General Introduction
Introduction:

Sexual reproduction in animal kingdom which empowers the organism to propagate and survive in the perpetually changing environment by enriching its genome through recombination includes two processes, one is the determination of the sex of organism and other is the production of gametes. Inception of studying the sex determining mechanism took a lead from 1930 with the discovery of variety of sex determination mechanisms for example in *Drosophila* (genic balance X:A ratio), in *Dinophilis* (size of larvae), in *Bonelias viridis* (parasitic association), in Hymenoptera insects (haplo-diploidy), in Lepidopteran (ZW/ZZ), in *Matsococcus gallicola* (male X₁X₂X₃X₄X₅X₆O), in fishes and amphibians variable patterns of sex determination system prevails, in reptiles (temperature dependent), in birds (ZW/ZZ system with female heterogamety), in mammals 2-factor (XX/XY with male heterogamety) system (Bull, 1983; Manalakou *et al*., 2006). Sexual plasticity in fish is due to its diverse biology and ecology, which influences these species to engage in various sex-determination mechanisms, thus empowering it to survive and maintain a stable sex ratio. Because they are amenable to artificial culture and experimental analysis in many cases, fish also provide unique opportunities to investigate and test theoretical concepts of sex determination, ranging from evolutionary mechanisms to biochemical processes. Similarly, in aquaculture systems, understanding and controlling reproduction is central to the efficient propagation of organisms due to differential growth rates of sexes and a need for synchronous and reliable maturation.
Gonadal development and differentiation:

A brief introduction related to gonadal development and differentiation of fish in comparison to higher vertebrates including mammals will help us appreciate the need to probe sex development in varied fish species. Gonadal (ovary/testis) development in vertebrates begins with the formation of a genital ridge (Balinsky, 1975), which appears as a longitudinal thickening of mesoderm protruding into the coelomic cavity ventral to the developing kidney and lateral to the dorsal mesentery. They are made up of two distinct type of cells 1) gamete forming cells and 2) supporting somatic cells, having different developmental origin. Gamete forming cells are derived from primordial germ cells (PGCs) that stem from the vegetal portion of the unfertilized egg rich in granular cytoplasmic (Wei and Mahowald, 1994) substances. PGCs subsequently get surrounded by somatomesodermal cell and are transferred to the genital ridge through morphogenetic movements of mesoderm and cell migratory activities (Hamaguchi, 1992). They do not differ in both sexes and remain undetermined until subjected to hormonal and other endogenous or exogenous factors (environment) driving them to transform into spermatogonia or oogonia. Somatic cells on the other side are derived from cortex epithelial layer, and they are also similar in presumptive males and females. In mammals, the Y linked sex-determination gene, Sry, recruits mesonephric cells into the gonad in males (Sinclair et al., 1990; Koopman, 1999). In medaka Y linked DMY is implicated with the putative role to induce development of somatic cell into Sertoli cells in male (Matsuda et al., 2002). Subsequently, the somatic cells surrounding PGCs differentiate into seminiferous tubules and supporting connective tissue, and into cells similar to Leydig and Sertoli cells found in mammals (Van Vuren and Soley, 1990). During ovarian
development, the somatic cells and PGCs differentiate to form follicles, comprised of oocytes surrounded by an inner granulosa and outer thecal layers. Histologically, ovarian development in females is first detectable with the proliferation of somatic and oogonial cells followed by early oocyte differentiation and the formation of the ovarian cavity. Testicular development is found later than ovarian differentiation, usually weeks or months after the onset of ovary development (Guraya, 1994; Nakamura et al., 1998).


