was drawn from the lateral ear vein for collecting pre-immune serum. Subcutaneous injection was done with 500 μg of antigenic peptide combined with Freund's complete adjuvant. This was followed by two booster doses (250 μg) of antigenic peptide mixed with Freund's incomplete adjuvant administered 14 days apart. The serum was then collected and used for immunohistochemistry after checking titre and antibody specificity.

2.8 Immunohistochemistry and Immunofluorescence

Localization of Sycp3 protein using anti-Sycp3 antibody was performed with IHC in catfish testis sections. In brief, catfish testis was dissected out and fixation was done using Bouin's fixative (saturated picric acid: formaldehyde: glacial acetic acid, 15:5:1) for 3-4 hours. After dehydration of tissue with a gradient series of alcohol and xylene, embedding was done in paraplast (Sigma) and testis sections of 6 μm were cut using a rotary microtome (Leitz 1512, Holly, MI, USA) which were fixed on to glass slides. Deparaffinized tissues in xylene were hydrated through a graded series of alcohol. Endogenous peroxidase was blocking was done

with 0.1 % H_2O_2 added to the slides for 15 min at room temperature (RT). Washing of slides was done using PBS pH 7.4 (0.1 M) for 10 min and 10% normal goat serum (Bangalore Genei) in 0.1 M PBS was used for blocking (1 h at RT). Anti-Sycp3 polyclonal antibody (1:1000) was added to the slides, and incubated in a humid chamber at 4 ^{o}C overnight. Pre-adsorbed antibody was used for negative control. Following incubation, the slides were washed with 1X PBS with 0.1% Tween (PBST). The sections were then incubated with HRP conjugated secondary antibody (Bangalore Genei) for 2 hours at room temperature. VECTASTAIN® Elite ABC reagent (Vector Laboratories, Burlingame, Calif. USA) was added to the slides, and incubated at RT for approximately 30 minutes. Washing of slides was done with PBS. Colour development was then performed with 3′3′-diaminobenzidine (DAB) as chromogen and H_2O_2 (Vector Laboratories) as a substrate. Hematoxylin (Qualigens Fine Chemicals, Worli, Mumbai, India) was used as a counterstain, and the slides were again dehydrated with a gradient alcohol series followed by xylene, and fixed mounting with DPX Mountant. Micropublisher 3.3 RTV-CCD camera in CX-41 Olympus Microscope (Olympus Corporation, Tokyo, Japan) was used to obtain the pictures along with Q capture Pro 6 software (Quantitative Imaging Corporation).

For IF, testes were dissected out from adult catfish and sections were prepared as described earlier. The tissue sections were washed several times with 0.1M 1X PBS and then fixed for about 3 h in 4 % paraformaldehyde prepared in 0.1 M PBS. Repeated washing was done with 0.1 M PBS. The testis tissues were then immersed in OCT compound (Leia, Buffalo Grove, IL, USA) and stored in -80 ^{o}C freezer for about 2–4 h. Tissue sections of 7 μm were cut using cryostat (Leica CM1850). Blocking of the sections was done with 10 % normal goat serum followed by overnight incubation with primary Sycp3-antibody. Pre-adsorbed antibody was

served as negative control. After this, FITC-conjugated anti-rabbit secondary antibody was added to the testis sections and kept for 2-3 h at RT. IF images were then taken with IX81 Olympus Microscope (Olympus Corporation), containing Cell Sens dimension software.

2.9 *In vitro* esiRNA-PEI transfection

Using the cDNA of *sycp3* cloned from catfish, endonuclease prepared small interfering RNA (esiRNA) was commercially manufactured and acquired (Sigma). Preparation of primary testicular cell culture of catfish was done as per the procedure described by Murugananthkumar and Senthilkumaran (2016). In brief, the testicular culture was prepared prior to the day of transfection and 1 X 10^6 cells of the culture were plated into several wells of Corning® Costar® 24 well cell culture plates (Sigma). Culture medium comprising of DMEM, 10 % FBS, 1X Glutamax prepared with antibiotics/antimycotics was added to each well of the culture plate and incubation was done for 24-48 h at 37 °C with constant supply of 5 % CO_2. Prior to transfection, this medium was carefully pipetted out and OPTI-MEM® I (~100 µl each) was pipetted into each well. Control siRNA (MISSION siRNA Fluorescent Universal Negative Control #1, Cat. no. SIC007 conjugated with 6-FAM, Sigma, 10ng/ µl) and different concentrations of *sycp3*-esiRNA (10, 20, 40 and 100 ng/ µl) were incubated with branched PEI (MW 25 K, Sigma) in sterile HEPES-NaCl buffer, pH 7.4 for ~30 min at RT to form a PEI-esiRNA complex. The control siRNA/*sycp3*-esiRNA-PEI complex of different concentrations were then added to the respective wells and incubated for 12 hours. PEI alone as well as *sycp3*-esiRNA with no PEI was also added as additional controls to rule out any off target effects. After the transfection, the mixture esiRNA-PEI and OPTI-MEM® I were then discarded. Fresh culture media were then added for each well. The cells were then collected after incubation for another 24 h. Total RNA was isolated from all samples (control

siRNA/esiRNA/PEI/*sycp3*-esiRNA-PEI). Relative expression of certain testis related genes post transfection were analyzed, and calculated as explained before. To analyze the transfection efficiency, microphotographs of the cells transfected with control siRNA were obtained from an IX81 Olympus Microscope (Olympus Corporation) and Cell Sens dimension software.

2.10 *In vivo* esiRNA-PEI transfection

About 3µg of *sycp3*-esiRNA was combined with PEI (Sigma) that had been dissolved in HEPES-NaCl, pH 7.4 to form a PEI-esiRNA complex as per the protocol described by Höbel and Aigner (2010). It was then kept at room temperature for ~30 minutes to form an esiRNA-PEI complex and then used for transfection (Falco *et al*., 2009; Zhou *et al*., 2012). Adult (one year and ten months old) catfish (n=5) during prespawning phase were taken for the experiment. The fishes were anesthetized on ice before dissection with 100 mg/L solution of MS-222 (Sigma). The ventral region of the catfish was sterilized with ethanol, and a small incision of about 10mm was made before the pelvic fin junction with a sterile scalpel so as to expose the testis. The esiRNA-PEI complex was then slowly injected directly into the testis with a sterile 1 ml syringe. Control siRNA-PEI complex or PEI was injected for control groups. The incision was carefully sutured with sterile 30 mm catgut and treated with antibiotics. The animals were monitored for 3 days in isolated tanks, and tissue samples were taken at different intervals by sacrificing the fishes at approximately 48 and 72 hours post transfection (to analyze the duration of the siRNA silencing that occurred *in vivo*) for qPCR analysis (snap frozen using liquid nitrogen and stored in -80^{o} C).

2.11 Statistical analysis

All the data were expressed as mean ± standard error of mean (SEM). Significance among groups was tested by ANOVA followed by Student's–Newman–Keuls' test using SigmaPlot 12.0 software (Systat Software Inc., Chicago, IL, USA). Differences amongst various groups were considered significant at $P < 0.05$.

3. Results

3.1 Identification of Sycp3 by two-dimensional gel electrophoresis and MALDI-TOF/TOF-MS analysis

To screen the differentially expressed proteins during gonadal development, 2D-electrophoresis was performed in order to isolate the differentially expressed spots. The 2D analysis was done a minimum number of three times and adult fish used were approximately 1 year old with fully matured testis and ovary, while for juvenile fish, gonads of <100 dph fish were pooled and used since the testes and ovaries are still not fully matured at these stages. This was important in identifying factors which come into play during the process of germ cell and gonadal development. For this particular experiment, juvenile fish testis of 70-100 dph were pooled (n=20) and juvenile fish ovaries of 50-80 dph were pooled (n=5) for protein extraction and for comparative analysis between adult and juvenile gonads, the same concentration of total protein extracted from each tissue were used. The comparative analysis between the 2D gel images of adult and juvenile testis and ovarian tissues showed many differentially expressed protein spots (Figure 1). Differential expression was calculated in fold changes, and only the protein spots which had fold changes greater than 2 were considered significant and selected for MALDI analysis. Lower molecular weight proteins

(>100 kDa) were favoured, to narrow down the search for factors essential for gonadal development considering encoded nucleotide sequence length**.** In males, among the total spots detected with Image Master 2 D Platinum (GE Healthcare), we isolated four spots which had significant differential expression in juvenile testis, taking adult testis as control. Among these, two spots were downregulated (DR1, DR2) in juvenile testis, and two spots were upregulated (UR1, UR2). In females, although several differentially expressed proteins were also identified, there were no factors that were directly linked to gonadal development as well as recrudescence except for vitellogenin, which is already an extensively studied protein in oogenesis. Hence, the work was more disposed towards factors identified in male fish. The list of proteins identified in female and male fish using MALDI-TOF analysis are shown in Table 2 and 3, respectively. Among these, one upregulated spot in juvenile testis (UR1) was identified as Sycp3, which was selected for further investigation as it is related to meiosis as per the aim of our study. The three other sports identified are P2X purinoceptor 1-like protein, annexin A and hemoglobin cathodic subunit alpha, which showed little or no evidence for their role in gametogenesis.

3.2 Molecular cloning of *sycp3* from catfish testis

To analyze the expression profile of the protein upregulated in juvenile testis, the *sycp3* gene of catfish was characterized. Cloning of a partial cDNA of 372 bp of *sycp3* from juvenile catfish testis was performed by RT-PCR with degenerate primers that were designed based on the Sycp3 peptide sequence data obtained by 2D MALDI-TOF/TOF mass spectrophotometry. RACE strategy was used to obtain both 5′ and 3′ ends, to get the full length cDNA of 723bp. The obtained ORF encodes a putative protein of 241 amino acids. The full length cDNA of *sycp3* was obtained by alignment of the partial cDNA sequences

and RACE cDNAs with Lasergene software. Details of the primers used are listed in Table 1. Sequence analysis revealed the presence and high homology of the peptide sequence identified through 2D proteomic and MALDI-TOF analysis.

3.3 Multiple sequence alignment and phylogenetic analysis

Multiple sequences of deduced amino acids were aligned using the Sycp3 sequences of various mammals and teleost species with the ClustalO alignment software from European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/msa/clustalo/) and phylogenetic analysis was done using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny) by neighbour-joining method (Figure 2 and 3). The different GenBank accession numbers used for Sycp3 are: *Homo sapiens* (NM_001177949.1), *Mus musculus* (NM_011517.2) *Xenopus tropicalis* (NM_001078758.1), *Danio rerio* (NM_001040350.1) *Monopterus albus* (AJP00088.1), *Oreochromis niloticus* (XP_003439417.1), *Onchorynchus mykiss* (NP_001117979.1), *Oryzias corvinotus* (BAE47002.1).

3.4 Expression analysis of *sycp3* in tissue distribution, reproductive cycle and during ontogeny in catfish

Tissue distribution of *sycp3* revealed exclusive expression in gonads of both male ($P < 0.01$) and female ($P < 0.01$) when compared to other tissues analyzed such as liver, kidney, brain, muscle and heart (Figure 4A and B, respectively). The expression of *sycp3* was found to be significantly ($P < 0.05$) elevated during the preparatory phase of the reproductive cycle of catfish, and moderate expression was found in the prespawning and spawning, while there was minimal expression during the resting phase (Figure 5). In the ontogeny study (Figure 6),

there was a stage-dependent increase in *sycp3* expression observed in testis at different age groups of catfish, with significantly ($P < 0.05$) elevated expression levels seen at 50 dph in the gonads of both male and female catfish. This high expression of *sycp3* is comparable to preparatory phase results where recrudescence of gonadal development is evident in annually reproducing teleosts.

3.5 ISH of *sycp3* in catfish testis

ISH to localize *sycp3* mRNA transcripts was performed in testis sections of adult catfish. Positive signals were detected in spermatogonia as well as in spermatocytes (Figure 7A - C). Sense probe as a negative control did not show any signal (Figure 7D), thereby signifying the specificity of the antisense probe used.

3.6 Immunolocalization of Sycp3 in testis of catfish

Localization of Sycp3 was done in testis of adult catfish using IHC and IF during late preparatory phase (Figure 8 and 9, respectively). Strong immunoreactivity was detected for sycp3 in the developing spermatogonia and spermatocytes (Figure 8A – C; 9A – C), while mature sperm/spermatids did not show any immunoreactivity (counterstained with hematoxylin). The negative controls with preabsorbed antibody of Sycp3 did not show any positive signals (Figure 8D and 9D). A similar result was also found in IF detection, where positive immunoreactivity were detected in the spermatogonia and spermatocytes.

3.7 Effects of *in vivo* and *in vitro* hCG induction on sycp3 expression

The expression of *sycp3* was found to be significantly upregulated ($P < 0.05$) after hCG induction in adult male catfish as compared to the control samples. In the *in vivo* induction,

an elevated expression of *sycp3* was observed after the period of induction, when compared to control (Figure 10A). For the *in vivo* study (Figure 10B), a significant elevation of *sycp3* expression was detected from 6 hours onwards when compared to the control samples analyzed.

3.8 *In vitro* transfection of *sycp3*-esiRNA in catfish testicular culture

In the *in vitro* transfection experiment (n=5), PEI mediated *sycp3*-esiRNA transfection indicated significant ($P < 0.05$) reduction in the expression levels of *sycp3* when compared to other control groups (Figure 11A). In addition, the expression levels *sycp3* post esiRNA transfection showed that downregulation varied with different doses of esiRNA transfection. The expression level of *sycp3* was significantly ($P < 0.05$) decreased by 65 % post transfection with 100 ng/µl of *sycp3*-esiRNA when compared to control, and this dose was selected for further experiments. The transient knockdown of *sycp3* showed a significant ($P < 0.05$) decrease in the expression levels of other important testis-related genes when compared to control (Figure 11B). The relative mRNA levels of *vasa*, *dmrt1*, *sox3*, *sox9* and *wt1* showed a decrease of 41, 71, 20, 23 and 82 % respectively, while there was no observable significant difference for *c-kit* in comparison to control. In addition, there were no significant changes observed in *sycp3* expression levels in primary testicular culture cells transfected with PEI alone and esiRNA without PEI, when compared to control (Figure 11A) which infers that both PEI alone nor esiRNA without PEI did not alter the gene expression levels. The primary testicular culture transfected with control siRNA survived normally (Figure 12A) and transfection of control siRNA with PEI in catfish primary testicular culture showed fluorescent signals confirming siRNA uptake in the cells (Figure 12B and C).

3.9 *In vivo* transfection of *sycp3*-esiRNA in adult catfish

Initially, PEI without any siRNA was injected into the testis of adult catfish, and tissue samples were collected for quantification of *sycp3* mRNA with qPCR. These were analyzed and compared with the gene expression levels in control siRNA (Sigma) injected as well as *sycp3*-esiRNA-PEI complex injected animals. There were no significant changes observed in the levels of *sycp3* in the PEI-only injected catfish (n=5) when compared to control which infers that neither PEI alone nor control siRNA did not alter the gene expression levels. However, a combination of PEI with *sycp3*-esiRNA injected into the testis significantly ($P < 0.05$) decreased the expression of *sycp3* mRNA expression levels in comparison with other groups (Figure 13A) which indicated that PEI mediated esiRNA transfection into the testis was capable of silencing *sycp3* mRNA expression. Expression levels of *sycp3* were also quantified for the tissues collected at two separate time intervals, 48 and 72 hours post transfection and compared to control groups at each time point (Figure 13B). The PEI mediated *sycp3*-esiRNA transfection resulted in significant decrease ($P < 0.05$) in the levels of *sycp3* expression both at 48 and 72 hours post transfection with 63 and 54% decrease respectively when compared to control. The percentage decrease had been found to be lowered after 72 hours indicating that transcripts might get restored to normal levels as shown earlier by Murugananthkumar and Senthilkumaran (2016). Histological HE stained images of testis injected with esiRNA compared to control (Figure 14) showed the transient nature of the PEI-esiRNA transfection was not enough to cause any significant phenotypic change post transfection. Testis morphology was not affected significantly, and sperm/spermatids were observed to develop as normal. Hence, transient knockdown may not impart phenotypic changes unlike gene based permanent knockdowns that can impart phenotypic changes.

In addition, the expression levels of various genes which are crucial for germ cell development were also analyzed with qPCR after 48 and 72 hours *sycp3* esiRNA silencing in the catfish testis as well as in the control groups (Figure 15). Various transcription factor genes such as *dmrt1*, *sox3*, *sox9* and *wt1* as well as RNA-binding protein *vasa*, which all play crucial roles in germ cell development and spermatogenesis, showed a significant downregulation ($P < 0.05$) in expression levels at both 48 and 72 hours after transfection. Tyrosine kinase receptor *c-kit* however did not show any significant downregulation. There were not much difference seen in the expression levels between 48 and 72 hours for most genes, except for *sox3* whose levels reverted back to normal at 72 hours.

S. No	Primer name	Primer sequence 5'-3'	Usage	GenBank Accession number
1.	*sycp3* F2	GAACATRGAGGARAYGGAG	RT-PCR	KY553233
2.	*sycp3* R	CCATCTCYTGYTGTTGHGYKTCC		
3.	5P	AGCAGAGCCATCGCCTTCTTCAGCTCC	RACE	
4.	5N	CTCCCACTGCTGAAGCACTGACAGCACC		
5.	3P	GGTGCTGTCAGTGCTTCAGCAGTGGGAG		
6.	3N	GGAGCTGAAGAAGGCGATGGCTCTGCT		
7.	*sycp3* RTfw	CCCAGTATAGCCAGAGGCAGAAGCTG	qPCR	
8.	*sycp3* RTR	GAACATGGAGGAGATGGAGAAGAGTC		
9.	*18s rRNA* F	GCTACCACATCCAAGGAAGGCAGC	qPCR	KM018296
10.	*18srRNA* R	CGGCTGCTGGCACCAGACTTG		
11.	*ckit* RTfw	GCACTGTTGGAGGTCCGACTCTGGTG	qPCR	submitted
12.	*ckit* RTrv	GGTGCTGGCTCTTACACAGACTGTG		
13.	*vasa* RTfw	GGTCTGAGTAAAGTTCGTTATCTG	qPCR	GU562470
14.	*vasa* RTrv	GCCAACCTTTGAATATCCTCTG		

15.	*dmrt1* RTfw	ATGGCCGCTCAGGTGGCTCTGCGG	qPCR	FJ596554
16.	*dmrt1* RTrv	GCGGCTCCCAGAGGCAGCAGGAGA		
17.	*sox3* RTfw	CACGGTATGAGTAGCCCACCA	qPCR	HQ680982
18.	*sox3* RTrv	GCGATGGCAGGTGGTGGTGAG		
19.	*sox9* RTfw	TCTGGCGGCTGCTGAATGAAGG	qPCR	HM149258
20.	*sox9* RTrv	CTCGGTATCCTCGGTTTCACC		
21.	*wt1* RTfw	ACGCGCACAGGGTGTTCGA	qPCR	JF510005
22.	*wt1* RTrv	GGTACGGTTTCTCTCCTTGTG		

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

Sl. No.	Protein name	Molecular weight in kDa	Accession number	Species identified	Score
1.	Phosphoglycerate kinase	41.6	gi|432901128	*Oryzias latipes*	62
2.	Creatine kinase	43.6	gi|124358786	*Fundulus heteroclitus*	62
3.	Alcohol dehydrogenase	36.9	gi|308321630	*Ictalurus furcatus*	77
4.	Vitellogenin	148.7	gi|159459934	*Clarias macrocephalus*	110
5.	Golgi-apparatus protein 1-like	130.3	gi|348545663	*Oreochromis niloticus*	41
6.	14-3-3 protein	22.6	gi|45602838	*Carassius auratus*	178
7.	Ring-finger 213 protein	23	gi|328797754	*Amblygobius phalaena*	41
8.	Somatotropin	23.2	gi|17368853	*Perca flavescens*	37

Table 2. List of identified protein spots by 2D-gel electrophoresis followed by MALDI-TOF/TOF from adult and juvenile ovary of female catfish.

Name of spot	Expression pattern	Molecular weight in kDa	Protein name	Accession number	Species identified	Score
UR1	Upregulated	27.9	synaptonemal complex protein 3	gi|217416450	*Onchorynchus mykiss*	60
UR2	Upregulated	47.5	P2X purinoceptor 1-like protein	gi|348541227	*Oreochromis niloticus*	55
DR1	Downregulated	35	Annexin A	gi|225705972	*Osmerus mordax*	45
DR2	Downregulated	15.3	Hemoglobin cathodic subunit alpha	gi|115502083	*Tetradon nigroviridis*	57

Table 3. List of identified protein spots by 2D-gel electrophoresis followed by MALDI-TOF/TOF from adult and juvenile testis of male catfish.

