Chapter 1

Introduction
Sex determination is a fundamental biological process of great importance, whereby bipotential gonads develop into a testis or into an ovary, essential for the propagation of the species. Nature has evolved an astonishing variety of genetic and epigenetic sex determining systems which all achieve the same result, the generation of two sexes, a male or a female. In the animal kingdom, a large number of sex determination mechanisms are seen. In vertebrates, this process is accomplished by highly specialized sex chromosomes (GSD - Genetic Sex Determination) to simple environmental cues (known as ESD - Environmental Sex Determination). Overall, GSD is the major mode of sex determination, seen in all five phyla of vertebrates including mammals, and is much better understood. In GSD species, the specialized sex chromosomes may differ: morphologically (heteromorphic or homomorphic), in numbers (from one pair to many pairs), and even in their effect due to the presence of several minor sex-determining genes (Bull 1983; Solari 1994; Grutzner et al., 2004). In comparison to GSD, very little is known regarding ESD and its underlying mechanism. The ESD species, in general, seem to lack apparent sex chromosomes or sex specific genes that could consistently produce a genetic difference among zygotes, and their sex appears to be mainly decided post fertilization by the simple cues like temperature, pH etc. of the environmental milieu of the developing embryos. Among different environmental cues seen in ESD species, the incubation temperature as the sex-determining factor is the most important and widespread. The latter is called Temperature-dependent Sex Determination (TSD) which is chiefly found in the reptilians; it occurs in all crocodilians, tuartaras studied till to date, and is common among turtles (Valenzuela & Lance 2004).

Sex determination is exclusively temperature dependent in all crocodilians. The particular period of embryonic development during which temperature appears to be critical for the sex determination is called Temperature Sensitive Period (TSP) (Bull 1983; Solari 1994). In TSD species, the sex ratios can be easily manipulated by changing the incubation temperature during TSP. The Indian mugger (Crocodylus palustris) is one such species in which exclusively males or females can be produced by manipulating the incubation temperature of fertilized eggs. It is demonstrated that the incubation temperature at and below 31.0°C and above 33.0°C results exclusively in female development whereas varying proportion of both males and females are produced only between the narrow range of 31.0°C-33.0°C with 32.5°C supporting exclusive male development (Lang et al., 1989). The embryonic phase wherein the sex determination process is sensitive to environmental temperature has been broadly delineated to developmental stages 21st to 25th (spanning approximately 21 - 25 days of
development) by temperature shift incubation studies (Lang & Anderson 1994). All the field studies and universal absence of sex chromosomes in crocodilians suggest that gene(s) responsible for primary sex determination are present in all embryos, but are differentially and selectively expressed at specific time points during development as a function of temperature, leading to the development of either males or females. The pattern of sex ratios vis-a-vis critical temperature range, suggests the female development being the default mechanism, but male development needing specific activation of factor(s) in response to temperature. Because of these unique features, Crocodylus palustris provides an ideal and attractive system to: a) gain insight into the molecular basis of TSD; and b) for identification and characterization of temperature sensitive biological control amenable to fine tuning by temperature. The possibility of such temperature sensitive biological control would be of great significance in both basic and applied biotechnology research.

Several studies done in the past (mostly in mammalian species) have helped in detailing the developmental/cellular events, as well as, identification of many of the candidate genes involved in- maintenance of bipotential gonad primordia, sex determination and early differentiation of gonads (Merchant-Larios & Moreno-Mendoza 2001; Pannetier et al., 2004; Swain & Lovell-Badge 1999; Lovell-Badge et al., 2002; Wilhelm et al., 2007). Major morphological events occurring during the embryonic gonad development are broadly conserved among vertebrates viz., formation of testicular cords immediately after sex-determination/decision, strengthening of cortex in developing female gonads and regression of Wolffian ducts (Morrish & Sinclair 2002; Yao & Capel 2005; Smith & Sinclair 2004). A similar broad conservation has also been evident even for the major sex determining genes. The Sry (Sex determining region of Y chromosome) is revealed as a key testis-determining gene in mammals. In addition, a number of other genes, Wt1, Sf1, Sox9, Dax1, Lim1, Emx2, Lhx9, Wnt4, Fgf9 GATA4, Amh and Dmrt1 have been identified and implicated either in the maintenance of the bipotential gonadal primordia and/or in the early differentiation of gonads after sex determination (Swain & Lovell-Badge 1999; Wilhelm et al., 2007). These genes generally show dimorphic expression in males and females. Genes like Sf1, Wt1, M33, Lim1, Emx2 etc. are shown to express early with a role in growth and survival of bipotential gonad, while Sox9, Dax1, Fgf9, Amh and other genes are involved in the differentiation and development of the gonads. Many of these genes appear to be conserved through evolution at least in their organization, although, the same may not be true for their role in sex-determination. It is now well documented that there exists conspicuous differences in the spatio-temporal expression of many such sex-related genes in different systems, e.g., in Gallus (having
ZZ/ZW sex determining system), Sox9 expresses after Amh, a situation just reverse to that of mammals wherein Amh is the transcriptional target of Sox9 (de Santa et al., 1998). Similarly, Sry is absent in the Gallus and it is the Dmrt1 gene (present on the Z chromosome) showing early expression in the bipotential gonads that is suggested to be the possible male sex deciding candidate gene (Smith et al., 2003; Raymond et al., 1999). Nevertheless, at this stage, our understanding of the putative genetic cascade underlying the sex determination mechanism remains poor, mainly because of lacking information regarding functional interplay between the known and many other unknown (yet to be identified) sex candidate gene(s). Filling these missing pieces is the challenge for future.