A plethora of gonadal differentiation mechanism are exhibited by ~28,000 (see Penman and Piferrer, 2008) species of teleosts, ranging from species that directly develop and finally possess only testis or ovary at sexual maturation termed as gonochoristic to species with synchronous or sequential hermaphroditism (Devlin and Nagahama, 2002; Price, 1984). Among gonochoristic species, Yamamoto (1969) described two forms, differentiated (indifferent gonad → testis/ovary) and undifferentiated (initially all develop ovary → ~50% masculinization occurs). On the other hand, hermaphrodites are species that
have functional ovarian and testicular tissues during the life-cycle and are further classified into sequential hermaphrodites; protogynous (mature female → male) and protandrous (mature male → female), synchronous hermaphrodite (intersex) and bidirectional hermaphrodites which can alter their sex in both directions (Devlin and Nagahama, 2002).

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

Gonadal differentiation occurs through a single development pathway to produce completely differentiated gonad in mammals (Capel et al., 1998) where as in fish, gonadal development may be controlled by intrinsic factors, steroids and growth factors (gonochorist) social behaviour (natural hermaphrodites) or extrinsic environmental factors such as temperature, endocrine disrupters or pollutants. Irrespective of the mode or type of
reproduction, maintenance of stable sex ratio ideally 1:1 drives the individual towards a strategy that help them to sustain a balance in their population against the varying selective pressures such as natural and anthropogenic. The prevalence of sex-related growth dimorphism is quite common (Parker, 1992) which in turn influence reproductive capacity and growth pattern of population before and after attaining sexual maturation. Hence, for a species or a small population in a species, sex is influenced either by a single sex factor or combination of several sex factors or environmental differences, and leading to evolutionary transitions involving changes in the relative abundance of these three factors (Bull, 1983).

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

Gonochorism in teleosts may solely be due to genetic differences between sexes initially or absence of genetic difference but respond to environmental conditions prevailing at the
time of fertilization (Bull, 1983; Valenzuela, 2008). The slides presented below adopted from a presentation on sex determination and differentiation in fish by Piferrer (2007) illustrate these mechanisms explicitly.
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Environmental Sex Determination (ESD)

- Sex is determined after fertilization according to the immediate environment
- Little influence of the genotype on gender determination
- Identical genotypes can produce different phenotypes depending on the environment
- All ESD systems have at least slight inherited effects on sex determination
- Selection favors sex ratio in response to environment, and the establishment of a mechanism

Conditions for ESD (The Charnov and Bull model)
1) Patchy environment during early life, differentially affecting male and female fitness
2) Little or no control of parents and offspring over environment selection
3) Random mating among patches

Environmental Factors
- Temperature (TSD)
- Many reptiles, some atherinid fish
- Some cichlid and poeciliid fish
- Gammarus duebeni (amphipod), some fish
- Mermithidae nematodes
- Conspecific interactions
- Borellia virois (marine worm)
- Ione thoracica (crustacean parasite)

Richard C. Lewontin
"The organism proposes and the environment disposes"

Adopted from Dr. Piferrer’s presentation on Determinacion Y Differenciacion Sexual En Los Pesces (www.reprofish.eu/.../Sex_determination_differentiation_Piferrer_2007. pdf)

Sexual development in mammals cannot be altered by treatment with exogenous steroids or environmental parameters suggesting that it is under the control of very stable genetic sex determination-XX/XY system with Sry being the master switch on Y chromosome. In contrast to mammals, both gonochorist and hermaphrodites are labile for sex change throughout their life (Pandian and Koteswaran, 1998; Devlin and Nagahama, 2002) which is explained on the basis of unique characteristic features in teleosts: the property of retaining bipotentiality by holding PGCs at many stages of gonadogenesis and their potentiality to redifferentiate throughout the reproductive lifespan. Among fishes a variety of sex determination/differentiation-related genes exist and this phenomenon is explained by the mechanism of chromosome evolution (Schartl, 2004; Volff et al., 2007). Genes downstream of the master regulatory gene that ultimately establish sexual dimorphism are
quite conserved in fish for example the steroidogenic enzyme, cytochrome P450 aromatase and the transcription factor dmrt1 which have pivotal role in ovarian and testicular differentiation, respectively (Guiguen et al., 2010; Wang et al., 2009). But there are many more candidate genes potentially implicated in sex differentiation which warrants a thorough study in different fish models.