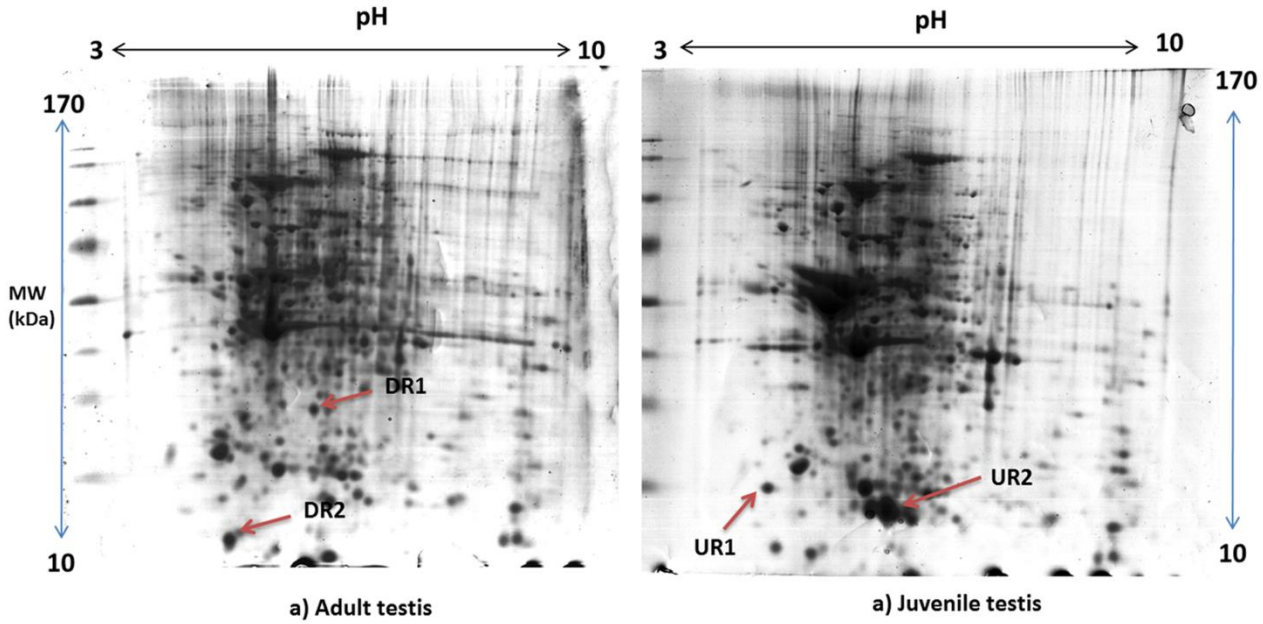


Fig. 1. Representative two-dimensional electrophoresis gels of adult and juvenile testis of catfish with adult testis taken as control. Spots indicated by arrows were either upregulated (UR1, UR2) or downregulated (DR1, DR2) in juvenile catfish. Spots identified are

synaptonemal complex protein 3 (UR1), P2X purinoceptor 1-like protein (UR2), Annexin A5 (DR1) and Hemoglobin cathodic subunit alpha (DR2).

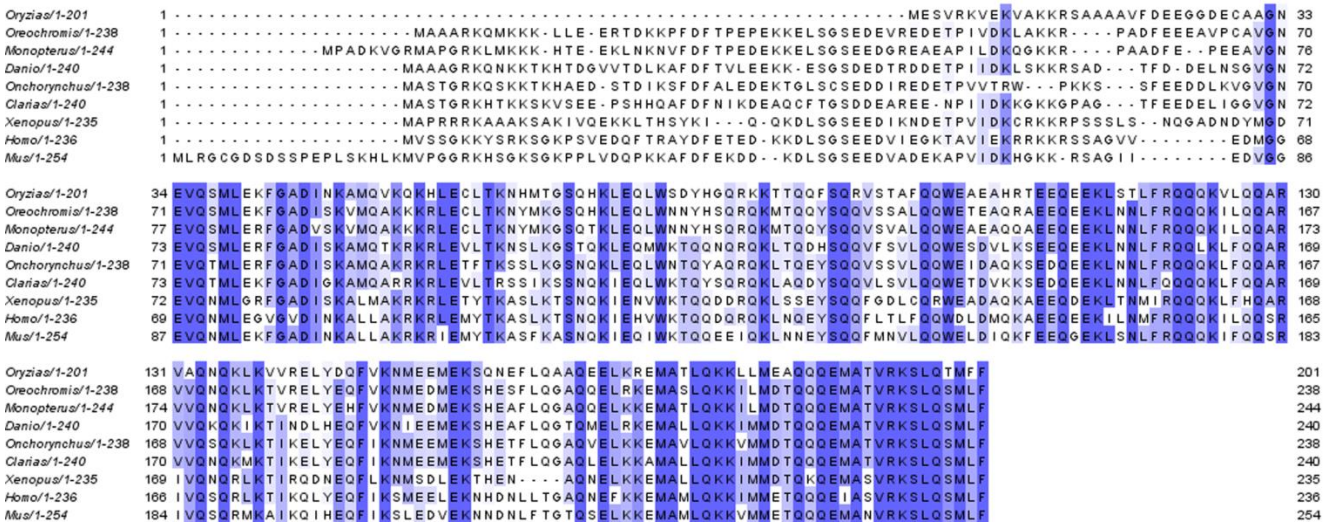


Fig. 2. Multiple sequence alignment of catfish Sycp3 with other mammalian and teleost species: *Homo sapiens* (NM_001177949.1), *Mus musculus* (NM_011517.2) *Xenopus tropicalis* (NM_001078758.1), *Danio rerio* (NM_001040350.1) *Monopterus albus* (AJP00088.1), *Oreochromis niloticus* (XP_003439417.1), *Onchorynchus mykiss* (NP_001117979.1), *Oryzias corvinotus* (BAE47002.1) using the ClustalO alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/)

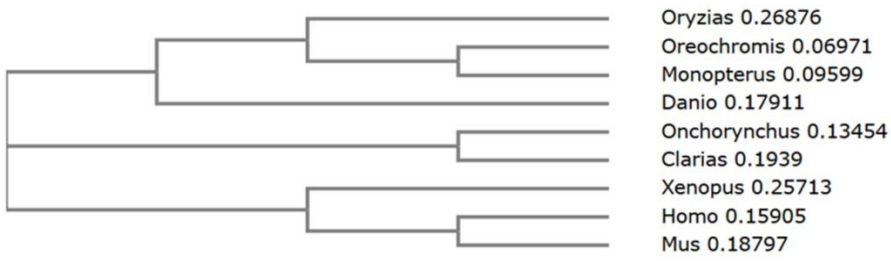


Fig. 3. Phylogenetic analysis of catfish Sycp3 with other mammalian and teleost species. The analysis was done using ClustalW2 by neighbor-joining method (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny).

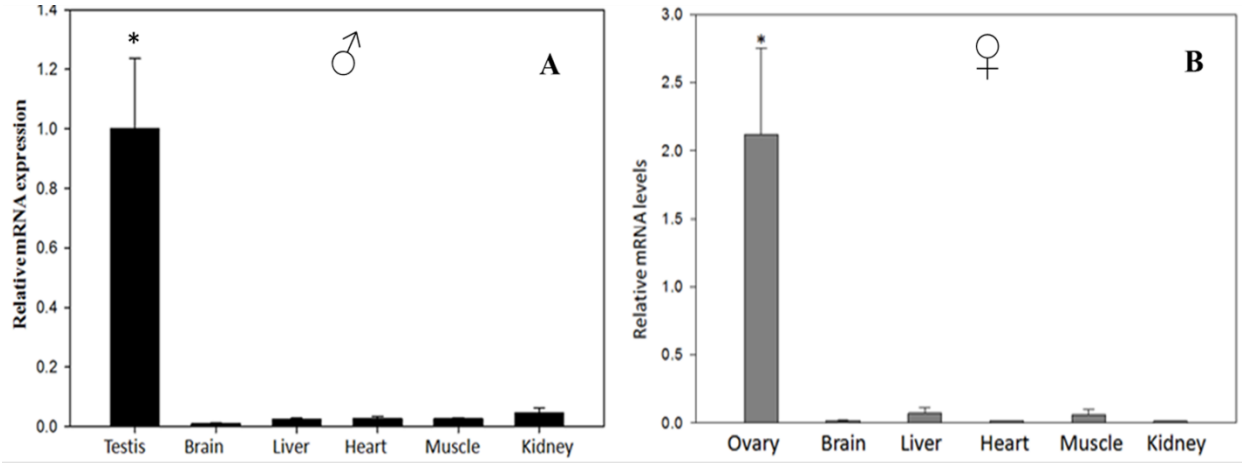


Fig. 4. qPCR analysis of *sycp3* expression in various tissues of catfish in male (A) and female (B). The relative expression of *sycp3* was normalized with *18S* rRNAand the values were calculated using $2^{-\Delta ct}$ method. Data (n = 5) were expressed as mean ± SEM. (*, $P < 0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

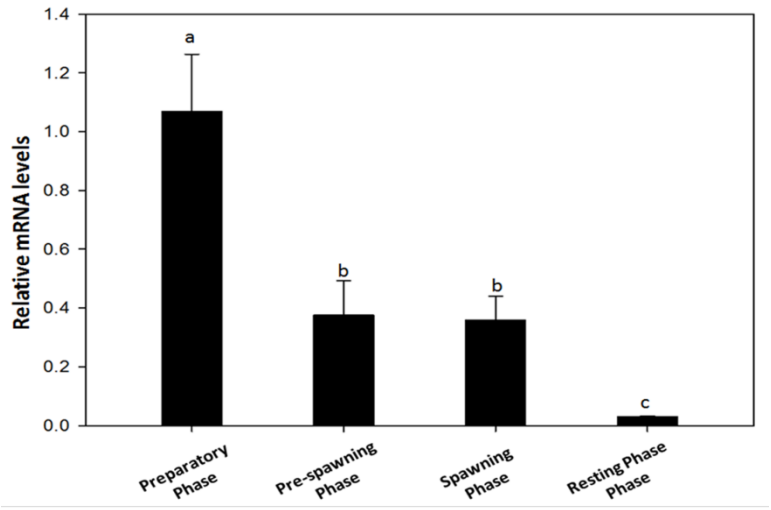


Fig. 5. qPCR analysis of *sycp3* expression during different phases of the testicular cycle in catfish, *Clarias gariepinus*. Data (n = 5) were expressed as mean ± SEM. Mean with different letters differs significantly while mean with same letters are not significant ($P< 0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

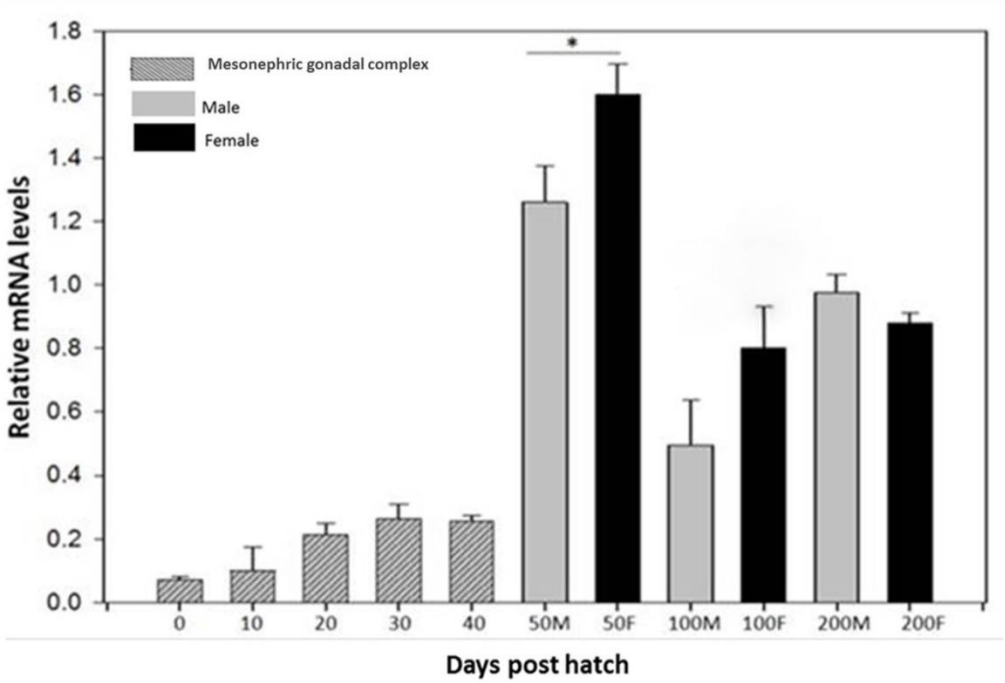


Fig. 6. qPCR analysis of *sycp3* expression during various stages of development of catfish. Data (n = 5) were expressed as mean ± SEM. (*, $P < 0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

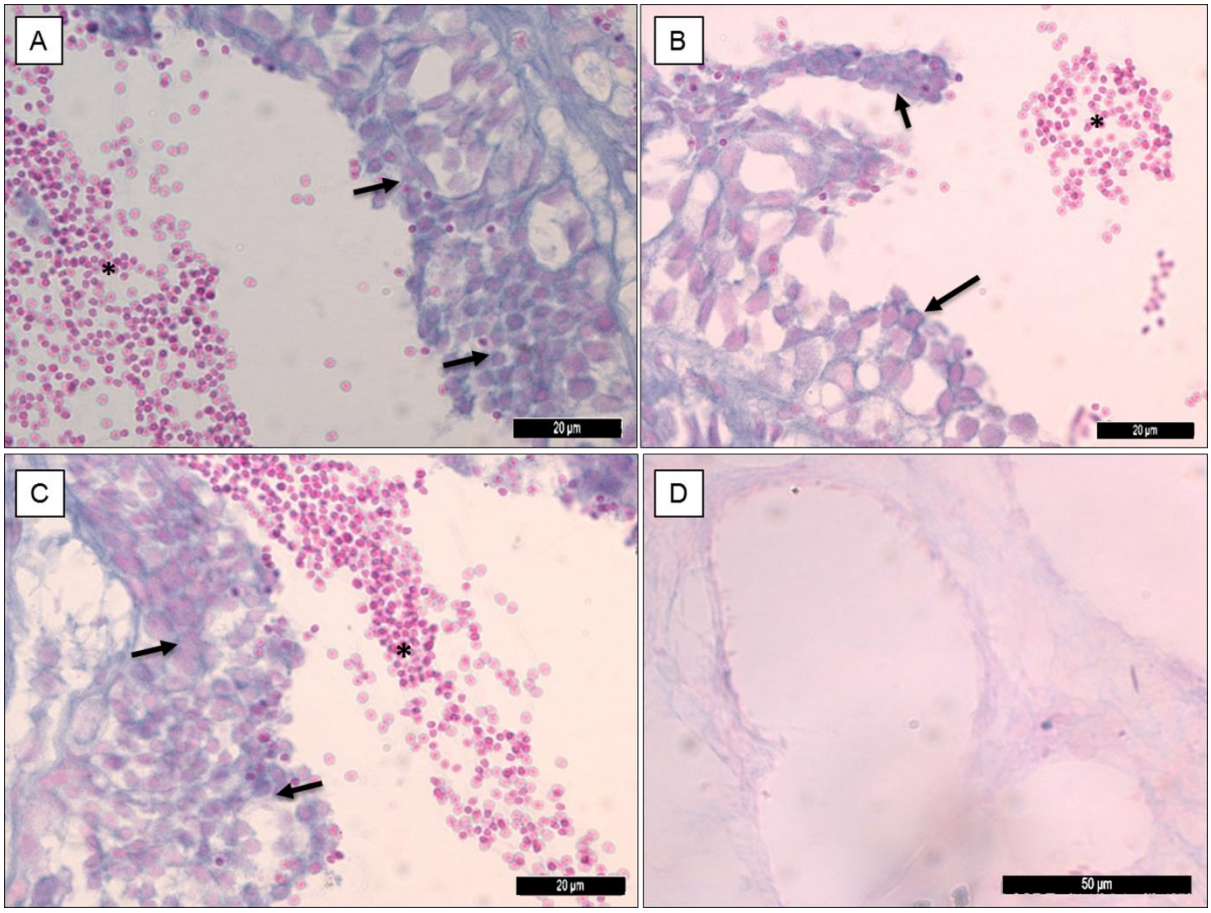


Fig. 7. Localization of *sycp3 m*RNA in adult testis of catfish by *in situ* hybridization. Testis of adult catfish displayed positive signals in spermatocytes and spermatogonia indicated by black arrows (A - C). Signals were not detected in mature sperm/spermatids (*). Sense probe of *sycp3* did not show any signal (D). Counterstain was done with nuclear red for antisense slides. Scale bars indicate A - C: 20µm; D: 50 µm

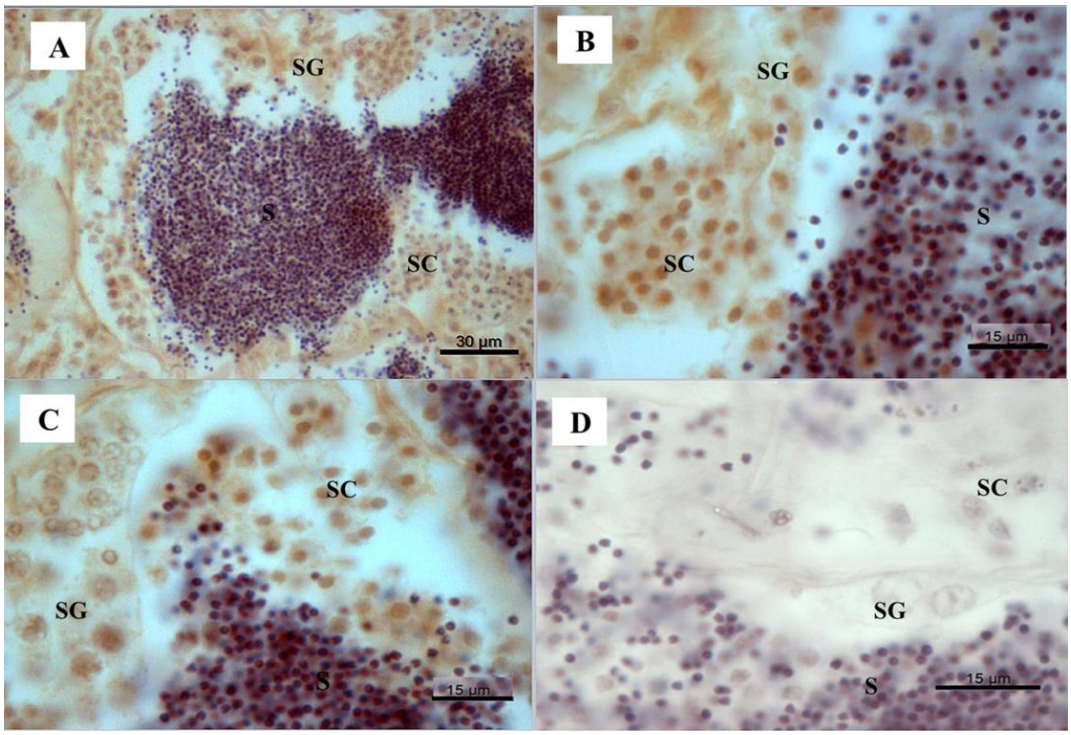


Fig. 8. Localization of Sycp3 protein in catfish testis. Immunoreactivity was observed in spermatogonia and spermatocytes (A-C), while negative control (D) did not show any signal (pre-adsorbed antibody). Abbreviation : SG- Spermatogonia, SC- Spermatocytes, S- Sperm/spermatids. Scale bars indicate A: 30µm, B, C and D: 15µm

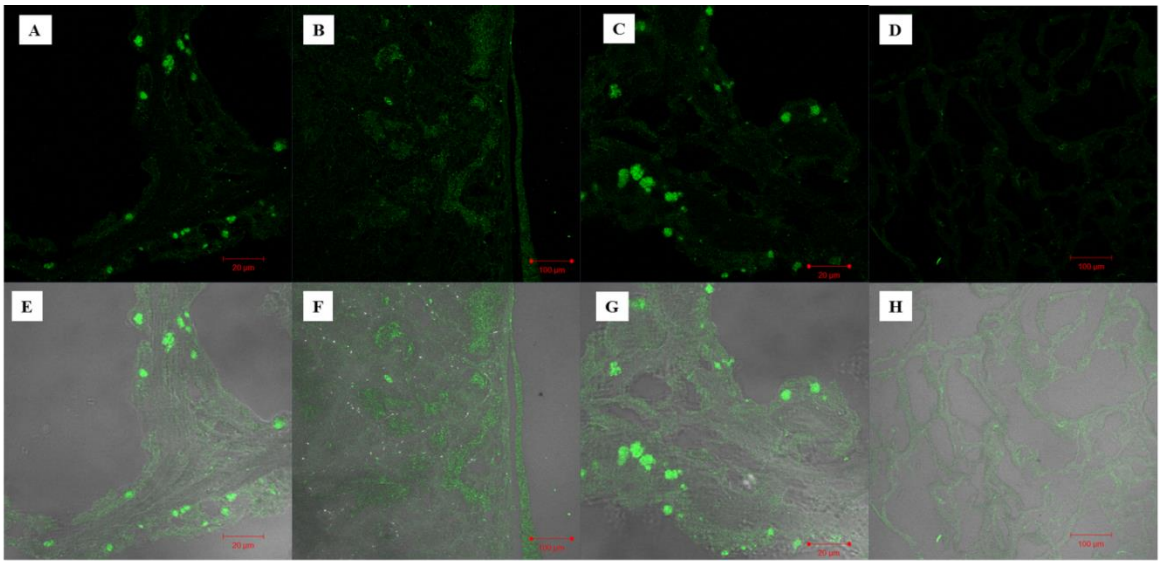


Fig. 9. Localization of Sycp3 protein in catfish testis using immunofluorescence stained with FITC which indicates positive signals for spermatogonia and spermatocytes (A-C) compared to negative control (D). E-H represent the corresponding phase contrast images. Scale bars indicate, A, C, E, and G: 20µm; B, D, F and H: 100µm

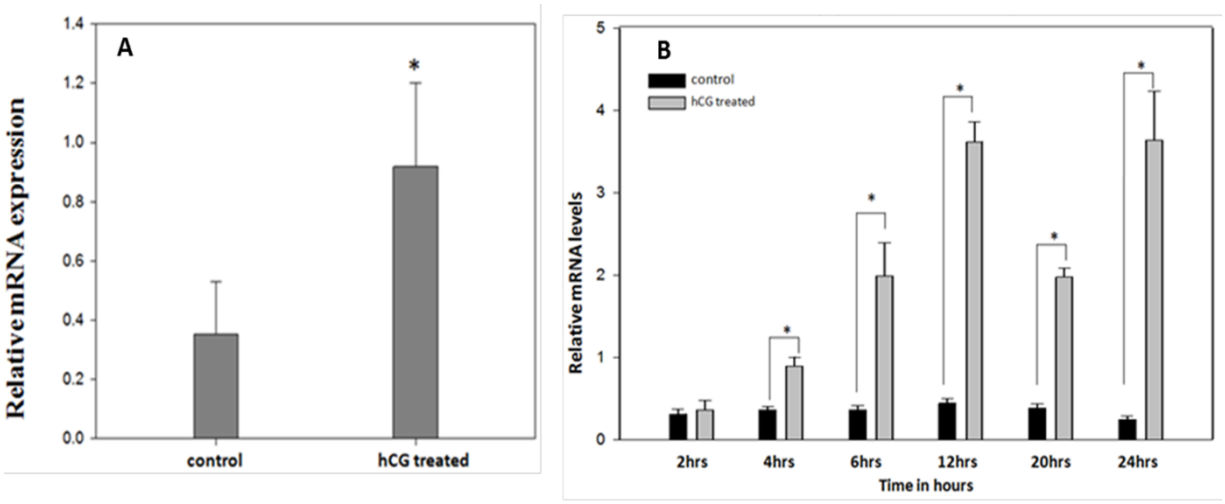


Fig. 10. qPCR analysis showing *sycp3* mRNA levels *in vivo* (A) and the testicular slices treatment with hCG *in vitro* (B) at different time intervals. *Sycp3* mRNA levels were significantly increased after hCG induction both *in vivo* and *in vitro* compared with the 0 h time point. Data (n = 5) were expressed as mean ± SEM. (*P< 0.05; ANOVA followed by Student-Newman-Keuls' post hoc test).

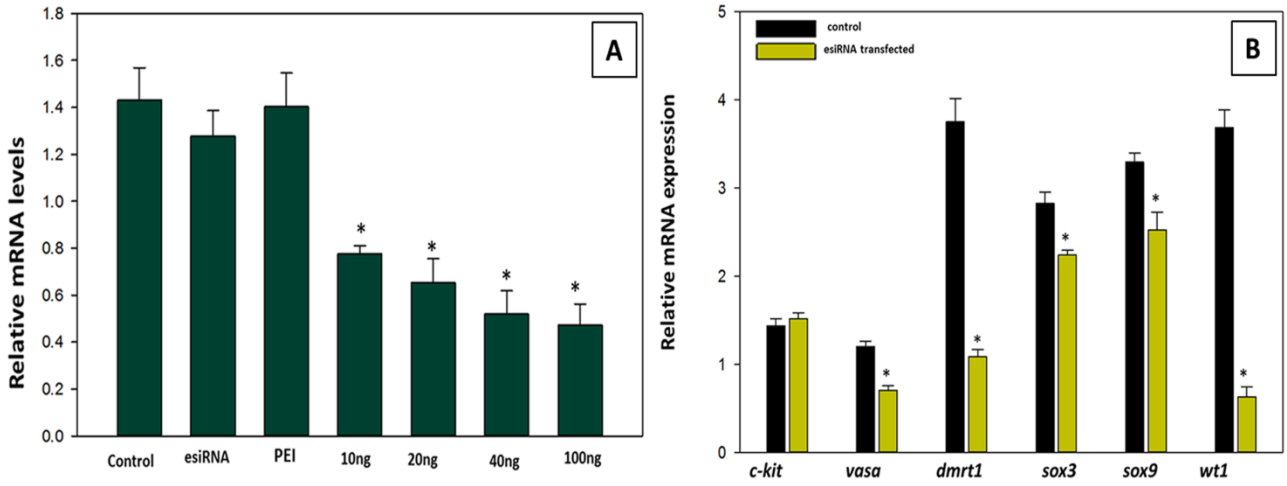


Fig. 11. *In vitro* transfection of PEI mediated *sycp3*-esiRNA in catfish testicular culture (mixed; n=5). Relative expression of *sycp3* was analyzed and was observed to be dose dependent after transfection with *sycp3*-esiRNA (A) and *sycp3* transcripts were quantified and compared between control siRNA, *sycp3*-esiRNA and PEI + esiRNA groups. Expression levels of certain testis related genes (B) were analyzed between control and *sycp3*-esiRNA transfected cultures. (*, $P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

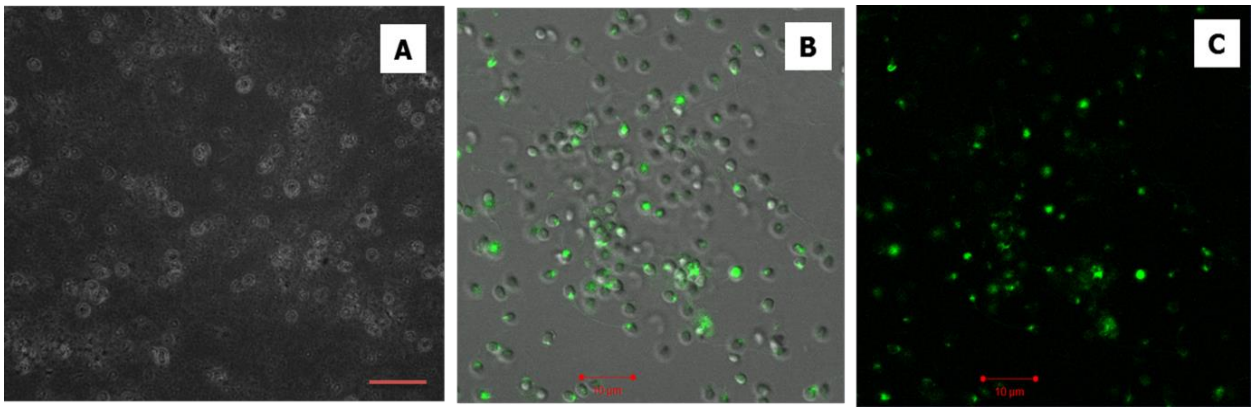


Fig. 12. Representative phase-contrast image of catfish primary testicular cells (A) and confocal microscope images (B and C) after PEI mediated transfection of control siRNA on catfish testicular culture (mixed) to check the efficiency.

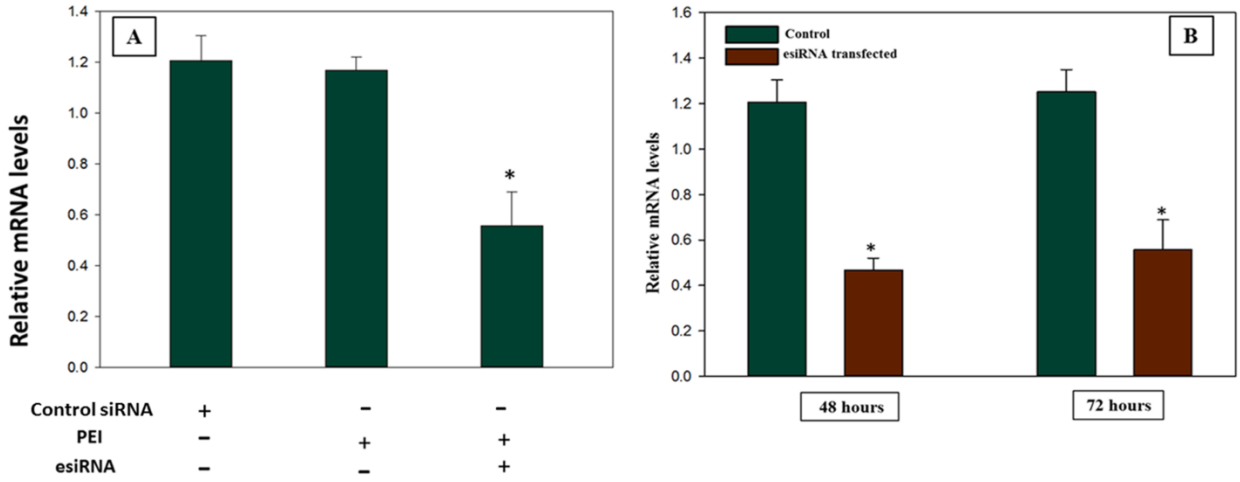


Fig. 13. qPCR analysis showing the *sycp3* levels after esiRNA transfection. (A) *In vivo* PEI mediated *sycp3*-esiRNA transfection in adult catfish (n=5). (B) Comparison of *sycp3* gene expression levels between control and *sycp3*-esiRNA-PEI complex transfected samples at different time points, i.e, 48 and 72 hours post transfection. (*, $P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

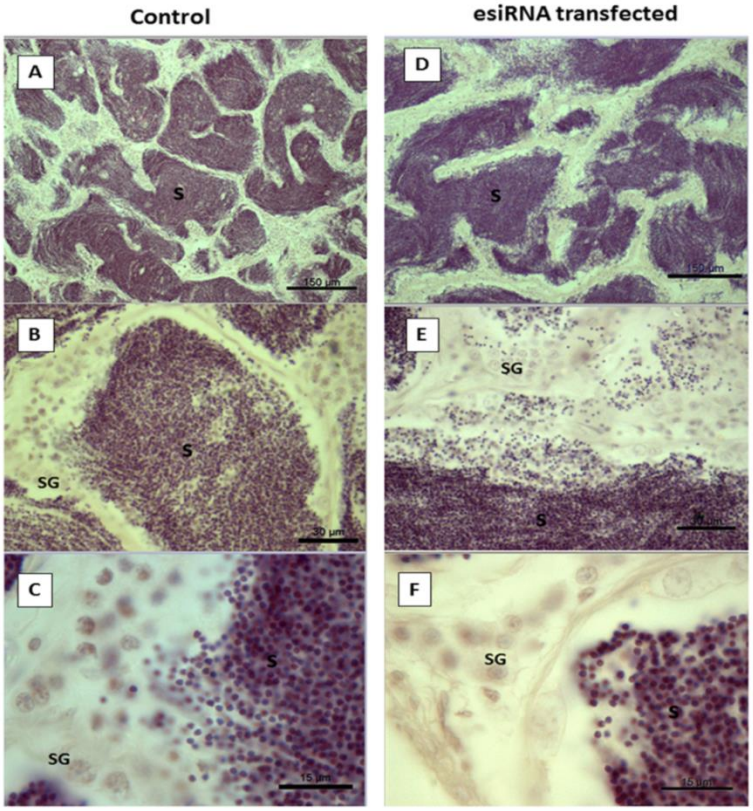


Fig. 14. Hematoxylin and Eosin (HE) staining of catfish testis between control siRNA and *sycp3*-esiRNA transfected tissues. In both control and esiRNA transfected tissues, the testis appeared to develop normally, and the lumen region was intact (A, B, D, E), filled with mature sperm/spermatids and developing spermatogonia (C, F). Abbreviation : SG- Spermatogonia, S- Sperm/spermatids. Scale bars indicate- A and D: 150µm; B and E: 30µm; C and F: 15µm.

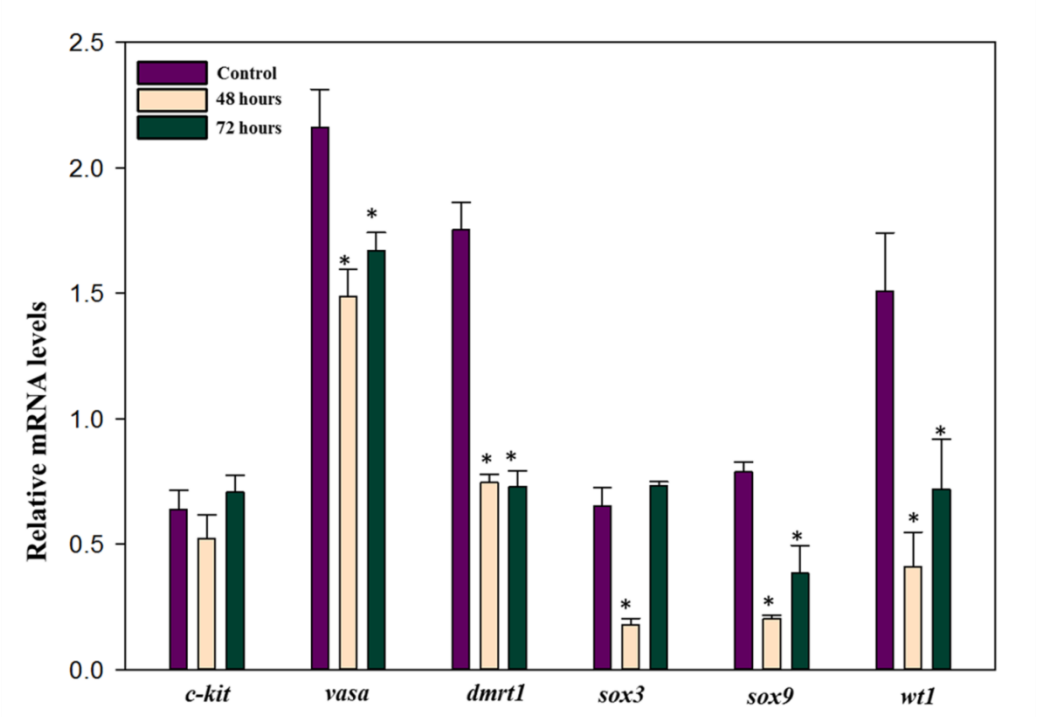


Fig. 15. Expression analysis of various genes which are crucial for germ cell development and spermatogenesis in the testis after *sycp3* esi-RNA silencing. Expression levels of each gene is compared to their respective control levels. (*, P < 0.05; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

4. Discussion

In this study, Sycp3 was identified from catfish testis using two-dimensional proteomic analysis, and by using the peptide sequence data the full length cDNA was cloned using RT-PCR and RACE strategy. Expression analysis was done during development stages, reproductive phases and after *in vivo* and *in vitro* induction with hCG. Using IHC and IF, localization of Sycp3 protein was found specifically in the spermatogonia and spermatocytes. ISH also revealed the presence of mRNA transcripts in the same type of testicular cells.

We had used a comparative proteomics approach to scan for key genes pertaining to germ cell development and meiosis during juvenile and adult stages of testis to set a more detailed insight into factors involved during critical stages of gonadal development, and spermatogenesis in particular (Chocu *et al*., 2011). Tissue distribution analysis demonstrated a tissue specific expression of *sycp3* in catfish testis, when compared to other tissues such as brain, liver, heart, kidney and muscles. This pattern of expression was also observed in female catfish, where *sycp3* expression was restricted to ovary.

The elevated levels of *sycp3* expression during preparatory phase as well as 50 dph onwards was also in correlation with the onset and progress of spermatogenesis in catfish, which implies that this gene is involved in germ cell development and gametogenesis during sexual development and recrudescence. There were minimally detectable levels of *sycp3* transcripts from 20 to 40 dph, and increased dramatically from 50 dph, which correspond to the various development stages of gonads as well as sex differentiation in catfish (Raghuveer *et al*., 2011) leading to an elevated number of germ cells, that was also seen in the levels of germ cell marker *vasa* (Raghuveer and Senthilkumaran, 2010). In the teleost, *Oreochromis niloticus*, the entry of germ cells into meiosis *in vivo* was found to occur in females around 35 dph, while in male gonads, meiotic cells were found to be differentiated around 85 dph (Kobayashi, 2010). In the protandrous black porgy *Acanthopagrus schlegelii,* it was observed that during gonadal development, the germ cells located in the ovary entered meiosis earlier when compared to the testis, and treatment with estradiol-17β (E2) that induced *cyp26* expression was found to decrease *sycp3* mRNA in the gonads (Lau *et al.*, 2013). Higher expression levels of *sycp3* in female catfish just after gonadal differentiation might indicate meiosis entry.

IHC and IF showed that Sycp3 was mainly localized in primary and secondary spermatogonia as well as spermatocytes, but not in sperm/spermatids. The same pattern of localization was also evident for *sycp3* transcripts in the ISH results, with expression seen mostly in developing spermatogonia and spermatocytes. This corroborates the results observed in zebrafish, where *sycp3* has been established as a molecular marker for spermatocytes in the testis, at different meiotic stages (Ozaki *et al*., 2011). Also in medaka, *sycp3* was expressed in primary spermatocytes but was not detected in secondary spermatocytes and spermatogenic cells, and signals were found to be diminished at the advanced meiotic stages (Iwai *et al*., 2006) which show a slight difference in the localization pattern observed in catfish. The expression patterns of *sycp3* have been examined in just a few fish species as most studies have been done in mammals. It is essential to reveal the expression patterns of germ cell/meiotic regulators in the reproductive systems of lower vertebrates. Considering this, the present study is the first of its kind to perform a dual approach with proteomics and genomics to corroborate the role of *sycp3* in gonadal development and recrudescence spanning two years of reproductive cycle.

In order to observe the impact of gonadotropins on the expression of *sycp3*, hCG induction was done by both *in vivo* and *in vitro* methods. The fishes used for both induction experiments were in late preparatory/pre-spawning phase, and hCG was found to upregulate the expression levels of *sycp3* even at those phases where the normal levels of this gene was found to be low. This might serve as an indication to infer that gonadotropin stimulates germ cell proliferation during recrudescence in other fishes like *Clarias batrachus* which have a much shorter breeding season (Rajakumar and Senthilkumaran, 2014) and upregulation of *sycp3* expression indicate its correlative response. The influence of androgens on this gene is

also being investigated and is still in progress. The expression level of the gene was found to be elevated after both types of induction. The processes of meiosis and the completion of various stages of spermatogenesis is dependent primarily on androgens, mainly 11-ketotestosterone in teleosts, which is produced by the Leydig cells under the influence of luteinizing hormone (LH), and secondarily dependent on the influence of follicle-stimulating hormone (FSH) which also facilitates pre-meiotic germ cell development (Schulz *et al*., 2001). However, the exact molecular mechanisms by which gonadotropins support the testicular production of steroids and gametes in lower vertebrates are less understood and the identification of the specific genes involved has proven difficult. Our study implies that *sycp3* may be directly or indirectly regulated by gonadotropins, although the exact mechanism has not yet been elucidated. In addition, the expression of *sycp3* during the testicular reproductive cycle correlated well with the levels of gonadotropins observed in catfish (Joy *et al*., 2000; Kirubagaran *et al*., 2005).

PEI mediated esiRNA silencing both *in vitro* and *in vivo*, has been well demonstrated for analysing transient effects in many organisms/vertebrates (Höbel and Aigner, 2010, 2013). In catfish, PEI mediated esiRNA silencing has been established in testis, ovary and brain (Prathibha and Senthilkumaran, 2016; Murugananthkumar and Senthilkumaran, 2016; Sudhakumari *et al*., 2017). The analysis also demonstrated that transfection of catfish testis with PEI mediated esiRNA targeted to *sycp3* significantly downregulated its expression levels both *in vitro* and *in vivo*. Further, the transient knockdown of this gene also resulted in the alteration of expression levels of various genes critical for germ cell and gonadal development such as *vasa*, *dmrt1*, *sox3*, *sox9* and *wt1*. The germ cell marker gene, *vasa*, as well as transcription factors *dmrt1*, *sox9* and *wt1* showed a significant decrease in their

expression levels after 48 and 72 hours, while *sox3* was transiently downregulated at 48 hours, but returned to normalcy at 72 hours. However, *c-kit* did not show any change in expression. Most of the knockdown/mutation experiments towards investigating the impact of the absence of this gene during gametogenesis had been done in mammals, but very few attempts were made in lower vertebrates including teleosts. In one such study, the targeted deletion of this gene in mice resulted in perturbed processes such as synaptic failure of chromosomes at meiotic prophase in males, extended meiotic chromosomes and male sterility (Kolas *et al*., 2004). Male mice with *Sycp3* null mutations have also resulted in complete sterility (Yuan *et al*., 2000). Consequently, the effect of the *in vivo* silencing on other testis related genes in our study might highlight the importance of this role during fish spermatogenesis, and meiosis, as can be observed in the significant downregulation of *vasa* in particular, which is a germ cell universal marker, and disruptions in this gene expressions have been shown to result in impaired meiosis and germ line stem cell loss (Hartung *et al*., 2014). Studies in mice where a null mutation was generated on the *Sycp3* gene had been shown to cause extensive apoptotic cell death resulting in sterility during prophase I of meiosis, failure of synapsis and formation of axial/lateral elements and SCs, and also affected expression of synaptonemal complex protein 1 (Yuan *et al*., 2000). Studies in human ovary and testis have also revealed the critical function of this gene during meiosis, and its relation to various reproductive problems (Yuan *et al*., 2002; Miyamoto *et al*., 2003; Wang and Höög, 2006). Taken together, the functional role of *sycp*3 during spermatogenesis and meiosis could be extrapolated to lower vertebrates such as teleosts where very little investigations have been done. The impaired expression levels of other testis-related transcription factors after *sycp3* esiRNA silencing might also imply a direct or indirect role of this gene in conjunction

with other factors to impact germ cell development. These results also warrant direct or indirect interactions and regulatory mechanisms that occur between the testes related genes analyzed and *sycp3*.