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

Adopted from Ferguson-Smith, R. Sex Dev. (2007), 1:2-11
Endocrine regulation of sex differentiation:

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

For example, the male determining $DMY$ gene in medaka, $Oryzias latipes$, evolved as a consequence of translocation and duplication of autosomal $dmrt1$ gene on another autosome, generating a new Y chromosome. However, this gene, $DMY$ is not an universal male sex determining gene in fish since it is found only in two species of medaka. Studies on platyfish, threespine stickleback, salmonids, tilapias and various other models have confirmed that the fish sex chromosomes are young and have evolved independently in different fish lineages. Hence, the current research focuses on comparing genes, i.e., $sox9$, $dmrt1$, $amh$, $nr5a1$, $nrob1$, $igf1$, $igf1ra$, $cyt19a$, $foxl2$, $fst$ and $lhr$ (Baron et al., 2005; Wang et al., 2007; Vizziano et al., 2007, 2008; Raghuveer and Senthilkumaran, 2009) that are involved in vertebrate sex determination. In addition, transcription factors, steroidogenic enzyme genes and the sex steroid receptors which ultimately regulate the sex steroids (androgens and estrogens) to exert their action on germ cells and other cell types and on
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organs involved in sex differentiation. Steroid production is correlated significantly with very early stages of gonadal differentiation (Nakamura, 1998) and any perturbation in the steroid-synthesizing capability can impair the sex determination/differentiation mechanism. Extensive work is done on diverse area covering most aspect of sex steroids: its production at the time of embryogenesis, larval development, and temporal expression of the steroidogenic enzyme producing it, the transcription factors regulating the expression of the steroidogenic gene and finally the receptors-mediated function by binding to appropriate protein/DNA.

For females, estradiol-17β (E₂) is supposed to be the major sex steroid responsible for inducing and maintaining ovarian development (Yamamoto, 1969). Various biochemical and histochemical evidences from fish belonging to different reproductive strata elucidate the role of E₂ and aromatase. First and foremost aromatase expression studied during early development revealed its role in female differentiation in vast number of teleosts (Kwon et al., 2001; Sudhakumari et al., 2005; Ijiri et al., 2008). Inhibition of E₂ synthesis in early developmental stages using aromatase inhibitors can lead to masculinization (Guiguen et al., 1999; Kitano et al., 1999; Nagahama, 2005). Further, immunohistochemical studies in the gonad of tilapia detected aromatase before the onset of sex differentiation in female with delayed occurrence in males (Nakamura et al., 1998). Corroboratively aromatase activity is enhanced at feminizing temperature and suppressed at masculinizing temperatures (D’cotta et al., 2001). In hermaphrodites, aromatase expression is the driving force for gonadal reorganization during sex change i.e., increase in aromatase mRNA levels during protandrous sex change (Kroon et al., 2005) and decrease during protogynous sex change (Bhandari et al., 2003). All these evidences indicate that ovarian
aromatase (cyp19a1) can be used as a molecular marker to infer ovarian differentiation in species lacking sex-linked color pattern or non-availability of mono-sex population.

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

Molecular analysis of steroidogenic enzyme genes from fish has revealed the existence of two forms of aromatase in most teleosts studied. For example in goldfish and zebrafish, (terminal enzyme producing E2) the brain and the ovarian isoform displays ~ 40% sequence homology with distinct spatiotemporal expression (Callard and Tchoudakova
1997, 2001; Trant et al., 2001). Similarly the presence of a novel isoform of 11β-hydroxysteroid dehydrogenase (HSD) type 2 gene (terminal enzyme producing 11-KT) in medaka, fugu, zebrafish-11β-HSD3 which is paralogous to mammalian 11β-HSD1 (Baker, 2004) and also the isolation of 11β-hydroxylase (11β-H, penultimate enzyme in 11-KT production) in the Nile tilapia (Zhang et al., 2010) revealed that though the biosynthetic pathway of sex steroid synthesis is conserved among fishes, the spatiotemporal expression of various isoforms vary in different fish models supporting the concept of independent evolution of sex genes in different fish lineages. Therefore, it would be highly relevant to explore the expression pattern of these steroidogenic enzyme genes during development in many more fish models to gain an insight into possible role of these steroids in endorsing the fish with the unique trait of plasticity in terms of sex determination/differentiation. This prompted us to study the ontogeny of these three steroidogenic enzyme genes involved in the synthesis of sex steroids and their putative role during gonadal differentiation and development in catfish Clarias gariepinus. This teleostean fish model has been chosen since it exhibits distinct recrudescence and quiescent phases during the reproductive cycle. It can be bred and reared easily from day 1 after hatch to adult in laboratory/natural conditions (outdoor tanks) for ontogeny and reproductive cycle studies.