In summary, present study aimed to identify factors involved in the crucial processes of germ cell development/meiosis through 2D proteomic analysis which identified the Sycp3 protein due to significant expression during gonadal development stages in juvenile catfish. This may be one of the few identified markers of meiosis which have been reported in lower vertebrates such as teleosts, specifically in annual breeders. Full length cDNA of *sycp3* was then cloned using peptide sequence data and its expression profile was analyzed during gonadal development, maturation as well as various phases of the annual reproductive cycle. This analysis presented the involvement of *sycp3* during the crucial stages of reproductive development via its expression pattern, as well as its specific localization only in the germ cells of catfish. In addition, the work also inferred the regulatory effects of gonadotropins on the process of meiosis perhaps by modulating *sycp3*. Transient silencing of *sycp3*, *in vivo* using esiRNA decreased the expression of various testis related genes which implied either a direct or indirect impact of this gene in testicular germ cell development and recrudescence.

References

Agbor V.A., Tao S., Lei N., Heckert L.L., 2013. A Wt1-Dmrt1 transgene restores DMRT1 to sertoli cells of Dmrt1(-/-) testes: a novel model of DMRT1-deficient germ cells. Biol. Reprod. 88: 51.

Botelho R.J., DiNicolo L., Tsao N., Karaiskakis A., 2001. The genomic structure of SYCP3, a meiosis-specific gene encoding a protein of the chromosome core. Biochim. Biophys. Acta 1518, 294–299.

Chocu S., Calvel P., Rolland A.D., Pineau C., 2012. Spermatogenesis in mammals: proteomic insights. Syst. Biol. Reprod. Med. 58, 179–190.

Di Carlo A.D., Travia G., De Felici M., 2000. The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. Int. J. Dev. Biol. 44, 241–244.

Falco A., Encinas P., Carbajosa S., Cuesta A., Chaves-Pozo E., Tafalla C., Estepa A., Coll J.M., 2009. Transfection improvements of fish cell lines by using deacylated polyethylenimine of selected molecular weights. Fish Shellfish Immunol. 26, 559–566.

Hartung O., Forbes M.M., Marlow F.L., 2014. Zebrafish *vasa* is required for germ-cell differentiation and maintenance. Mol. Reprod. Dev. 81, 946–961.

Höbel S., Aigner A., 2010. Polyethylenimine (PEI)/siRNA-mediated gene knockdown *in vitro* and *in vivo*. Methods Mol. Biol. 623, 283–297.

Höbel S., Aigner A., 2013. Polyethylenimines for siRNA and miRNA delivery *in vivo*. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 5, 484-501.

Iwai T., Yoshii A., Yokota T., Sakai C., Hori H., Kanamori A., Yamashita M., 2006. Structural components of the synaptonemal complex, SYCP1 and SYCP3, in the medaka fish *Oryzias latipes*. Exp. Cell. Res. 312, 2528–2537.

Joy K.P., Singh M.S., Senthilkumaran B., Goos H.J., 2000. Pituitary-gonadal Relationship in the Catfish *Clarias batrachus* (L): A study correlating gonadotropin-II and sex steroid dynamics. Zool. Sci. 17, 395–404.

Kirubagaran R., Senthilkumaran B., Sudhakumari C.C., Joy K.P., 2005. Seasonal dynamics in gonadotropin secretion and E2-binding in the catfish *Heteropneustes fossilis*. Fish Physiol. Biochem. 31, 183–188.

Kobayashi T., 2010. *In vitro* germ cell differentiation during sex differentiation in a teleost fish. Int. J. Dev. Biol. 54, 105–111.

Kolas N.K., 2004. Male mouse meiotic chromosome cores deficient in structural proteins SYCP3 and SYCP2 align by homology but fail to synapse and have possible impaired specificity of chromatin loop attachment. Cytogenet. Genome Res. 105, 182–188.

Lau E.L., Lee M.F., Chang C.F., 2013. Conserved sex-specific timing of meiotic initiation during sex differentiation in the protandrous black porgy *Acanthopagrus schlegelii*. Biol. Reprod. 88, 1–13.

Liu T.D., Yu B.Y., Luo F.H., Zhang X.L., Wu S.C., Liu L.H., Wu Y.J., 2012. Gene expression profiling of rat testis development during the early post-natal stages. Reprod. Domest. Anim. 47, 724–731.

Miyamoto T., Hasuike S., Yogev L., Maduro M.R., Ishikawa M., Westphal H., Lamb D.J., 2003. Azoospermia in patients heterozygous for a mutation in SYCP3. The Lancet 362, 1714–1719.

Murugananthkumar R., Prathibha Y., Senthilkumaran B., Rajakumar A., Kagawa H., 2016. *In vivo* induction of human chorionic gonadotropin by osmotic pump advances sexual maturation during pre-spawning phase in adult catfish. Gen. Comp. Endocrinol. http://dx.doi.org/10.1016/j.ygcen.2016.09.015

Murugananthkumar R., Senthilkumaran B., 2016. Expression analysis and localization of wt1, ad4bp/sf-1 and gata4 in the testis of catfish, *Clarias batrachus*: Impact of wt1-esiRNA silencing. Mol. Cell Endocrinol. 431, 164–176.

Ozaki Y., Saito K., Shinya M., Kawasaki T, Sakai N., 2011. Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. Gene Expr. Patterns 11, 309–315.

Page J., Viera A., Parra M.T., de la Fuente R., Suja J.A., Prieto I., Barbero J.L., Rufas J.S., Berríos S., Fernández-Donoso R., 2006. Involvement of synaptonemal complex proteins in sex chromosome segregation during marsupial male meiosis. PLoS Genet. 2: e136.

Prathibha Y., Senthilkumaran B., 2016. Involvement of pax2 in ovarian development and recrudescence of catfish: a role in steroidogenesis. J. Endocrinol. 231, 181-195.

Raghuveer K., Senthilkumaran B., 2010. Cloning and differential expression pattern of vasa in the developing and recrudescing gonads of catfish, *Clarias gariepinus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 79–85.

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.

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.

Schulz R.W., Vischer H.F., Cavaco J.E., Santos E.M., Tyler C.R., Goos H.J., Bogerd J., 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 407–417.

Sudhakumari C.C., Anitha A., Murugananthkumar R., Tiwari D.K., Bhasker D., Senthilkumaran B., Dutta-Gupta A., 2017. Cloning, localization and differential expression of Neuropeptide-Y during early brain development and gonadal recrudescence in the catfish, *Clarias gariepinus*. Gen. Comp. Endocrinol. https://doi.org/10.1016/j.ygcen.2017.03.006.

Wang H., Höög C., 2006. Structural damage to meiotic chromosomes impairs DNA recombination and checkpoint control in mammalian oocytes. J. Cell Biol. 173, 485–495.

Yuan L., Liu J.G., Zhao J., Brundell E., Daneholt B., Höög C., 2000. The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. Mol. Cell 5, 73–83.

Yuan L., Liu J.G., Hoja M.R., Wilbertz J., Nordqvist K., Hoog C., 2002. Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3. Science 296, 1115–1118.

Zhou X., Laroche F., Lamers G.E.M., Torraca V., Voskamp P., Lu T., Chu F., Spaink H.P., Abrahams J.P., Liu Z., 2012. Ultra-small graphene oxide functionalized with polyethylenimine (PEI) for very efficient gene delivery in cell and zebrafish embryos. Nano Res. 5, 703-709.

Abstract

C-kit receptor is a member of a family of growth factor receptors that have tyrosine kinase activity, and are involved in the transduction of growth regulatory signals across the plasma membrane through the activation by its ligand, kitl/scf. The present study analyzed the mRNA and protein expression profiles of *c-kit* in the gonads of catfish, *Clarias gariepinus*, using *in situ* hybridization, immunohistochemistry and real time PCR. The tissue distribution analysis revealed higher expression mainly in the gonads of catfish. Ontogeny studies showed that the expression is minimal during early stages of development and highest during 50-75 dph, and the dimorphic expression in gonads decreased gradually till adulthood, which might suggest an important role for this gene during critical stages of sex differentiation and gonadal development. The expression of *c-kit* throughout various phases of gonadal cycle were analyzed in both male and female, which showed minimal expression during resting phase, and the difference in the expression levels between male and females during the pre-spawning phase may be due to the difference in hormonal regulation at this stage. The effect of human chorionic gonadotropin induction both *in vivo* and *in vitro* on *c-kit* showed higher expression suggesting the regulatory influence of hypothalamo-hypophyseal axis. In addition, *in vivo* transient silencing using *c-kit*-esiRNA in adult catfish during recrudescence showed a decrease in c-*kit* expression, which also affected the expression level of germ cell meiotic marker *sycp3*, as well as several steroidogenic enzyme genes and factors involved in germ cell maturation. The findings of this study suggest that *c-kit* receptor seems to be crucial for germ cell proliferation, development and maturation during recrudescence in catfish.

1. Introduction

Various stages of spermatogenesis that constitute germ cell migration, proliferation, maturation and survival as well as testicular development and function, are under the influence of the tyrosine kinase receptor protein, c-Kit and its ligand, stem cell factor (SCF) (Loveland and Schlatt, 1997; Zhang *et al*., 2013). The proto-oncogene *c-kit* is allelic to the *dominant white spotting* (W) locus on chromosome 5 in mouse and belongs to a family of growth factor receptors with intrinsic tyrosine kinase activity that is crucial for transduction of growth regulatory signals across the plasma membrane (Besmer *et al*., 1993, Yoshinaga *et al*., 1991; Galli *et al*., 1993). The KIT receptor comprises of three main functional regions: the outer extracellular domain that consists of five immunoglobin-like structures essential for ligand binding and dimerization, a transmembrane hydrophobic region which anchors the receptor to the cell membrane and the intracellular kinase domain required for transduction of the signal conveyed by the specific ligands that results in subsequent autophosphorylation of several tyrosine residues (Sette *et al*., 2000; Roskoski, 2005). The ligand SCF is expressed in Sertoli cells that lie in close association with germ cells (Yoshinaga *et al*., 1991). Apart from its role in reproductive physiology, c-Kit signalling has been found to be critical for other biological processes like hematopoiesis, pigmentation, gut movement, as well as the nervous system. Further, impairments of the receptor kinase activity has been found to result in allergies and cancer (Isozaki *et al*., 1995; Zhang and Fedoroff, 1997; Puxeddu *et al*., 2003; Alexeev and Yoon, 2006; Edling and Hallberg, 2007; Stankov *et al*., 2014). In gametogenesis, *c-kit* expression has been shown to be crucial for the development and maintenance of primordial germ cells (PGCs) in both male and female gonads, from embryonic to post-natal stages (Besmer *et al*., 1993; Nakatsuji and Chuma, 2001; Hutt *et al*.,

2006). During spermatogenesis in particular, numerous studies have been done in mammals that have elucidated the critical role of *c-kit* at all stages of development (Mauduit *et al*., 1999). Mutations in the loci of c-Kit or its ligand SCF have resulted in aberrant PGC development and sterility in mice (De Miguel *et al*., 2002). The expression of c-Kit receptor has been observed mainly in spermatogonia and Leydig cells of adult mouse testis (Manova *et al*., 1990; Zhang *et al*., 2013) and expression has been detected at post-natal day 5 exclusively in differentiating type A and B spermatogonia and primary spermatocytes (Manova *et al*., 1993; Sette *et al*., 2000). A truncated c-kit receptor with an absent extracellular, transmembrane and part of the intracellular tyrosine kinase domain, has also been detected in spermatids and spermatozoa (Albanesi *et al*., 1996). One of the key roles of *c-kit* expression is maintenance of the ratio between self-renewal and differentiation of spermatogonial stem cells which is normally maintained at 1.0, and any deviations from this ratio may lead to tumours in the seminiferous epithelium (Bokemeyer *et al*., 1996; Zhang *et al*., 2011). In addition, the activation of c-Kit signalling has a decisive role in spermatogenesis by activating meiosis at specific time points, and it then leads to subsequent expression of early meiotic markers such as *Dmc1* and *Sycp3* (Vincent *et al*., 1998; Di Carlo *et al*., 2000).

Although the function and expression of *c-kit* has been well studied in mammalian systems, very few reports have been documented on its role in fish reproduction. Earlier studies in model organisms such as zebrafish, *Danio rerio*, were restricted mostly pertaining to the processes such as haematopoiesis, melanogenesis and oogenesis (Ransom *et al*., 1996; Weinstein *et al*., 1996; Parichy *et al*., 1999, Rawls and Johnson, 2000; Yao and Ge, 2010, 2013). *C-kit* has been identified as a male germ cell marker in spermatogonial cells of the

Japanese medaka *Oryzias latipes*, dogfish *Scyliorhinus canicula* and rohu *Labeo rohita* (Hong *et al*., 2004; Loppion *et al*., 2008; Bosseboeuf *et al*., 2013) and there have been no detailed study on the involvement of this gene in germ cell development, proliferation and maturation especially in annual breeders such as catfish. Annually breeding teleosts show distinguished pattern of germ cell maturation and recrudescence during first and cyclic stages of gametogenesis.

Present study aims to analyse the role of *c-kit* during various stages of germ cell development with spermatogenesis in particular, using the African air-breathing catfish, *Clarias gariepinus* as a model. Initially, cDNA of *c-kit* was cloned from catfish testis, which was followed by real-time PCR (qPCR) analysis of expression in various tissues, during gonadal ontogeny and seasonal reproductive phases. The response of this gene to gonadotropins using human chorionic gonadotropin (hCG) was analyzed in both *in vitro* and *in vivo*. Localization of *c-kit* gene and protein in the testis was done using *in situ* hybridisation (ISH) and immunohistochemistry (IHC)/Immunofluorescence (IF), respectively. Further, *in vitro* and *in vivo* transient silencing using *c-kit*-esiRNA by polyethylenimine (PEI) mediated transfection was performed in adult catfish during recrudescence to analyze the effect of *c-kit* on various germ cell markers, steroidogenic enzyme genes and others factors related to germ cell maturation and spermatogenesis in catfish.

2. Materials and Methods

2.1 Animal breeding and sample collection

Breeding and rearing of *C. gariepinus* used for the experiment was performed as per the process that was detailed in the Chapter 1a. After the required number of days post hatch,

samples were collected from fishes of different age groups from embryo stages as well as juvenile and adult stages. Catfish were anesthetized prior to dissection with 100 mg/L of MS-222, (Sigma). Gonads of male and female catfish (adult, 400 dph and juvenile, 100 dph) were then dissected out, snap frozen in liquid nitrogen and stored at -80^{o}C for total RNA isolation. Sampling for fish was carried out as per the general guidelines and with the approval of the Institutional Animal Ethics Committee, University of Hyderabad.

2.2 Molecular cloning of *c-kit* from testis of catfish

Cloning of a partial fragment of *c-kit* was done as per the method described in the Chapter 2. In brief, total RNA was isolated from catfish testis was done with TRI-reagent (Sigma) adult and RNA concentration and quality was measured using NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). First strand cDNA was synthesized using Primescript RT-PCR using the required amount of total RNA. PCR amplification using the degenerate primers designed for *c-kit* (Table 1) was performed with Taq 2X Master Mix (New England Biolabs Inc., Ipswich, MA, USA). Primers were designed by aligning the existing sequences of *c-kit* of siluriformes available in NCBI data base using Lasergene software (DNASTAR, Madison, WI, USA). The amplified PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) after gel purification, then sequenced bidirectionally, analyzed and confirmed as catfish *c-kit* partial cDNA through BLAST. Gene specific primers were designed using the nucleotide sequence of partial cDNA of *c-kit* to obtain the full length sequence using RACE strategy. After preparation of both 5` and 3` RACE cDNA templates with SMARTer^{TM} RACE cDNA amplification kit (Clontech, Mountainview, CA, USA), touchdown PCR was done with the designed RACE primers (Table 1) at recommended conditions along with the necessary constituents supplied along

with the kit. All thermal cycling conditions were done according to the instructions given by the manufacturer. The resulting RACE products were cloned into pGEM®-T easy vector (Promega), sequenced bidirectionally and sequence analysis was done using NCBI-BLAST.

2.3 Quantitative RT-PCR (qPCR)

Gene expression analysis was determined using qPCR with SYBR green detection method as per the protocol described in the Chapter 2. In brief, total RNA from testis was prepared with TRI-reagent® (Sigma) followed by cDNA synthesis. All specific primers used for the experiments are listed in Table 1 and qPCR primers for *c-kit* with amplicon length of ~200 bp were designed. The reactions were set up in triplicates in MicroAmp® 96-Well pates with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, Calif., USA) in an ABI Prism® 7500 fast thermal cycler (Applied Biosystems) as per the universal thermal cycling conditions provided by the manufacturer. Cycle threshold (Ct) values from the exponential phase of PCR amplification were used for generating ΔCt value with *18S rRNA* taken for the normalization against the expression of target gene. For calculation of the relative expression of genes, $2^{-\Delta Ct}$ method was used.

2.4 Quantitative analysis of *c-kit* for tissue distribution, reproductive cycle and ontogeny studies with qPCR

Both adult male and female catfishes (n=5), from late preparatory phase were sacrificed and different tissues (brain, heart, muscle, liver, kidney, testis, ovary) were then dissected out. Testis samples were collected for phase expression analysis at different time intervals of the reproductive cycle. In catfish, gonads differentiate into either a testis or ovary and are morphologically distinguishable only after ~35-50 days post hatch (dph). In 0, 10, 20 and 40

dph fish, mesonephric-gonadal complex was dissected out, and developing gonads from both male and female were then collected at 50, 75, 100, 200 dph as well as adults. In addition, embryo samples were also collected after IVF at different hours post fertilization. All samples were stored in -80^{o}C. Total RNA extraction, cDNA synthesis and qPCR (performed in triplicates for each sample) were done as described earlier.

2.5 hCG induction *in vitro* and *in vivo*:

Gonadotropin induction *in vitro* and *in vivo* using hCG at early prespawning phase on adult catfish were performed as per the procedure described in the Chapter 2. In brief, testicular slices of about 50 µm thickness were prepared which were then cultured in tissue culture plates added with 2 ml of L-15 medium, 10 mM HEPES and antibiotics (penicillin, 100 IU/ml; streptomycin, 0.1 mg/ml). The testicular slices were maintained carefully at 20–22 °C for ~24 h with 100 IU/ml of hCG (Pubergen; Sanzyme Ltd., Shameerpet TS, India) added to each well. Physiological saline (0.6 % NaCl w/v) served as control. Testis samples were then collected from both hCG treated and control at different time intervals of 2, 4, 6, 12, 20, and 24 h. This was followed by total RNA isolation used for cDNA synthesis, and relative expression analysis for *c-kit* was done as previously explained.

For in *vivo* induction (long term effect), fishes were anesthetized with 100 mg/L of MS-222 (Sigma), 5000 IU of hCG dissolved in 100 µl of saline was injected carefully into an osmotic pump (ALZET® osmotic pumps, Cupertino, 116 CA, USA) and then implanted into the peritoneal cavity through a small incision of about 8mm cut in the intraperitoneal region and then placed close to the gonads. The fishes were sacrificed after 21 days and samples were

taken for analysis. Detailed procedure has been explained earlier in the Chapter 2, and also by Murugananthkumar *et al*. (2016).

2.6. Western blot analysis

Western blot analysis was performed using C-KIT polyclonal antibodies (Cat. No. LS-C160001; Life Span Biosciences, Seattle, WA, USA) raised against the conserved C-terminal regions of human C-KIT which was ~90% homologous with the catfish conserved region. The homologous amino acid residue region of C-KIT for human and catfish are NH2-KICDFGLARDITTDSNYVVKGNARLPVKW-COOH and NH2-KICDFGLARDIKNDSNYVVKGNARLPVKW-COOH respectively. Catfish testis was homogenized with a buffer containing 1.5 M Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, and ProteoBlock™ protease inhibitor cocktail (Thermo Scientific) on ice to obtain a homogenate which was centrifuged at 12,000 x g at 4 ^{o}C for 30 minutes. Supernatant was pipetted out and protein concentration was estimated using Bradford method. The homogenate containing a protein concentration of 100 µg was run on a 12 % SDS-polyacrylamide gel and then transferred overnight at 4 ^{o}C onto a nitrocellulose membrane (Pall Life sciences, Port Washington, NY, USA). After blocking the membrane with 5 % skimmed milk in Tris-buffered saline (TBS) for 1 h at RT, it was washed thoroughly in TBS with 0.1 % Tween 20 (TBST), and then incubated with a 1:5000 dilution of polyclonal anti-C-KIT antibody overnight at 4 °C. The membrane was washed several times with TBST and then incubated for 1h goat anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase (Merck Bangalore Genei, Bengaluru, India) at RT. The membrane was again washed and visualization of the protein bands was done with BCIP-NBT substrate-catalyzed detection (Roche, Roche Diagnostics GmbH, Mannheim, Germany).

2.7. *In situ* hybridization (ISH)

As per the method described in the Chapter 2, localization of the mRNA transcripts of *c-kit* in adult catfish testis was performed with ISH. After preparation of the testis sample by fixation and sectioning in a cryostat with freezing medium, about 1 µl each of sense and antisense cRNA probes (heat denaturation done for 5 min at 80 °C after dilution with ~200 µl of hybridization buffer) were added to the slides and then incubated overnight at 50 °C in a sterile RNase free incubator. After this, the slides were washed and again incubated overnight with anti-DIG-ALP antibody diluted 1:1000 in maleic acid buffer (Roche) at 4 °C. After incubation with detection buffer (Roche), colour development was done with BCIP-NBT (Roche) as substrates and nuclear red as a counter stain. The slides were washed after adequate colour development, followed by dehydration in a gradient alcohol series and mounting with DPX mountant. Images were obtained with Leica DM6 B digital research microscope (Leica Microsystems Gmbh, Wetzlar, Germany).

2.8 Immunohistochemistry and Immunofluorescence

Localization of c-kit in adult catfish testis sections using the anti-C-Kit antibody was done with IHC. The detailed protocol is given in the Chapter 2. In brief, adult catfish testis fixed in Bouin's fixative for 3-4 hours followed by embedding in paraplast (Sigma) after dehydrating with a gradient series of ethanol followed by xylene treatment. About 6 µm paraplast sections were prepared and fixed on to Poly-L-Lysine coated glass slides. After deparaffinization with xylene and rehydration in a gradient series of ethanol, blocking was done with 0.1 % H_2O_2 added to the slides for ~10 min at room temperature (RT) followed by washing with 0.1 M phosphate-buffer saline (PBS) pH 7.4 and blocking with 10 % normal goat serum (Bangalore

Genei) in 0.1 M PBS for 1 h at RT. Anti-C-KIT polyclonal antibody (1:500) or pre-adsorbed antibody with excess antigen of c-Kit peptide (as a negative control) were then added on the slides followed by incubation at 4^{o}C in a humid chamber overnight. After washing the slides with 1X PBS with 0.1% Tween (PBST), incubation for 2 hours with HRP conjugated secondary antibody (Bangalore Genei) was performed at RT, washed and VECTASTAIN® Elite ABC reagent (Vector Laboratories, Burlingame, Calif. USA) was added. The slides were developed, counterstained with hematoxylin and mounted. Images were obtained using Leica DM6 B digital research microscope (Leica Microsystems Gmbh, Wetzlar, Germany).

For IF, testis dissected from adult catfish were fixed using 4% paraformaldehyde in 0.1 M 1X PBS for about 2–3 h and then immersed in OCT compound (Leia, Buffalo Grove, IL, USA) which was stored in -80^{o}C freezer for about 2–4 h. Tissue sections of 7 μm were cut using cryostat (Leica CM1850), followed by blocking with normal goat serum and overnight incubation with primary C-KIT antibody. FITC-conjugated anti-rabbit secondary antibody was added and IF images were obtained as described in the Chapter 2.

2.9 Preparation of catfish primary testicular culture

Preparation of primary testicular culture of catfish was performed as per the protocol described by Murugananthkumar and Senthilkumaran (2016). In brief, prior to dissection, anaesthesia was administered to an adult male catfish was using MS-222 (Sigma) on ice. The abdominal area was thoroughly disinfected with 70 % ethanol and testes were dissected out. After repeated washing with sterile PBS, testicular slices were carefully cut in a sterile cell culture plate containing Leibovitz (L-15) medium and washed with PBS for 5 times after which ~0.20-0.30 % collagenase type I and 2μl DNase were added and then incubated for 15-

20 min at RT. The testicular slices were then gently pressed with the end of a sterile syringe to create a cell suspension in the medium which was then filtered with a 40 µm cell strainer. This suspension was centrifuged at 200 xg at 4 °C. Resuspension of the pellet was done in Dulbecco's modified Eagle's medium (DMEM) and washed several times with DMEM. The culture medium containing ~1 X 10^6 cells were plated into several wells of Corning® Costar® 24 well cell culture plates (Sigma) and a freshly prepared medium which includes DMEM, 10 % FBS, 1X Glutamax with antibiotics/antimycotic solution was then added. Incubation was done at 30 °C with a steady supply of 5 % CO_2 for 24 h prior to use in transfection experiments. Trypan blue staining was used for cell viability assessment.

2.10 *In vitro* esiRNA-PEI transfection

PEI mediated *in vitro* transfection with *c-kit-esiRNA* was done as per the method described in the Chapter 2. Briefly, *c-kit* esiRNA was synthesized (Sigma) and obtained commercially using the ~500 bp cDNA of *c-kit* cloned from catfish. PEI (Sigma) dissolved in sterile HEPES-NaCl buffer, pH 7.4 was complexed with control siRNA (MISSION siRNA Fluorescent Universal Negative Control #1, Cat. no. SIC007, Sigma, 10ng/ µl) and varying concentrations of *c-kit*-esiRNA (10, 20, 40 and 100 ng/ µl) by incubation for 30 min at RT which were then added to the respective culture wells and maintained for 12 h at 37 ^oC. The mixture of OPTI-MEM®I and control siRNA/esiRNA-PEI were then discarded after the transfection and fresh cell culture medium was as previously described. After transfection, the cells were allowed to grow for another 24 h before sample collection. Total RNA preparation, analysis of relative expression of certain testis related genes post transfection with qPCR, assessment of transfection efficiency with control siRNA and obtaining microphotographs are as explained in the Chapter 2.

2.11 *In vivo* esiRNA-PEI transfection

For *in vivo* transfection, the method described in the Chapter 2 was followed. Adult (~2 year old) catfish (n=5) undergoing prespawning phase were used. *C-kit*-esiRNA (3 µg) complexed with PEI in sterile HEPES-NaCl pH 7.4 (Höbel and Aigner, 2010, 2013) was used for transfection (Falco *et al.*, 2009; Zhou *et al.*, 2012). A small incision of about 10 mm was cut near the pelvic fin junction of the fish with a sterile scalpel after anaesthesia and the *c-kit*-esiRNA-PEI complex was then injected into the testis with a sterile 1 ml syringe. Control siRNA-PEI complex or PEI alone was injected for control groups. The incision was then sutured with sterile 30 mm catgut and treated with antibiotics. The fishes were monitored and maintained for 3 days in individual tanks, and tissue samples were taken at different intervals by sacrificing the fishes at approximately 48 and 72 hours post transfection and then snap frozen and stored in -80^{o} C.

2.12. Statistical analysis

All the data were expressed as mean ± standard error of mean (SEM). Significance among groups was tested by Analysis of variance (ANOVA) followed by Student's–Newman–Keuls' test using SigmaPlot 12.0 software (Systat Software Inc., Chicago, IL, USA). A probability of $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Molecular cloning of *c-kit* from testis of catfish

Initially, a partial cDNA of 372 bp of *c-kit* was cloned from testis of juvenile catfish using RT-PCR. The cDNA fragments of 5′ and 3′ ends were then acquired with RACE strategy to obtain a partial cDNA sequence of 1.5 kb that encodes a putative protein of 530 amino acids

which contains signature conserved tyrosine kinase domains that is adequate to perform expression studies required for this analysis. Further, the almost full length cDNA with deduced amino acid sequence was confirmed as *c-kit* homologue through BLAST.

3.2 Tissue distribution, reproductive cycle and ontogeny expression analysis of *c-kit* in catfish

The expression levels of *c-kit* revealed variability in different tissues, but showed significantly ($P < 0.05$) higher expression in gonads of both male and female, and moderate expressions in kidney and low in liver and muscle (Figure 1A and B). *C-kit* expression during various phases of gonadal cycle in both male and female showed minimal expression during resting phase, with significantly elevated levels during the preparatory to spawning phases in males (Figure 2A), while this expression levels are low during pre-spawning phase in females when compared to males (Figure 2B). The difference in the expression levels between male and females during the pre-spawning phase may be due to the difference in hormonal regulation during this stage. Ontogeny studies showed minimal expression during early stages of development and highest during 50-75 dph, where males showed a significantly higher expression during 50 and 75 dph. The dimorphic expression decreased gradually by adulthood (Figure 3). This high expression of *c-kit* during this stage is comparable to preparatory phase results where recrudescence of gonadal development is evident in annually reproducing teleosts. The *c-kit* expression during embryonic stages post fertilization shows a gradual and constant expression until 20 h and expression significantly ($P < 0.05$) increases after 24 h at the hatched larva stage (Figure 4).

3.3 hCG induction *in vitro* and *in vivo*

Induction with gonadotropin using hCG, both *in vitro* and *in vivo*, showed a positive effect on the expression levels of *c-kit*. *In vitro* induction of catfish testis with hCG indicated a significant ($P < 0.05$) elevation of *c-kit* expression from 4 h onwards when compared to the control samples analyzed (Figure 5A). In the *in vivo* induction, there was a significant increase in *c-kit* expression after the period of induction (21 days), when compared to control (Figure 5B).

3.4 ISH of *c-kit* in catfish testis

ISH was performed to confirm the specific localization of *c-kit* mRNA transcripts in the adult catfish testis sections. The spermatogonia and spermatocytes (Figure 6A - C) displayed positive signals which revealed a heterogeneous pattern similar to that observed in the IHC analysis and this localization pattern was constantly seen in most of the testis sections analyzed. Sense probe as a negative control did not show any signal (Figure 6D), which confirmed the specificity of the antisense probe used.

3.5 *C-kit* immunolocalization in catfish testis

Localization of c-kit with both IHC and IF were done in adult catfish testis during late preparatory phase (Figure 7 and 8, respectively). Pronounced immunoreactivity for c-kit protein was seen in the developing spermatogonia lining the basement membrane and as well as spermatocytes (Figure 7A – C; 8A – D). However, the signals were heterogeneous across the tubule, indicating stage specificity. Hence, immunoreactivity was not observed in mature sperm/spermatids counterstained with hematoxylin as well as in the negative controls with pre-adsorbed antibody of c-kit (Figure 7D). IF detection also displayed a fairly similar result

as IHC where positive signals (FITC stain) were seen spermatogonia and spermatocytes. Western blot analysis using anti-C-KIT antibody on adult testis sample detected a positive band (~60 kDa) which correspond to the cytoplasmic domain of the protein (Figure 7E)

3.6 *In vitro* transfection of *c-kit*-esiRNA in catfish testicular culture

In the *in vitro* transfection analysis (n=5), a significant ($P < 0.05$) decrease was observed in the levels of *c-kit* expression in the samples transfected by PEI complexed *c-kit*-esiRNA when compared to other control groups (Figure 9A). The expression levels of *c-kit* after esiRNA transfection also showed varied downregulation with different concentrations. There was a significant ($P < 0.05$) decrease in the expression level of *c-kit* by 78 % after transfection with 100 ng/µl of *c-kit*-esiRNA when compared to control, and this dose was used for further experiments. In addition, the transient gene silencing of *c-kit* showed a significant ($P < 0.05$) decrease in the expression levels of other important testis-related factors and steroidogenic enzyme genes when compared to control (Figure 9B). Transfection of only PEI and *c-kit*-esiRNA separately on catfish testicular cells did not indicate any substantial alteration in *c-kit* expression levels when in comparison with control. The primary testicular culture transfected with control siRNA complexed with PEI were monitored carefully (Figure 10A and B) and showed fluorescent signals confirming siRNA uptake in the cells (Figure 10C - F).

3.7 *In vivo* transfection of *c-kit*-esiRNA in adult catfish

Initially, PEI alone without any esiRNA was injected directly into the testis of adult catfish and survival of the animal was carefully monitored. Testis samples were then dissected from all transfected groups and collected for quantification of *c-kit* mRNA expression using qPCR.

Gene expression levels were measured and compared for PEI, control siRNA (Sigma) as well as *c-kit*-esiRNA-PEI complex injected animals. There were no significant changes observed in the expression levels of *c-kit* in the PEI-only injected catfish (n=5) when compared to control which infers that neither PEI alone nor control siRNA did negatively alter the gene expression levels. However, PEI complexed with *c-kit*-esiRNA injected into the testis significantly ($P < 0.05$) decreased the expression level of *c-kit* mRNA expression levels when compared to the other groups (Figure 11A). This implied the capacity of PEI complexed *c-kit*-esiRNA in silencing *c-kit* mRNA expression. Quantification of *c-kit* expression levels of were also done for the tissues collected at two distinct time intervals, 48 and 72 hours post transfection and compared to control groups (Figure 11B). Transfection using *c-kit*-esiRNA combined with PEI caused a significant decrease ($P < 0.05$) in the levels of *c-kit* expression both at 48 and 72 hours post transfection when compared to control, with 64 and 57 % decrease, respectively. The change in percentage decrease after 72 hours indicates that the transcript levels might get restored to normal levels due to transient silencing nature of the experiment as reported earlier by Murugananthkumar and Senthilkumaran (2016). The expression levels of *c-kit* was also measured in kidney to rule out any off-target effects of the transfection, and gene expression levels did not show any significant change (Figure 11C) indicating the specificity of transient silencing.

In addition, the expression levels of various testis related factors and steroidogenic enzyme genes crucial for germ cell development, were also evaluated with qPCR after 48 and 72 hours post esiRNA transfection in the catfish testis as well as in the control groups (Figure 12). Interestingly, most of the genes showed a significant decrease ($P < 0.05$) in expression levels at both 48 and 72 hours post transfection except for activin where no significant

change was evident. The expression levels between 48 and 72 hours for most genes remained fairly constant, except for *vasa* whose levels reverted back to normal at 72 hours.

Sl. No	Primer name	Primer sequence 5'-3'	Usage	GenBank Accession number
1.	*c-kit* Dgfw	GGCHCTRATGTCRGARCTRAAGG	RT-PCR	submitted
2.	*c-kit* Dgrv	GGCSACYTGGTASGAGAAGC		
3.	5P	GCTGAGGAGGTCTTCAGTGTCCAG	RACE	
4.	5N	CAGAGTCGGACCTCCAACAGTGCA		
5.	3P	GCCTGCACTGTTGGAGGTCCGACTC		
6.	3N	GGCTCTGGACACTGAAGACCTCCTC		
7.	5P#2	CCTCCCGTTCCCAAAGCACTGGTGC		
8.	5N#2	CTGCAGAGGCCTGCATGCAAGCTTC		
9.	3P#2	GCCCGTCTGCCGGTGAAGTGGATGT		
10.	3N#2	GCAGTCCTTACCCGGGTGTACCTGT		
11.	5P#3	CAGGATGAAGCAAAGGATGGCTGC		
12.	5N#3	GGCTCTCCACCGCTCCCTTCCCAAA		
13.	3P#3	CCCGTCTGCCGGTGAAGTGGATGTC		
14.	3N#3	CATGCGGTCTTGTTGGGAGGCAGA		
15.	*c-kit* RTfw	GCACTGTTGGAGGTCCGACTCTGGT	qPCR	
16.	*c-kit* RTrv	GGTGCTGGCTCTTACACAGACTGTG		
17.	*18s rRNA*	GCTACCACATCCAAGGAAGGCAGC	qPCR	AB105163
18.	*18srRNA*	CGGCTGCTGGCACCAGACTTG		
19.	*sycp3* RTfw	CCCAGTATAGCCAGAGGCAGAAGC	qPCR	KY553233
20.	*sycp3* RTrv	GAACATGGAGGAGATGGAGAAGAG		
21.	*vasa* RTfw	GGTCTGAGTAAAGTTCGTTATCTG	qPCR	GU562470
22.	*vasa* RTrv	GCCAACCTTTGAATATCCTCTG		
23.	*dmrt1* RTfw	ATGGCCGCTCAGGTGGCTCTGCGG	qPCR	FJ596554
24.	*dmrt1* RTrv	GCGGCTCCCAGAGGCAGCAGGAGA		
25.	*sox3* RTfw	CACGGTATGAGTAGCCCACCA	qPCR	HQ680982
26.	*sox3* RTrv	GCGATGGCAGGTGGTGGTGAG		
27.	*sox9* RTfw	TCTGGCGGCTGCTGAATGAAGG	qPCR	HM149258
28.	*sox9* RTrv	CTCGGTATCCTCGGTTTCACC		
29.	*wt1* RTfw	ACGCGCACAGGGTGTTCGA	qPCR	JF510005
30.	*wt1* RTrv	GGTACGGTTTCTCTCCTTGTG		
31.	*3β-hsd* RTfw	GAGGTAAATGTGAAAGGTACCAA	qPCR	HQ680983
32.	*3β-hsd* RTrv	TAGTACACAGTGTCCTCATGG		
33.	gata4 RTfw	CAGAGACGACTCTCTGCTTCCAG	qPCR	KT031389
34.	gata4 RTrv	CCCCGTGGAGCTTCATGTAGAG		
35.	*11β-h* RTfw	GGCAGTGGAGCGAATGCTGAA	qPCR	HQ680986
36.	*11β-h* RTrv	GCACCCCGGGGAACCTTGAGC		

37.	*17β-hsd12*	AGCCATCGAGAGCAAGTACCATGT	qPCR	JN848590
38.	*17β-hsd12*	AAGCCGAGTCATCTGACAAACCGA		
39.	*activin*	TCGCAGAGCCTGTTGATGCT	qPCR	KF956110
40.	*activin*	GCTGACACTCAGGGTCTGCA		

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

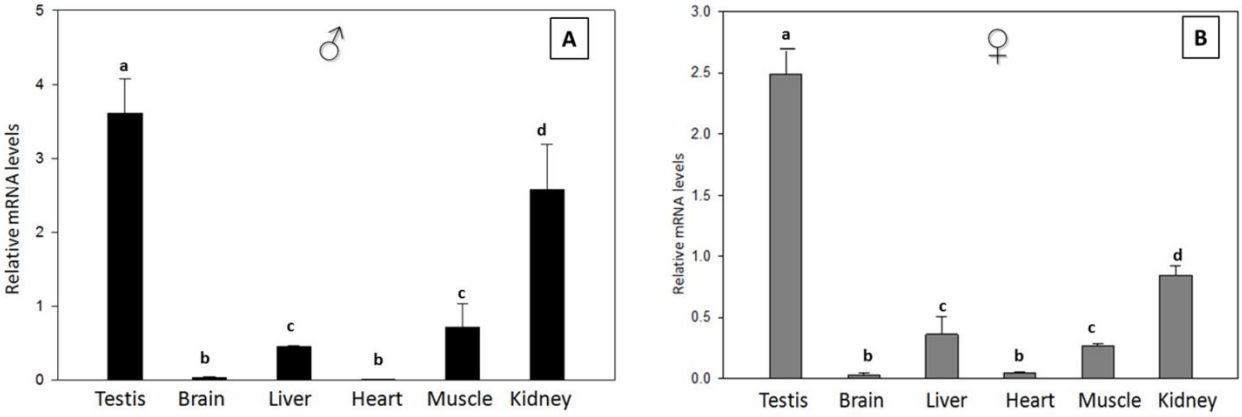


Fig. 1. qPCR analysis of *c-kit* expression in various tissues of catfish in male (A) and female (B). The relative expression of *c-kit* was normalized with *18S* rRNA and the values were calculated using $2^{-\Delta ct}$ method. Data (n = 5) were expressed as mean ± SEM. Mean with different letters differs significantly while mean with same letters are not significant (*, $P <$ 0.05; ANOVA followed by Student-Newman-Keuls' post hoc test).