**Hormonal regulation of gametogenesis in fish:**

The functional unit of testis in teleosts is the spermatogenic cyst formed by group of Sertoli cells surrounding and nourishing synchronously developing germ cells whereas the functional unit within the ovary is ovarian follicle consisting of developing oocyte surrounded by follicular cell layers. The granulosa and thecal layers are separated by a basement membrane.
Spermatogenesis is a complex and highly coordinated process of producing haploid spermatozoa from diploid spermatogonia. In males, it consists of three phases: 1) mitotic phase with different generations of spermatogonia which are genetically determined 2) the meiotic phase that includes the primary and secondary spermatocytes and 3) the spermiogenic phase with the haploid spermatids emerging from meiosis and differentiating into spermatozoa (Schulz et al., 2010). Oogenesis on the other hand, is the process by which primordial germ cells become ova and it can be broadly divided into six major steps: (1) formation of primordial germ cells (germline segregation), (2) transformation of primordial germ cells in to oogonia (sex differentiation), (3) transformation of oogonia into oocytes (onset of meiosis), (4) growth of oocyte under meiotic arrest (vitellogenesis), (5) resumption of meiosis (maturation), and (6) ovulation (Patino and Sullivan, 2002).
A complex cross-talk between the gonad and pituitary occurs via endocrine and paracrine control after perceiving visual, social and chemical cues by brain. This leads to the secretion of gonadotropins (follicle stimulating hormone-FSH and Lutenizing hormone-LH) and growth factors which triggers the production of sex steroids in gonads facilitating the development of mature ova and sperm from spermatogonial and oogonial cells.
Pioneering reproductive biologist, Nagahama (1994) elucidated the production of $E_2$ in teleosts by a two-cell type model where the outer thecal cell layer under the influence of gonadotropin secretes the androgen substrate (testosterone) which diffuses into the granulosa cell layer where the aromatase is localized (Kagawa et al., 1982; 1985) to get converted into $E_2$. Likewise 11β-hydroxytestosterone is synthesized by the Leydig cells under the influence of gonadotropin (FSH) which activate the Sertoli cells to produce activin B that acts on the spermatogonial cells to undergo mitosis leading to proliferation, differentiation and production of spermatocytes (Nagahama, 1994; Miura and Miura, 2001).