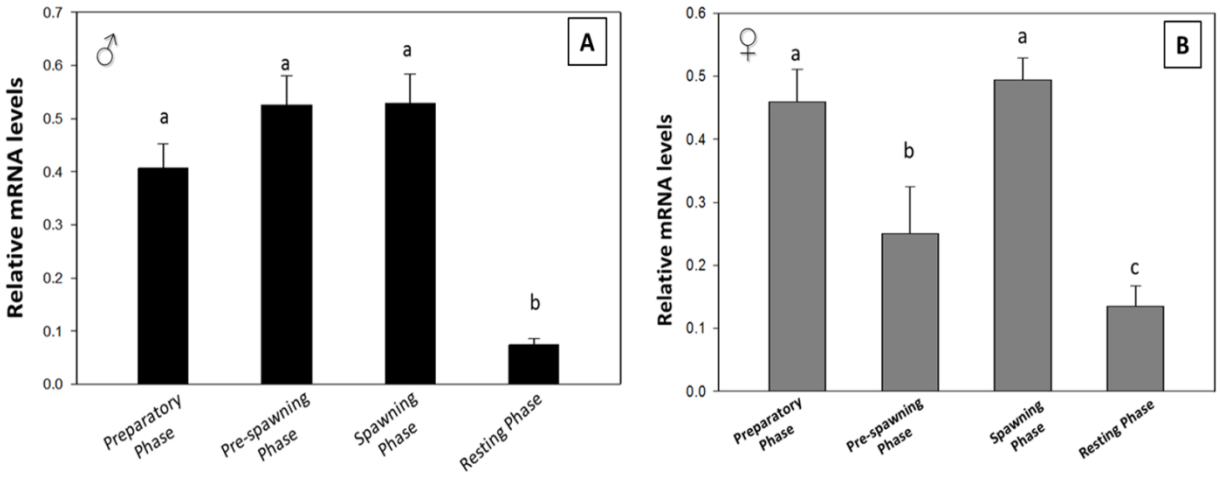


Fig. 2. qPCR analysis of *c-kit* expression during different phases of the reproductive cycle in catfish, *Clarias gariepinus* in male (A) and female (B). Data (n = 5) were expressed as mean ± SEM. Mean with different letters differs significantly while mean with same letters are not significant ($P< 0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

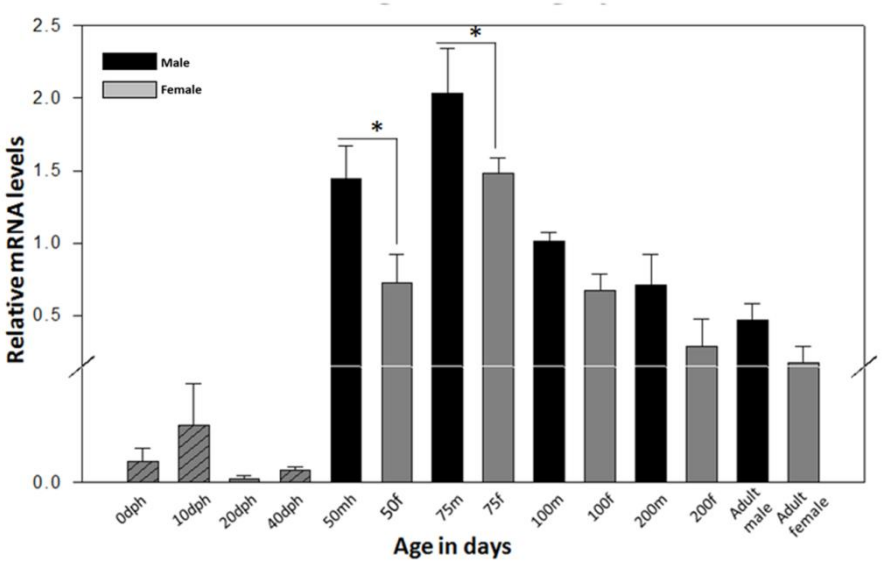


Fig. 3. qPCR analysis of *c-kit* expression during various stages of development of catfish. Data (n = 5) were expressed as mean ± SEM. (*, $P < 0.05$; ANOVA followed by Student-Newman-Keuls' post hoc test).

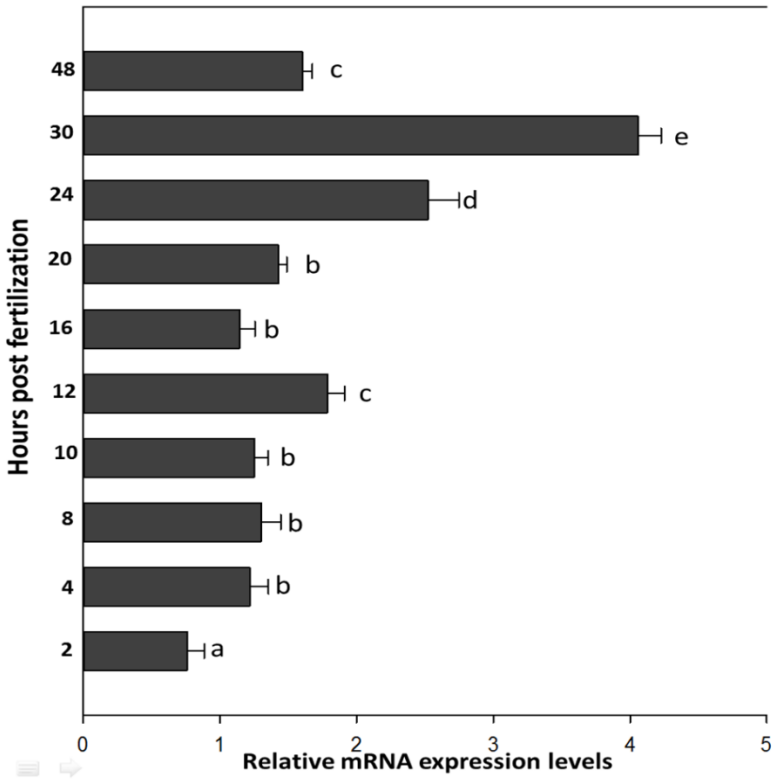


Fig. 4. qPCR analysis of *c-kit* expression during various stages of embryological development in catfish. Data (n = 5) were expressed as mean ± SEM (*, P < 0.05; ANOVA followed by Student-Newman-Keuls' post hoc test).

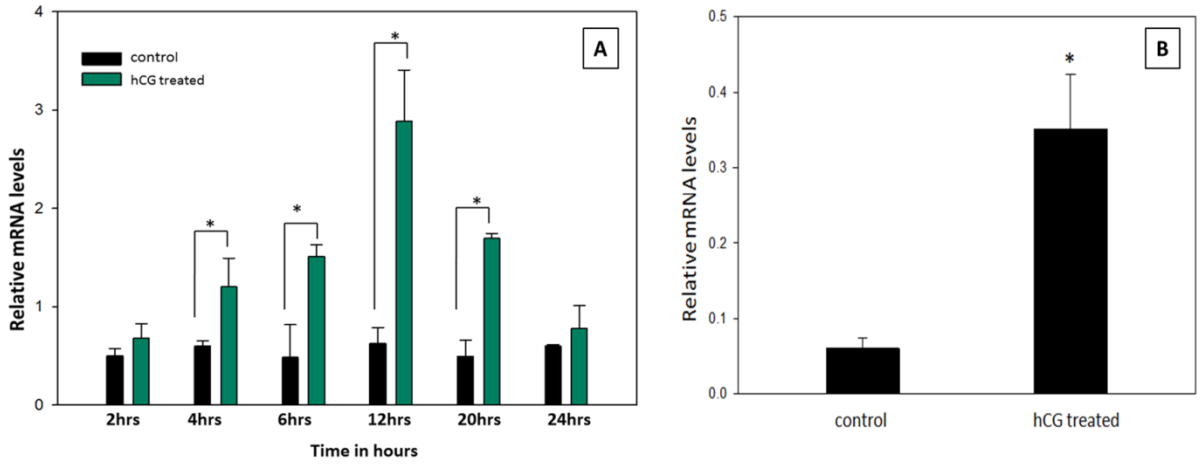


Fig. 5. qPCR analysis of *c-kit* mRNA expression levels after treatment with hCG *in vitro* in testicular slices (A) and *in vivo* (B) at different time intervals. Data (n = 5) were expressed as mean ± SEM. (*P< 0.05; ANOVA followed by Student-Newman-Keuls' post hoc test).

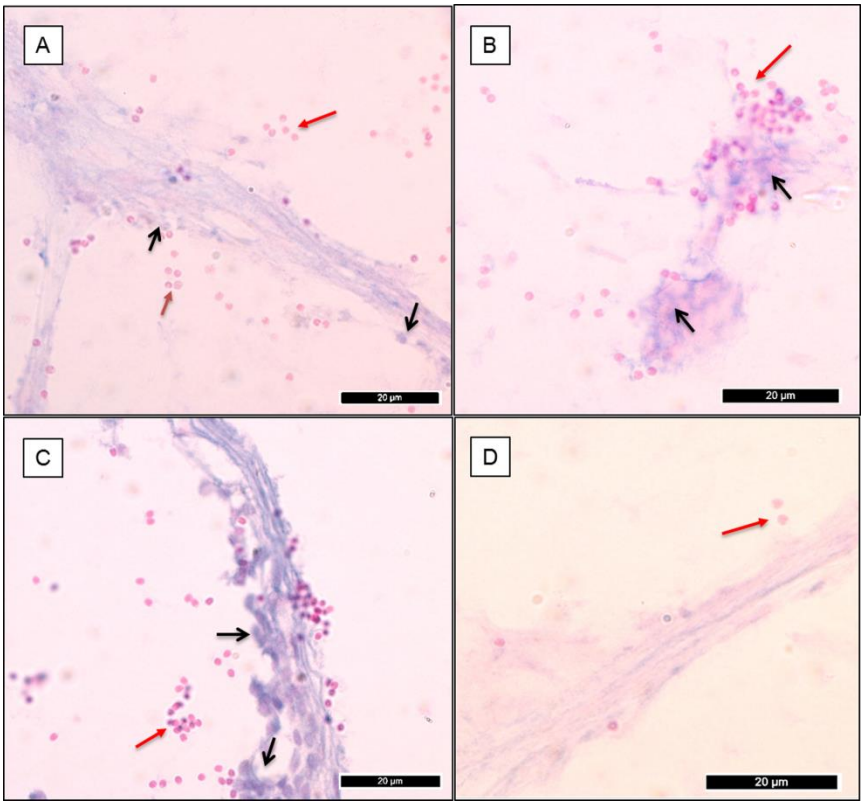


Fig. 6. *In situ* hybridization of *c-kit m*RNA in adult testis of catfish. Testis of adult catfish displayed positive signals in spermatocytes and spermatogonia indicated by black arrows (A-C). Signals were not detected in mature sperm/spermatids (red arrows). Sense probe of *c-kit* did not show any signal (D). Counterstain was done with nuclear red. Scale bars indicate 20 µm for all images.

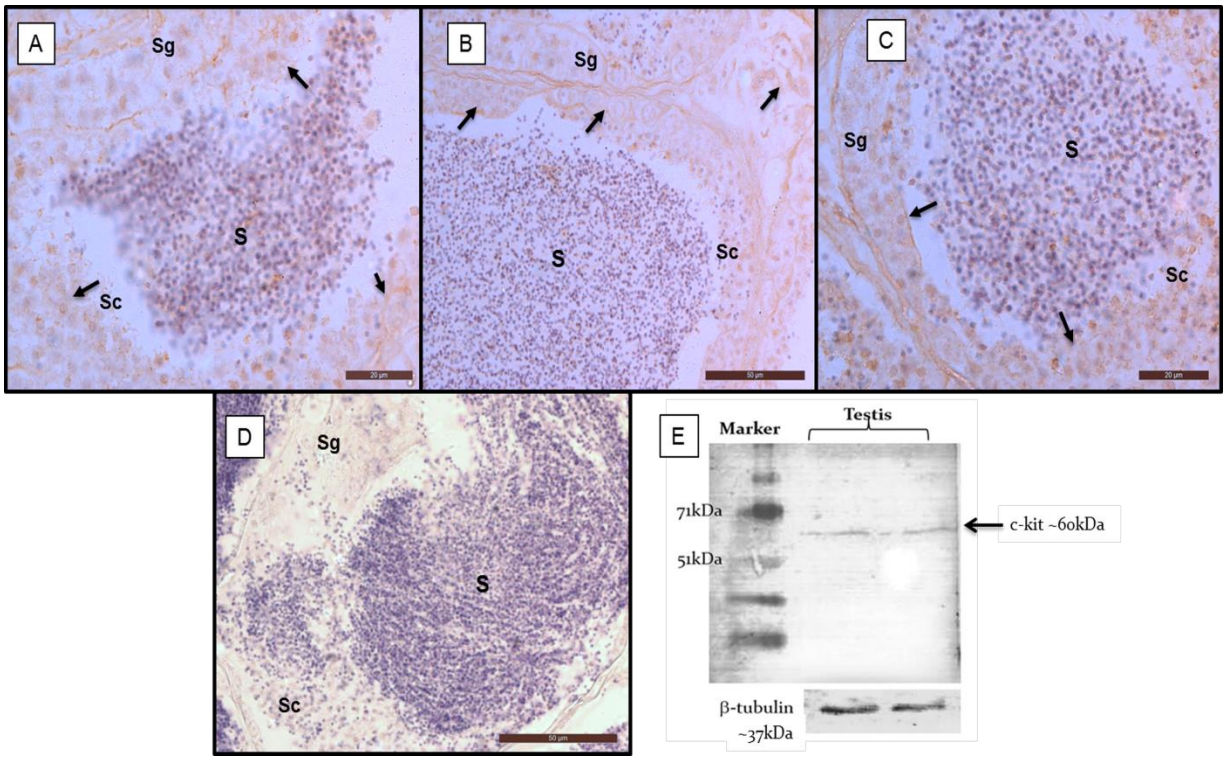


Fig. 7. C-kit immunohistochemical staining in catfish testis. Immunoreactivity was observed in spermatogonia and spermatocytes (A - C), while negative control with pre-adsorbed antibody (D) did not show any positive signal. Abbreviation : Sg - Spermatogonia, Sc - Spermatocytes, S - Sperm/spermatids. Scale bars indicate, A and C: 20 µm, B and D: 50 µm

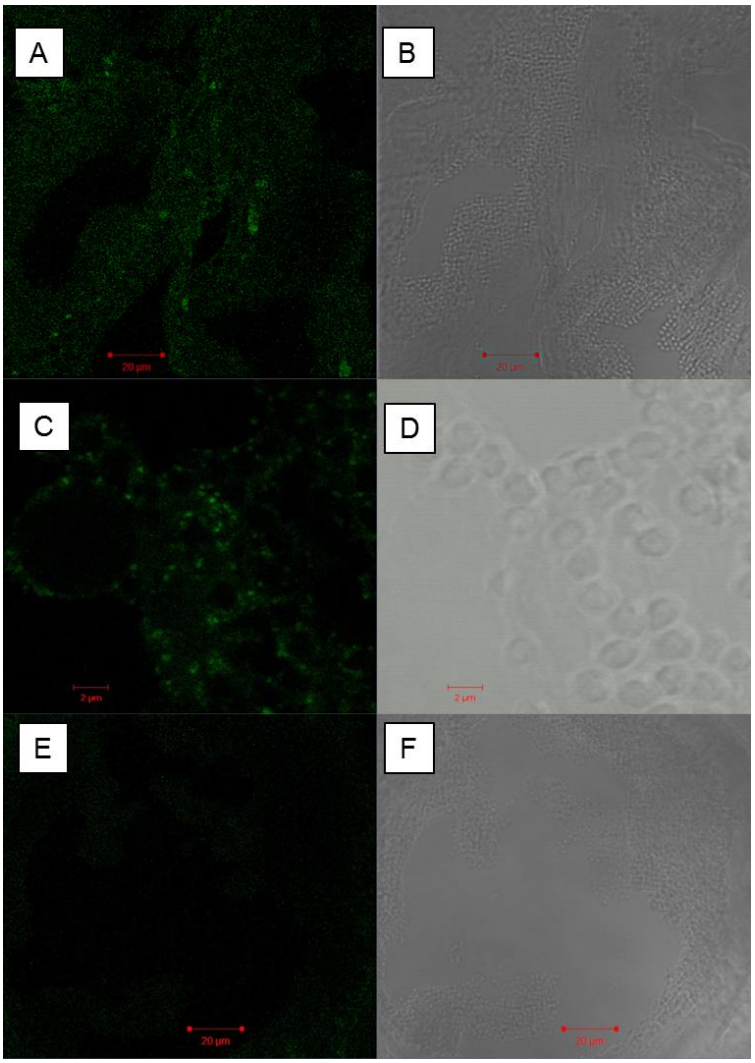


Fig. 8. Localization of c-kit protein in catfish testis using immunofluorescence stained with FITC which indicates positive signals for spermatogonia and spermatocytes (A and C) compared to negative control (E). B, D and F represent the corresponding phase contrast images. Scale bars indicate, A, B, E, and F: 20µm; C and D: 2µm

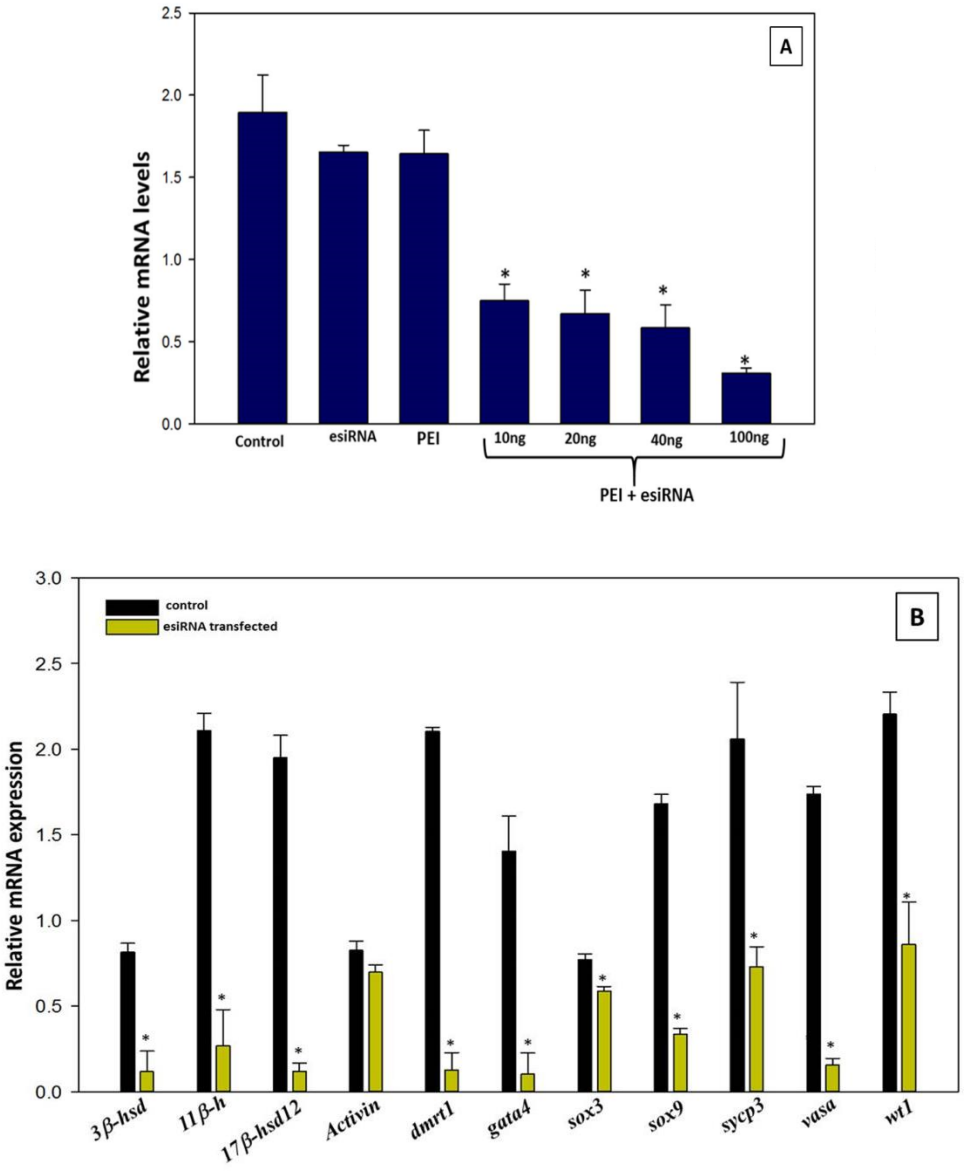


Fig. 9. *In vitro* transfection of PEI mediated *c-kit* -esiRNA in catfish testicular culture (mixed; n=5). Relative expression of *c-kit* was analysed and was observed to vary with the dose of transfection with *c-kit*-esiRNA (A) and *c-kit* transcripts were quantified and compared between control siRNA, *c-kit*-esiRNA and PEI + *c-kit*-esiRNA groups. Expression levels of certain testis related factors and steroidogenic enzyme genes (B) were analysed between control and *c-kit* -esiRNA transfected cultures. (*, $P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

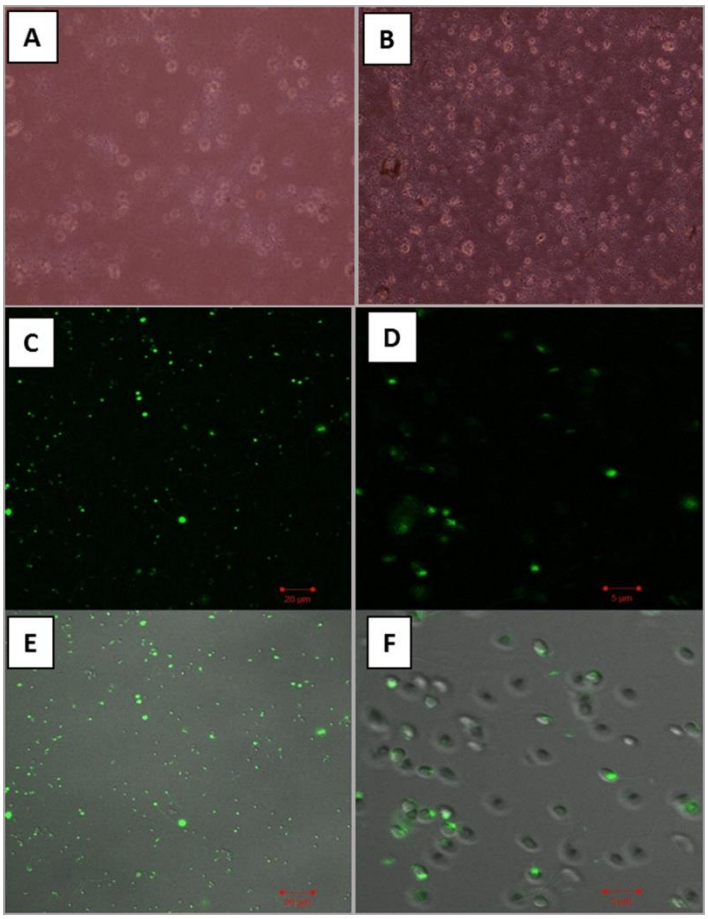


Fig. 10. Representative phase-contrast image of catfish primary testicular cells (A and B) and confocal microscope images (C - F) after PEI mediated transfection of control siRNA on catfish testicular culture (mixed) to check the efficiency.

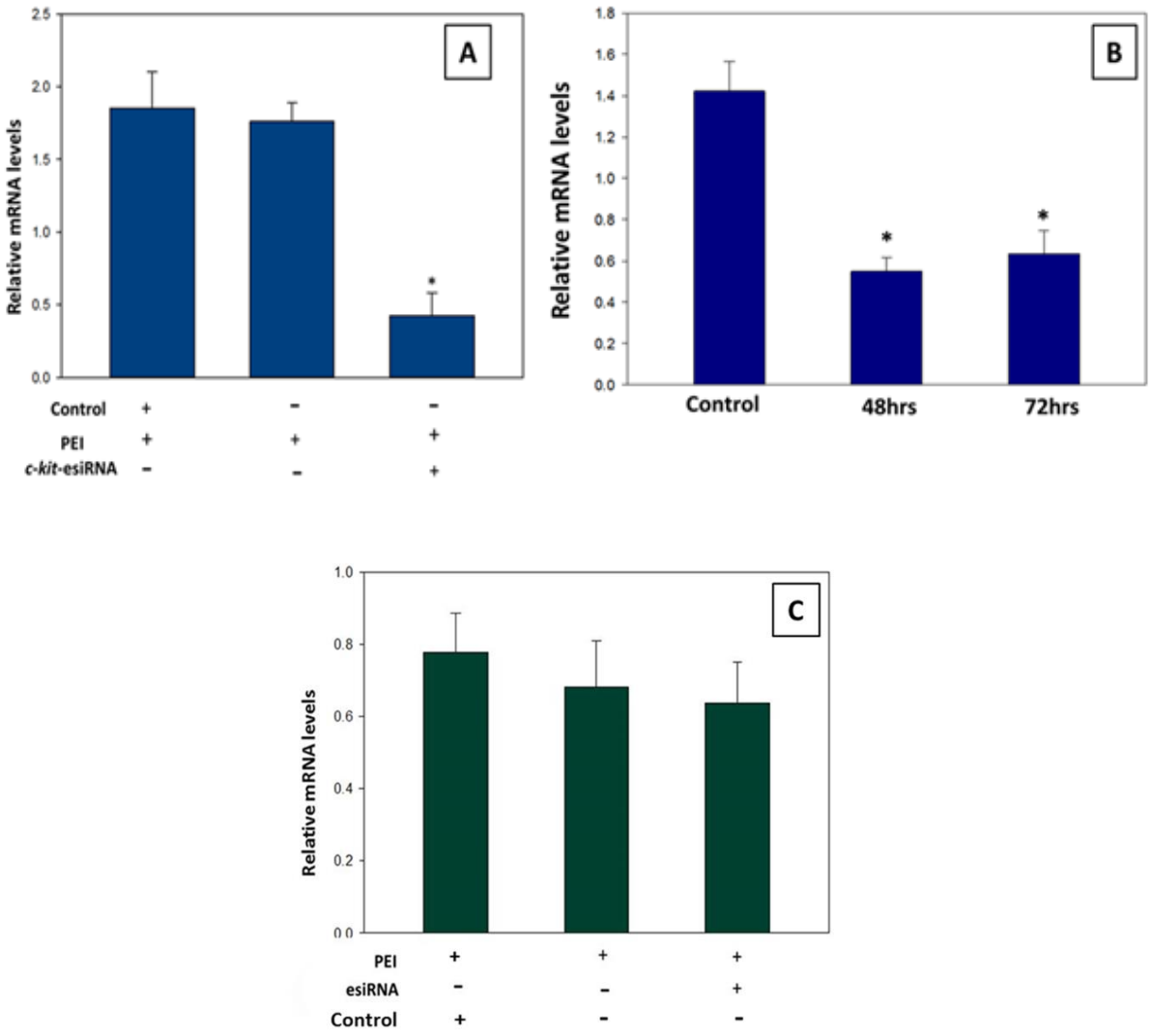


Fig. 11. qPCR analysis of the *c-kit* expression levels after esiRNA transfection. (A) *In vivo* PEI mediated *c-kit*-esiRNA transfection in adult catfish (n=5). (B) Analysis of *c-kit* gene expression levels between control and *c-kit* -esiRNA-PEI complex transfected samples at different time points, i.e, 48 and 72 hours post transfection. (C) Expression levels of c-kit in kidneys, which rules out any off-target effects of transfection process (*, $P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

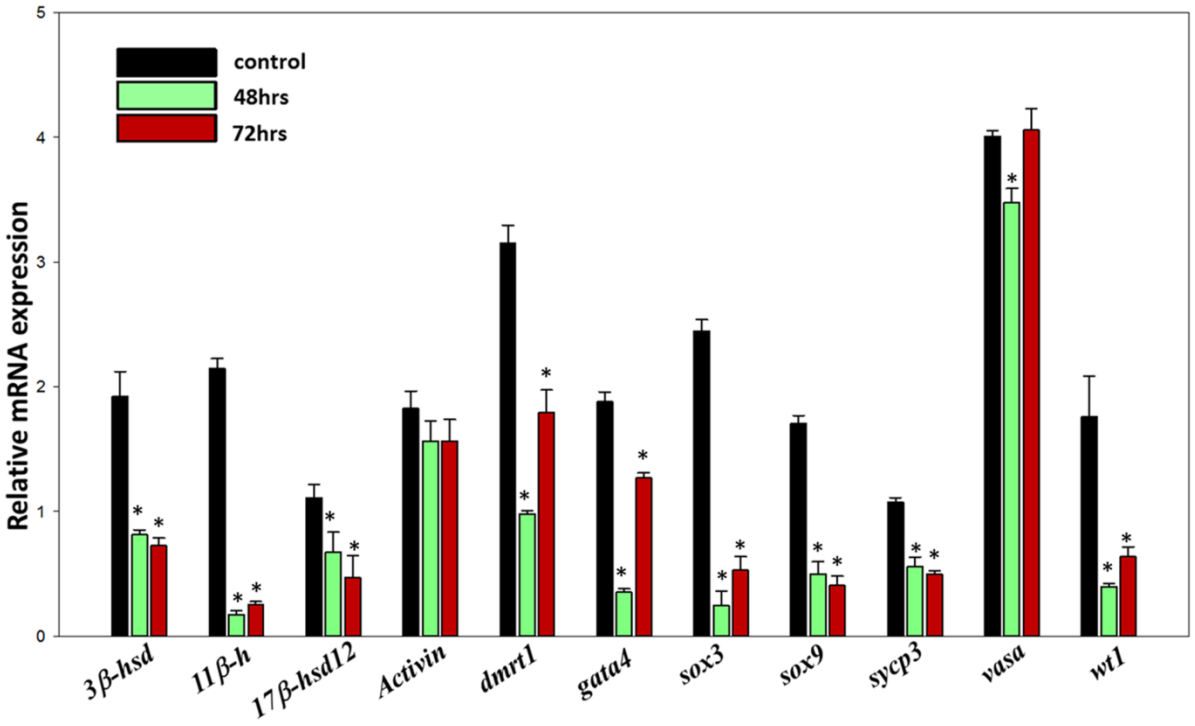


Fig. 12. Expression analysis of various testis-related factors and steroidogenic enzyme genes which are crucial for germ cell development and spermatogenesis after *c-kit*-esiRNA transient silencing. Expression levels of each gene are compared to their respective control levels (*, $P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

4. Discussion

Present study reports the cloning of a cDNA fragment harbouring the signature domain of *c-kit* from catfish, *C. gariepinus* testis using RT-PCR and RACE strategy. Expression analysis of *c-kit* during embryonic development stages and ontogeny showed elevated expression levels during critical periods of gonadal cycle. Tissue distribution and reproductive phase expression analysis also showed elevated levels of *c-kit* in adult gonads as well as during gonadal recrudescence. ISH revealed the localization of mRNA transcripts in the spermatogonia and spermatocytes of catfish testis and the same pattern was observed for c-kit protein using IHC and IF.

C-kit is a proto-oncogene and its expression has been established in a variety of cells such as germ cells, melanocytes, and hematopoietic cells of several mammalian species (Besmer *et al*., 1993; Sette *et al*., 2000; Shin *et al*., 2014). Extensive studies on its expression and detection have been carried out in mammals such as mouse (Yoshinaga *et al*. 1991), rat (Wershil *et al*., 1992), and primates (Galli *et al*., 1993; Scalercio *et al*., 2105), but the studies have been extremely limited in lower vertebrates such as teleosts as far as current literature is concerned. The present study corroborated with these observations, by initially cloning a partial *c-kit* fragment (1.5 kb) from catfish testis by RT-PCR followed by RACE that includes signature domain regions and a protein band of ~60 kDa molecular mass was also detected with a specific polyclonal antibody against c-kit in the protein lysate isolated from testis, which may correspond to the cytoplasmic terminal part of the receptor or a truncated isoform (Rossi *et al*., 1992; Albanesi *et al*., 1996). Tissue distribution analysis demonstrated higher *c-kit* expression in gonads of both male and female, when compared to moderate expression in kidney and low in liver and muscle. The role of *c-kit* in gonadal development and gametogenesis has been well documented in humans and mice (Besmer *et al*., 1993; Lammie *et al*., 1995; Loveland and Schlatt; Hutt *et al*., 2006) as well as in zebrafish (Yao and Ge, 2010, 2013).

The expression of *c-kit* showed a significant increase during preparatory, pre-spawning and spawning phases in males while there is a decrease in the pre-spawning phase in females which might be due to the difference in hormonal regulation pattern (Senthilkumaran *et al*., 2004; Martyniuk *et al*., 2009). The expression was minimal during the resting phase where spermatogenesis is relatively quiescent. In embryonic stages, *c-kit* expression analysis showed a marginal but constant expression level up to 20 h and increased significantly at 30 h

(the hatched larval stages) which is essentially the period of migration of PGCs to the genital ridge (Nishimura and Tanaka, 2014). Although there are limited reports of stage specific *c-kit* expression in teleost species, PGCs have been shown to express *c-kit* in embryonic stages of humans and mice (Manova and Bachvarova, 1991; Pesce *et al*., 1997; Høyer *et al*., 2005). In mice embryo, *c-kit* mRNA were detected in PGCs as early as 6.5–7 days post coitus (dpc) which continued during their subsequent proliferation and migration to the genital ridge (Manova *et al*., 1991). Various studies have also shown that mutations in the genes encoding either c-Kit or its ligand display abnormal germ cell migration and proliferation during this period (Besmer *et al*., 1993; Bernex *et al*., 1996, Chen *et al*., 2013).

Ontogeny studies at different time points showed marginal levels of *c-kit* expression from 0 to 40 dph, with a dramatic increase from 50 dph, which correspond to the onset and progression of gonadal development and sex differentiation in catfish (Raghuveer *et al*., 2011). This is also marked by an increase in germ cell population leading to elevated expression of germ cell markers such as *vasa* (Raghuveer and Senthilkumaran, 2010a). This pattern may be in minor parallel to the reports on mice where male PGCs, after their arrest at the G0/G1 of the cell cycle at around 13.5 days post-coitum (dpc), resume mitotic division again around 3 days after birth which results in a dramatic reduction of *c-kit* expression and can be detected again only in differentiated spermatogonia (Orr-Urtreger *et al*., 1990; Yoshinaga *et al*., 1991). Such a pattern of expression of *c-kit* in catfish corroborates these findings. In addition, the dimorphic expression pattern where males display a higher level of expression during 50-75 dph may be due to the difference in timing of meiotic entry of germ cells in fish (Martínez *et al*., 2014), where males begin to show a higher proliferation rate of spermatogonia only after ~40 dph as found in medaka (Nishimura and Tanaka, 2014).

The reproductive seasonality and gonadal activity in catfish are under the influence of gonadotropins which also exhibit seasonal pattern (Joy *et al*., 2000; Kirubagaran *et al*., 2005). In view of this, the effects of gonadotropins on *c-kit* expression were examined using *in vitro* and *in vivo* induction with hCG. The induction resulted in significant elevations in *c-kit* expression levels which implied that this gene may also be under the influence of gonadotropins, albeit indirectly through stimulation of Sertoli cells that in turn activate signalling cascades such as BMP4 and retinoic acid to increase *c-kit* levels in the developing germ cells as in mice (Zhang *et al*., 2011). This contention requires additional studies in catfish to correlate BMP4 and retinoic acid. Nevertheless, gonadotropin dependency of *c-kit* expression is well validated under *in vitro* and *in vivo* conditions for the first time in any lower vertebrate.

Using an antibody directed against the C terminus, intense immunoreactivity of c-kit was detected in primary and secondary spermatogonia as well as in spermatocytes, but no significant signals were seen in spermatids/sperm in the lumen. A similar pattern of localization was also observed in ISH for *c-kit* transcripts in the mice testis, with expression seen mostly in developing spermatogonia and spermatocytes (Manova *et al*., 1990). Additionally, the pattern of signals for both localization experiments showed minor heterogeneity where some areas within the same tubule showed more prominent signals than others. These results were in agreement with localization in mammalian species where the c-kit receptor expression has been detected in the early spermatogenic cells, but differ in the fact that localization is also seen in the late stages of spermatogenesis particularly in the acrosomal granules in human and rodent testis and sperm (Sandlow *et al*., 1997; Prabhu *et al*., 2006; Unni *et al*., 2009), which is not the case in catfish. To the best of our knowledge

based on current available literature, there have been no previous reports of such stage-specific expression of c-kit in the spermatogonial cells in teleosts.

The present study also aimed to examine the influence of PEI-mediated transient silencing of *c-kit* in both *in vitro* and *in vivo* systems. As explained earlier in the Chapter 2, transient gene silencing effects can be well demonstrated using PEI mediated esiRNA silencing *in vitro* and *in vivo* (Höbel and Aigner, 2010, 2013). The results obtained in the studies in catfish also validate the protocol (Prathibha and Senthilkumaran, 2016; Murugananthkumar and Senthilkumaran, 2016; Sudhakumari *et al*., 2017). Transection with *c-kit*-esiRNA via PEI delivery resulted in a significant downregulation of *c-kit* expression levels in both *in vitro* and *in vivo* transient gene silencing. Consequently, the treatment of *c-kit* esiRNA also resulted in the alteration of expression levels of several testis-related factors and steroidogenic enzyme genes critical for germ cell and gonadal development, either directly or indirectly. In mice, siRNA-mediated silencing of *c-kit* in mouse primary spermatogonial cells with anti-*c-kit* siRNA was found to result in DNA fragmentation and cell cycle arrest at G(2)/M phase that diminished cell viability and proliferation (Sikarwar and Reddy, 2008). However, knockdown/mutation experiments that explore in detail the impact of the silencing of this gene during gametogenesis is lacking in both mammals as well as teleosts in particular. Down-regulation of germ cell markers crucial for meiosis, *sycp3* and *vasa*, implies the role of *c-kit* in spermatogonial differentiation and meiotic progression in agreement with studies in humans and rodents (Rossi *et al*., 2008; Medrano *et al*., 2012, Syrjänen *et al*., 2014). The activation of c-Kit signalling has a decisive role in mice spermatogenesis through meiosis activation at specific time points, which results in the ensuing expression of early meiotic markers such as *Dmc1* and *Sycp3* (Vincent *et al*., 1998; Di Carlo *et al*., 2000). The expression

levels of *wt1* and *gata4*, genes that are known to play a key role during testicular development of catfish (Murugananthkumar and Senthilkumaran, 2016), were also found to be significantly decreased. Transient silencing experiments of these genes in catfish have also negatively affected the expression levels of several steroidogenic enzyme genes involved in androgen production (Murugananthkumar and Senthilkumaran, 2016), which may indirectly implicate the involvement of *c-kit* in germ cell development and gametogenesis by affecting important testis-related transcription factors. This may reflect a similar situation in the down-regulation of other transcription factors such as *dmrt1*, *sox3* and *sox9*, whose critical roles have been well documented in gonadal development as well as spermatogenesis in catfish (Raghuveer and Senthilkumaran, 2009, 2010b; Raghuveer *et al*., 2011; Rajakumar and Senthilkumaran, 2014a). In addition, analysis of *c-kit* transient silencing also decreased the expression levels of several analyzed steroidogenic enzyme genes, either directly or indirectly. Although the exact mechanism has not yet been elucidated, aberrations in expression of such key enzyme genes related to testicular function (Raghuveer and Senthilkumaran, 2012; Rajakumar and Senthilkumaran, 2014b, 2015) may indirectly associate the role of *c-kit* in germ cell development and gametogenesis. Activin may have a different mode to enhance spermatogenesis as it is known to induce proliferation of spermatogonia in eel (Miura *et al*., 1995). Lack of effect on activin also shows no global effect supporting *c-kit* dependent effect of esiRNA silencing. However, further studies are required to establish the exact cascade of molecular events that occur between these genes.

In summary, this study described the cloning, localization and expression analysis of *c-kit* receptor in catfish testis during germ cell development and recrudescence. This may be one of the few detailed reports of this gene in lower vertebrates such as teleosts, specifically in

annual breeders. This study implicated the plausible involvement of *c-kit* during the critical stages of reproductive development and gametogenesis via its expression pattern and localization in the germ cells of catfish. The regulatory effects of gonadotropins on the gene were also evident in the *in vitro* and *in vivo* induction studies. In addition, in *vitro* and *in vivo* transient gene silencing of *c-kit* using esiRNA caused a significant downregulation in the expression levels of various testis related genes analyzed, which may infer the role of this gene in testicular germ cell development and recrudescence, either directly or indirectly.

References

Albanesi C., Geremia R., Giorgio M., Dolci S., Sette C., Rossi P., 1996. A cell- and developmental stage-specific promoter drives the expression of a truncated c-kit protein during mouse spermatid elongation. Development 122, 1291-1302.

Alexeev V., Yoon K., 2006. Distinctive role of the c-Kit receptor tyrosine kinase signaling in mammalian melanocytes. J. Invest. Dermatol. 126, 1102-1110.

Bernex F., De Sepulveda P., Kress C., Elbaz C., Delouis C., Panthier J.J., 1996. Spatial and temporal patterns of c-kit-expressing cells in WlacZ/+ and WlacZ/WlacZ mouse embryos. Development 122, 3023-3033.

Besmer P., Manova K., Duttlinger R., Huang E.J., Packer A., Gyssler C., Bachvarova R.F., 1993. The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. Dev. Suppl. 125-137.

Bokemeyer C., Kuczyk M.A., Dunn T., Serth J., Hartmann K., Jonasson J., Pietsch T., Jonas U., Schmoll H.J., 1996. Expression of stem-cell factor and its receptor c-kit protein in normal testicular tissue and malignant germ-cell tumours. J. Cancer Res. Clin. Oncol. 122, 301-306.

Bosseboeuf A., Gautier A., Auvray P., Mazan S., Sourdaine P., 2013. Characterization of spermatogonial markers in the mature testis of the dogfish (*Scyliorhinus canicula* L.). Reproduction 147, 125-139.

Chen L., Faire M., Kissner M.D., Laird D.J., 2013. Primordial germ cells and gastrointestinal stromal tumors respond distinctly to a cKit overactivating allele. Hum. Mol. Genet. 22, 313-327.

De Miguel M.P., Cheng L., Holland E.C., Federspiel M.J., Donovan P.J., 2002. Dissection of the c-Kit signaling pathway in mouse primordial germ cells by retroviral-mediated gene transfer. Proc. Natl. Acad. Sci. USA. 99, 10458-1063.

Di Carlo A.D., Travia G., De Felici M., 2000. The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. Int. J. Dev. Biol. 44, 241-244.

Edling C.E., Hallberg B., 2007. c-Kit--a hematopoietic cell essential receptor tyrosine kinase. Int. J. Biochem. Cell Biol. 39, 1995-1998.

Falco A., Encinas P., Carbajosa S., Cuesta A., Chaves-Pozo E., Tafalla C., Estepa A., Coll J.M., 2009. Transfection improvements of fish cell lines by using deacylated polyethylenimine of selected molecular weights. Fish Shellfish Immunol. 26, 559–566.

Galli S.J., Iemura A., Garlick D.S., Gamba-Vitalo C., Zsebo K.M., Andrews R.G., 1993. Reversible expansion of primate mast cell populations *in vivo* by stem cell factor. J. Clin. Invest. 91, 148–152.

Galli S.J., Tsai M. Wershil B.K., 1993. The c-kit receptor, stem cell factor, and mast cells. What each is teaching us about the others. Am. J. Pathol. 142, 965–974.

Höbel S., Aigner A., 2010. Polyethylenimine (PEI)/siRNA-mediated gene knockdown *in vitro* and *in vivo*. Methods Mol. Biol. 623, 283–297.

Höbel S., Aigner A., 2013. Polyethylenimines for siRNA and miRNA delivery *in vivo*. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 5, 484-501.

Hong Y., Liu T., Zhao H., Xu H., Wang W., Liu R., Chen T., Deng J., Gui J., 2004. Establishment of a normal medakafish spermatogonial cell line capable of sperm production *in vitro*. Proc. Natl. Acad. Sci. USA. 101, 8011-8016.