Numerous studies have demonstrated the fluctuation in the 11-KT levels and the shift in steroidogenesis to $17\alpha, 20\beta$-dihydroxy-4-pregnen-3-one ($17\alpha, 20\beta$-DP) during transition.
of testis from stage I to stage IV in cyclic reproducing teleosts (Sakai et al., 1987; Cavaco et al., 1997). Further discovery of microarray technique and transcriptome study of 9152 contigs from trout using both spermiating testis and isolated testicular cells have provided an insight on the temporal changes in the expression of varied genes (DNA repair gene, meiotic recombination etc.) during the transformation of spermatogonial stem cells to spermatids (Schulz et al., 2010). But none have reported simultaneous change in expression and protein activity of the enzyme producing 11-KT during testicular transition. Further reports exist from salmonids species on the stimulatory role of gonadotropins on 11-KT synthesis and up-regulation of the penultimate and terminal steroidogenic enzyme genes $11\beta$-$H$ and $11\beta$-$HSD2$ synthesizing it when administered in the immature fish (Jiang et al., 1996; 2003) but no data exist on stage-dependent responsiveness of these genes to gonadotropin induction. Besides gonadotropins, thyroxine is also essential for the normal functioning of teleost testis and alteration in the thyroxine levels caused marked biochemical and histological changes in the testis of Nile tilapia and catfish (Matta et al., 2002; Swapna et al., 2006; Blanton and Specker, 2007). Therefore to elucidate the significance of thyroxine during testicular recrudescence, we studied the expression of $11\beta$-$H$ and $11\beta$-$HSD2$, by treating the fish with goitrogenic agent, thiourea, during testicular recrudescence. In this study, we also analyzed several other steroidogenic enzyme genes, $3\beta$-hydroxysteroid dehydrogenase (enzyme involved in the rate limiting step), cytochrome p450c17α enzyme and $20\beta$-hydroxysteroid dehydrogenase (which synthesizes the maturation inducing hormone in teleosts) the former gene was chosen to study if triiodothyronine depletion blocked the initial steps of steroidogenesis and the latter two genes transcripts were measured to observe if there is a shift in steroidogenic
pathway leading to the synthesis of 17α, 20β-DP, (the steroid required for resumption of prophase-I arrested oocytes in teleosts) eventually causing precocious maturation. Taken together, an attempt was made to study the gene expression and activity of 11β-H and 11β-HSD2 during different phases of catfish testicular cycle to delineate specific role of steroidogenic enzyme genes, gonadotropins and thyroid hormone in maintaining the seasonal reproductive cycle.

Similarly during oogenesis plasma FSH levels induced follicular E₂ production, which in turn, stimulated hepatic vitellogenin synthesis (Specker and Sullivan, 1994) followed by a rise in plasma LH levels, (well known as pre-ovulatory LH surge) coinciding the production of pre-ovulatory follicle (Khan and Thomas, 1999). Subsequently gonadotropin binds to its receptor on granulosa cells (Oba et al., 1999; Kumar et al., 2000) and stimulates a sequence of events including shift in steroidogenesis, acquisition of oocyte maturation competence, production of maturation-inducing hormone (MIH), MIH-dependent resumption of meiosis and cytoplasmic maturation (Kanamori and Nagahama, 1988; Nagahama, 1994; Senthilkumaran et al., 2004; Lubzens et al., 2010). Seasonal fluctuations in aromatase expression and activity levels are well documented in female sea bass and the Nile tilapia where they have attributed these fluctuations accountable for follicular growth and maturation (Chang et al., 1997; Dalla Valle et al., 2002; Gonzalez and Piferrer, 2003; Chang et al., 2005). But this was not attempted simultaneously from the same fish which may not depict a correct picture of the change in the transcription and translation capacity of the ovary during that phase of follicular growth. Therefore, we attempted to simultaneously analyze the expression profile and rate of production of E₂ during ovarian cycle. We also monitored the responsiveness of catfish ovarian
aromatase gene and enzyme activity after induction with gonadotropin (hCG) to substantiate early findings from amago salmon where gonadotropins potentiated oocyte growth and maturation (Kagawa, 1982; Young, 1983). However in catfish, single form of gonadotropin has been purified, which showed seasonal variation and the interactions between fish gonadotropin and their receptors, appear to be less discriminatory (Koide et al., 1992; Bogerd, 2005; Kirubagararan et al., 2005). Apart from gonadotropin, several other hormones such as thyroid hormones, insulin and growth hormone have been implicated in the regulation of vitellogenesis (Patino and Sullivan, 2002). This prompted us to study the effect of thyroid hormone depletion by using thiourea during gonadal recrudescence.

In the backdrop of this existing state of knowledge, the present thesis is an effort to understand the role of terminal enzymes responsible for the production of E$_2$ and 11-KT at molecular level, and further monitor the implications of thyroid hormone depletion and hCG administration on gonadal recrudescence. These aspects have been studied as four major chapters discussing the results and findings.

References


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