Høyer P.E., Byskov A.G., Møllgård K., 2005. Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries. Mol. Cell Endocrinol. 234, 1-10.

Hutt K.J., McLaughlin E.A., Holland M.K., 2006. Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. Mol. Hum. Reprod. 12, 61-69.

Isozaki K., Hirota S., Nakama A., Miyagawa J., Shinomura Y., Xu Z., Nomura S., Kitamura Y., 1995. Disturbed intestinal movement, bile reflux to the stomach, and deficiency of c-kit-expressing cells in Ws/Ws mutant rats. Gastroenterology. 109, 456-464.

Joy K.P., Singh M.S., Senthilkumaran B., Goos H.J., 2000. Pituitary-gonadal relationship in the catfish *Clarias batrachus* (L): A study correlating gonadotrophin-ii and sex steroid dynamics. Zoolog. Sci. 17, 395–404.

Kirubagaran R., Senthilkumaran, B., Sudhakumari C.C., Joy K.P., 2005. Seasonal dynamics in gonadotropin secretion and E(2)-binding in the catfish *Heteropneustes fossilis*. Fish Physiol. Biochem. 31, 183-188.

Lammie A., Drobnjak M., Gerald W., Saad A., Cote R., Cordon-Cardo C., 1994. Expression of c-kit and kit ligand proteins in normal human tissues. J. Histochem. Cytochem. 42, 1417-1425.

Loppion G., Crespel A., Martinez A.S., Auvray P., Sourdaine P., 2008. Study of the potential spermatogonial stem cell compartment in dogfish testis, *Scyliorhinus canicula* L. Cell Tissue Res. 332, 533–542.

Loveland K.L., Schlatt S.J, 1997. Stem cell factor and c-kit in the mammalian testis: lessons originating from Mother Nature's gene knockouts. J. Endocrinol. 153, 337-344.

Manova K., Bachvarova R.F., 1991. Expression of c-kit encoded at the W locus of mice in developing embryonic germ cells and presumptive melanoblasts. Dev. Biol. 146, 312-324.

Manova K., Huang E.J., Angeles M., De Leon V., Sanchez S., Pronovost S.M., Besmer P., Bachvarova R.F., 1993. The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia. Dev. Biol. 157, 85–99.

Manova K., Nocka K., Besmer P., Bachvarova R.F., 1990. Gonadal expression of c-kit encoded at the W locus of the mouse. Development 110, 1057-1069.

Martínez P., Viñas A.M., Sánchez L., Díaz N., Ribas L., Piferrer F., 2014. Genetic architecture of sex determination in fish: applications to sex ratio control in aquaculture. Front. Genet. 5: 340.

Martyniuk C.J., Kroll K.J., Porak W.F., Steward C., Grier H.J., Denslow N.D., 2009. Seasonal relationship between gonadotropin, growth hormone, and estrogen receptor mRNA expression in the pituitary gland of largemouth bass. Gen. Comp. Endocrinol. 163, 306-317.

Mauduit C., Hamamah S., Benahmed M., 1999. Stem cell factor/c-kit system in spermatogenesis. Hum. Reprod. Update 5, 535–545.

Medrano J.V., Ramathal C., Nguyen H.N., Simon C., Reijo Pera R.A., 2012. Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived *in vitro*. Stem Cells. 30, 441-451.

Miura T., Miura C., Yamauchi K., Nagahama Y., 1995. Human recombinant activin induces proliferation of spermatogonia *in vitro* in the Japanese eel *Anguilla japonica*. Fisheries Sci. 63, 434–437.

Murugananthkumar R., Senthilkumaran B., 2016. Expression analysis and localization of wt1, ad4bp/sf-1 and gata4 in the testis of catfish, *Clarias batrachus*: Impact of wt1-esiRNA silencing. Mol. Cell Endocrinol. 431, 164–176.

Nakatsuji N., Chuma S., 2001. Differentiation of mouse primordial germ cells into female or male germ cells. Int. J. Dev .Biol. 45, 541-548.

Nishimura T., Tanaka M., 2014. Gonadal Development in Fish. Sex. Dev. 8, 252-261.

Orr-Urtreger A., Avivi A., Zimmer Y., Givol D., Yarden Y., Lonai P., 1990. Developmental expression of c-kit, a proto-oncogene encoded by the W locus. Development. 109, 911-923.

Parichy D.M., Rawls J.F., Pratt S.J., Whitfield T.T., Johnson S.L., 1999. Zebrafish sparse corresponds to an orthologue of c-kit and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. Development 126, 3425-3436.

Pesce M., Di Carlo A., De Felici M., 1997. The c-kit receptor is involved in the adhesion of mouse primordial germ cells to somatic cells in culture. Mech. Dev. 68, 37-44.

Prabhu S.M., Meistrich M.L., McLaughlin E.A., Roman S.D., Warne S., Mendis S., Itman C., Loveland K.L., 2006. Expression of c-Kit receptor mRNA and protein in the developing, adult and irradiated rodent testis. Reproduction 131, 489-499.

Prathibha Y., Senthilkumaran B., 2016. Involvement of pax2 in ovarian development and recrudescence of catfish: a role in steroidogenesis. J. Endocrinol. 231, 181-195.

Puxeddu I., Piliponsky A.M., Bachelet I., Levi-Schaffer F., 2003. Mast cells in allergy and beyond. Int. J. Biochem. Cell Biol. 35, 1601-1607.

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

Raghuveer K., Senthilkumaran B., 2010a. Cloning and differential expression pattern of *vasa* in the developing and recrudescing gonads of catfish, *Clarias gariepinus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 79–85.

Raghuveer K., Senthilkumaran B., 2010b. Isolation of *sox9* duplicates in catfish: localization, differential expression pattern during gonadal development and recrudescence, and hCG-induced up regulation of *sox9* in testicular slices. Reproduction 140, 477-487.

Raghuveer K., Senthilkumaran B., 2012. Cloning and expression of 3β-hydroxysteroid dehydrogenase during gonadal recrudescence and after hCG induction in the air-Breathing catfish, *Clarias gariepinus*. Steroids 77, 1133-1140.

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.

Rajakumar A., Senthilkumaran B., 2014a. Expression analysis of *sox3* during testicular development, recrudescence and after hCG induction in catfish, *Clarias batrachus*. Sex. Dev. 8, 376-386.

Rajakumar A., Senthilkumaran B., 2014b. Molecular cloning and expression analysis of 17b-hydroxysteroid dehydrogenase 1 and 12 during gonadal development, recrudescence and after *in vivo* hCG induction in catfish, *Clarias batrachus*. Steroids 92, 81-89.

Ransom D.G., Haffter P., Odenthal J., Brownlie A., Vogelsang E., Kelsh R.N., Brand M., van Eeden F.J., Furutani-Seiki M., Granato M., Hammerschmidt M., Heisenberg C.P., Jiang Y.J., Kane D.A., Mullins M.C., Nüsslein-Volhard C., 1996. Characterization of zebrafish mutants with defects in embryonic hematopoiesis. Development 123, 311-319.

Rawls J.F., Johnson S.L., 2000. Zebrafish kit mutation reveals primary and secondary regulation of melanocyte development during fin stripe regeneration. Development 127, 3715-3724.

Roskoski R. Jr., 2005. Signaling by Kit protein-tyrosine kinase – the stem cell factor receptor. Biochem. Biophys. Res. Commun. 337, 1–13.

Rossi P., Lolicato F., Grimaldi P., Dolci S., Di Sauro A., Filipponi D., Geremia R., 2008. Transcriptome analysis of differentiating spermatogonia stimulated with kit ligand. Gene Expr. Patterns. 8, 58-70.

Rossi P., Marziali G., Alanesi C., Charlesworth A., Geremia R., Sorrentino V., 1992. A novel c-kit transcript, potentially encoding a truncated receptor, originates within a kit gene intron in mouse spermatids. Dev. Biol. 152, 203–207.

Sandlow J.I., Feng H.L., Sandra A., 1997. Localization and expression of the c-kit receptor protein in human and rodent testis and sperm. Urology 49, 494-500.

Scalercio S.R., Brito A.B., Domingues S.F., Santos R.R., Amorim C.A., 2015. Immunolocalization of growth, inhibitory, and proliferative factors involved in initial ovarian folliculogenesis from adult common squirrel monkey (*Saimiri collinsi*). Reprod. Sci. 22, 68-74.

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

Sette C., Dolci S., Geremia R., Rossi P., 2000. The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. Int. J. Dev . Biol. 44, 599-608.

Shin J.Y., Hu W., Naramura M., Park C.Y., 2014. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. J. Exp. Med. 211, 217-231.

Sikarwar A.P., Reddy K.V., 2008. siRNA-mediated silencing of c-kit in mouse primary spermatogonial cells induces cell cycle arrest. Oligonucleotides. 18, 145-160.

Stankov K., Popovic S., Mikov M., 2014. C-KIT signaling in cancer treatment. Curr. Pharm. Des. 20, 2849-2880.

Sudhakumari C.C., Anitha A., Murugananthkumar R., Tiwari D.K., Bhasker D., Senthilkumaran B., Dutta-Gupta A., 2017. Cloning, localization and differential expression of Neuropeptide-Y during early brain development and gonadal recrudescence in the catfish, *Clarias gariepinus*. Gen. Comp. Endocrinol. https://doi.org/10.1016/j.ygcen.2017.03.006.

Syrjänen J.L., Pellegrini L., Davies O.R., 2014. A molecular model for the role of SYCP3 in meiotic chromosome organisation. Elife 20: 3.

Unni S.K., Modi D.N., Pathak S.G., Dhabalia J.V., Bhartiya D., 2009. Stage-specific localization and expression of c-kit in the adult human testis. J. Histochem. Cytochem. 57, 861-869.

Vincent S., Segretain D., Nishikawa S., Nishikawa S.I., Sage J., Cuzin F., Rassoulzadegan M., 1998. Stage-specific expression of the Kit receptor and its ligand (KL) during male gametogenesis in the mouse: a Kit-KL interaction critical for meiosis. Development 125, 4585-4593.

Weinstein B.M., Schier A.F., Abdelilah S., Malicki J., Solnica-Krezel L., Stemple D.L., Stainier D.Y., Zwartkruis F., Driever W., Fishman M.C., 1996. Hematopoietic mutations in the zebrafish. Development 123, 303-309.

Wershil B.K., Tsai M., Geissler E.N., Zsebo K.M., Galli S.J., 1992. The rat c-kit ligand, stem cell factor, induces c-kit receptor-dependent mouse mast cell activation in vivo. Evidence that signaling through the c-kit receptor can induce expression of cellular function. J. Exp. Med. 175, 245-255.

Yao K., Ge W., 2010. Kit system in the zebrafish ovary: evidence for functional divergence of two isoforms of kit (kita and kitb) and kit ligand (kitlga and kitlgb) during folliculogenesis. Biol. Reprod. 82, 1216-1226.

Yao K., Ge W., 2013. Spatial distribution and receptor specificity of zebrafish Kit system--evidence for a Kit-mediated bi-directional communication system in the preovulatory ovarian follicle. PLoS One 8: e56192.

Yoshinaga K., Nishikawa S., Ogawa M., Hayashi S., Kunisada T., Fujimoto T., Nishikawa S., 1991. Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development. 113, 689-699.

Zhang L., Tang J., Haines C.J., Feng H.L., Lai L., Teng X., Han Y., 2011. c-kit and its related genes in spermatogonial differentiation. Spermatogenesis 1, 186–194.

Zhang L., Tang J., Haines C.J., Feng H., Lai L., Teng X., Han Y., 2013. c-kit expression profile and regulatory factors during spermatogonial stem cell differentiation. BMC Dev. Biol. 13, 38.

Zhang S.C., Fedoroff S., 1997. Cellular localization of stem cell factor and c-kit receptor in the mouse nervous system. J. Neurosci. Res. 47, 1-15.

Zhou X., Laroche F., Lamers G.E.M., Torraca V., Voskamp P., Lu T., Chu F., Spaink H.P., Abrahams J.P., Liu Z., 2012. Ultra-small graphene oxide functionalized with polyethylenimine (PEI) for very efficient gene delivery in cell and zebrafish embryos. Nano Res. 5, 703-709.

The current research work aimed to identify new factors which are involved in gonadal development using a proteomic approach, by analysing the differential expression patterns of various proteins, in both altered (endocrine disruption) and normal physiological (juvenile and adult) conditions. This resulted in the identification of a germ-cell marker *sycp3* which was upregulated during juvenile stages. Further, from the results obtained in the investigation, another related gene, *c-kit* was also selected for the study due to its potential relation to *sycp3*. The role of *sycp3* along with *c-kit* in germ cell development and spermatogenesis were then examined during juvenile and adult stages using localization and gene expression pattern analysis in various ontogenic stages, tissue-wise and during seasonal reproductive cycle. The possible interactions of these two genes, either directly or indirectly, were also studied by transient silencing both genes using polyethylenimine mediated transfection of esiRNA, followed by analysis of the impact of their knockdown on various testis-related transcription factors and steroidogenic enzyme genes.

Each chapter has been provided with their separate bibliography to correlate previous findings with our current study.

Chapter 1: Proteome analysis of gonads: endocrine disruption and during juvenile and adulthood

A large-scale analysis of proteins that are involved in important physiological processes such as gonadal and germ cell development using proteomics can contribute greatly to identify novel factors. The present study analyzed the differential expression patterns of various proteins, in both altered (endocrine disruption) and normal physiological (juvenile and adult) conditions. The first approach was the use of endocrine disruption as a model to alter the normal physiological conditions in adult (recrudescing) gonads, to observe any changes in the

expression patterns of proteins that may be crucial for reproductive competency. Endocrine disruption using pesticides such as endosulfan and malathion, have been well established in their capacity to insult normal testicular and ovarian development as evidenced by previous reports. Through this approach, the present study identified numerous proteins that were differentially expressed in the pesticide-treated gonad when compared to the control. This has been described in detail.

1a. Two-dimensional proteomic analysis of gonads of air-breathing catfish, *Clarias batrachus* after the exposure of endosulfan and malathion

Endocrine disrupting chemicals have raised public concern, since their effects have been found to interfere with the physiological systems of various organisms, particularly during important stages of development and reproduction. Endosulfan and malathion, pesticides widely used for agricultural purposes, have been known to disrupt physiological functions in aquatic organisms. The work analyzed the effects of endosulfan (2.5 parts per billion [ppb]) and malathion (10 ppb) on the reproductive physiology of catfish (*C. batrachus*) by evaluating protein expression profiles after 21 days of exposure. The proteomic profile of testis and ovary after exposure to endosulfan showed downregulation of proteins such as ubiquitin and Esco2, and upregulation in melanocortin-receptor-2 respectively. Malathion exposed ovary showed upregulated prolactin levels. Such identification of differentially expressed proteins in gonads due to the exposure to these pesticides may serve as key indications to denote their disruptive effects at the level of proteins.

Unfortunately, since this approach did not yield any significant results in identification of factors directly related to germ cell or gonadal development, with the exception of ubiquitin, which is a well-known factor related to apoptosis that partially determines progression of spermatogenesis. The second approach was the use of normal physiological conditions to

examine the differential expression of proteins, comparing adult gonads with juvenile stages of *C. gariepinus*. Since the fecundity, hatchability and growth process of *C. gariepinus* are better than *C. batrachus* in laboratory conditions, the former catfish model has been used for further studies. This resulted in the identification of a germ-cell marker Synaptonemal complex protein-3 (Sycp3) which was upregulated in juvenile catfish testis when compared to adult.

Chapter 2: Identification, cloning and expression profile of *sycp3* during gonadal cycle and after esiRNA silencing in catfish

Sycp3 is a meiosis-specific component, essential for the synapsis of homologous chromosomes, and also a marker for the event. Although prior studies have established its role in meiosis and gametogenesis in the process of gonadal germ cell development during critical stages mostly in mammals, additional information about its involvement is important to elucidate its roles. Degenerate primers were designed based on the identified Sycp3 protein sequence using MALDI-TOF, and the full length cDNA of *sycp3* was cloned from catfish testis. The spatio-temporal expression pattern of *sycp3* was analyzed during the critical time period of gonadal development and recrudescence in catfish using real-time PCR. Polyclonal antibodies (based on deduced amino acid sequence) were raised to localize the protein using immunohistochemistry (IHC) while transcripts in testis were identified using *in situ* hybridization (ISH). Endonuclease prepared small interfering RNAs (esiRNA) targeted against *sycp3* gene in catfish testis was used to transiently silence *sycp3* gene *in vitro* and *in vivo*, and analyzed its transient knockdown effects as well as the impact of the silencing on other genes crucial for germ cell and testicular development. Tissue distribution in adult testis and ontogeny expression analysis indicated elevated expression levels in developing as well

as adult testis. Localization of Sycp3 protein and transcripts in the spermatogonia and spermatocytes of adult testis implies its involvement in spermatogenesis. Induction using human chorionic gonadotropin (hCG) was found to upregulate the expression levels of *sycp3* even at those phases where the normal levels of this gene expression was found to be low. This might serve as an indication to infer that gonadotropin stimulates germ cell proliferation during recrudescence. Transient silencing of *sycp3* using *sycp3*-esiRNA decreased the expression of *sycp3* as well as genes which are crucial for gonadal development and spermatogenesis either directly or indirectly. The present report suggests that *sycp3* might have a potential role in the development and maintenance of testicular function in catfish.

Chapter 3: Expression analysis of *c-kit* during germ cell development, recrudescence and after esiRNA silencing

C-kit is a member of a family of growth factor receptors that have tyrosine kinase activity, and studies in mammals have shown its involvement in spermatogonial proliferation, differentiation, survival and their subsequent entry into meiosis. In addition, the activation of *c-kit* during spermatogonial differentiation resulted in the activation of early meiotic markers. Since *sycp3* transient silencing showed that the *sycp3* knockdown did not have any significant influence on *c-kit* expression though the latter is known to have a role in spermatogenesis, the role of this gene was then examined in germ cell development and recrudescence. The tissue distribution analysis in adult catfish showed higher expressions mainly in the gonads of both male and female, and moderate expression in kidney and low in liver and muscle. The relative mRNA levels in the catfish embryos post fertilization showed a consistent expression during various stages of development of the post hatch stages. Ontogeny studies showed minimal expression during early stages of development and highest

during 50-75 dph, and the dimorphic expression decreased gradually by adulthood. *C-kit* expression during various phases of reproductive cycle in both male and female showed minimal expression during resting phase, and the difference in the expression levels between male and females during the pre-spawning phase might be due to the difference in hormonal regulation during this stage in both sexes. The enhanced expression of *c-kit* upon hCG induction suggests that this gene is under the regulation of hypothalamo-hypophyseal axis, most importantly by gonadotropins. C-kit protein was localized in the testis using IHC and transcripts were identified using ISH, which both showed localization in spermatogonia and spermatocytes, but no positive signals in spermatids/sperm. *In vivo* and *in vitro* transient silencing using *c-kit*-esiRNA during recrudescence showed a decrease in *c-kit* expression, which also affected the expression level of germ cell meiotic marker *sycp3*, as well as certain steroidogenic enzyme genes and factors related to germ cell maturation/gametogenesis. The present observations suggest that *c-kit* may have a crucial role in germ cell proliferation, development and maturation during gonadal recrudescence in catfish.

Conclusions

- Sycp3 identified via proteomic approach, was found to have a potential role in germ cell development as well as spermatogenesis of catfish, through analysis of its expression in various stages of ontogeny, seasonal reproductive phases, tissues and localization patterns in the testis.
- Transient silencing of *sycp3* studies using esiRNA downregulated the expression levels of other testis related genes which suggests that it might have a critical role in the germ cell development in catfish.

- The role of *c-kit* during germ cell development and recrudescence was examined by analyzing its expression in various stages of ontogeny, seasonal reproductive phases, tissues and localization patterns in the testis.
- Transient silencing of *c-kit* using esiRNA downregulated expression levels of several testis related factors, steroidogenic enzyme genes and germ cell marker *sycp3*.
- It is possible that *c-kit* may directly or indirectly affect the expression of an important germ cell marker *sycp3*, vis-à-vis having a crucial role in the development and proliferation of germ cells along with various testis related factors and steroidogenic enzyme genes.

Research publication from thesis work:

- **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.**

***Other chapters are in the process of submission to peer-reviewed journals.*

Research publication with members of Prof. B. Senthilkumaran's laboratory:

1. 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**.

2. Rajakumar A, Singh R, Chakrabarty S, Murugananthkumar R, **Laldinsangi, C**, Prathibha Y, Sudhakumari CC, Dutta-Gupta A, Senthilkumaran B. (**2012**). Endosulfan and flutamide impair testicular development in the juvenile Asian catfish, *Clarias batrachus*. **Aquat. Toxicol. 110, 123– 132**

Review chapter:

3. Prathibha, Y.*, **Laldinsangi, C**.* and Senthilkumaran, B. (**2013**). Perspectives on gonadotropin-releasing hormone–gonadotropins-monoamines: Relevance to gonadal differentiation and maturation in teleosts. In: "***Sexual Plasticity and Gametogenesis in Fishes***" (B. Senthilkumaran, Ed.), Nova Biomedical, USA (A Review) Chapter XXI, 341-362. (* *equal contribution*)