In contrast, relatively few studies have been carried out in TSD species that too in recent years and these are mainly limited to identification and expression analysis of conserved sex determining genes earlier identified in GSD species/mammals and hormonal effect on TSD. These studies, in general, have revealed ‘hormonal interplay’ as an important component of the underlying sex-determination mechanism(s), and homologues of many of the sex-related genes have been identified such as, Sf1, Wt1, Sox9, Amh, Dmrt1, and Aromatase gene of hormonal cascade (Shoemaker et al., 2007; Sarre et al., 2004; Western & Sinclair 2001). Presence of such genes even in TSD species exhibiting sex-specific quantitative variation in their expression during gonadal development (Morrish & Sinclair 2002; Sinclair et al., 2002), suggests a broad evolutionary and functional conservation between the genetic and hormonal machinery underlying the GSD and TSD. However, none of these studies done to date provides any insight about the nature/mode of the molecular machinery in embryo that senses the external temperature and triggers the process of sex-determination. Our understanding of the factor(s) involved in TSD and modulated by the temperature trigger is completely wanting. TSD thus, remains an enigmatic problem of developmental biology.

Unlike the mammalian sex-determination, a master regulator like Sry has not been detected in TSD species. On the other hand, another gene Sox9 (one of the Sry related HMG box carrying gene) that has been proposed to be the “master” testis-determining factor downstream of Sry in mammals, is highly conserved among vertebrates and unlike Sry, is also suggested to have an important role in sexual development even in birds and reptiles. The Sox9 gene is necessary for male development in mammals and a fortuitous insertional mutation has shown that constitutive Sox9 expression is sufficient to direct full male development in the mouse in the absence of Sry (Bishop et al., 2000). Inactivation of Sox9 in XY embryos leads to sex reversal of male to female, while exogenous
expression of Sox9 in XX gonads can induce testis formation in the absence of Sry (Vidal et al., 2001). Sox9 is also known to interact with Sf1 and activates transcription of Anti-Mullerian hormone gene Amh (de Santa et al., 1998). All these studies suggest Sox9 to be the "master" testis-determining factor downstream of Sry in mammals. However, few recent studies in other non-mammalian species showing confounding differential expression pattern of Sox9, appear to suggest otherwise; that Sox9 role as "master" testis effector gene may not be conserved in TSD and possibly other GSD species. In some studies in chicken (GSD species) and alligator and turtle (TSD species), Sox9 expression is observed to lack male/testis-specificity until testicular structures are established (Oreal et al., 1998; Western et al., 1999), while contrary to it, Sox9 expression is found to be male-specific preceding testis organization in another TSD sea turtle Lepidochelys olivacea (Torres Maldonado et al., 2002; Moreno-Mendoza et al., 2001) more closely resembling the pattern in mice. More recently, the work done in CCMB on detailed expression profiling of cpSox9 in another TSD crocodilian i.e., Indian mugger, amply demonstrate that the above referred observations about lack of male-sex specific expression prior to testis organization, may be a fall-out of the constraints of experimental design, as discerning male-specific expression becomes difficult unless critically analyzed due to Sox9 transcriptional diversity during gonadogenesis (Agrawal 2006). The in-house work at CCMB, further suggests an important role for Sox9 in male sex development/differentiation, albeit not as the candidate gene for primary sex-determination in TSD.

Lately, other major genes (other than Sry, Sox9) originally identified and implicated in the GSD in mammals are also being explored for their presence and possible role in TSD. Some such studies involving preliminary spatio-temporal expression analysis have revealed sex determining genes like, Wt1, Dmr1, Sf1 and Aromatase, as possible candidates having important role in TSD (Yao & Capel 2005). Among these, Dmr1 is a highly conserved gene that is shown to play an important role in GSD from being a master regulator in the teleost Medeka, candidate master regulator for male gonad development in birds, to a downstream gene required for the male gonad development in mammals (Raymond et al., 2000; Shan et al., 2000; Matsuda et al., 2002; Hodgkin 2002). Dmr1 is also shown to express in early stages of embryonic development including the indeterminate bipotential gonadal primordial tissue in some studies in TSD/HSD vertebrates (Ferguson-Smith 2007; Sreenivasulu et al., 2002; Sreenivasulu et al., 2002; Murdock & Wibbels 2006). Similar to Dmr1, Wt1 and Sf1 are also largely conserved and are implicated for an early role in bipotential gonad maintenance and sex determination in the mammals and few other GSD species. An expression analysis study
in the turtle also indicates an early role for these genes (Yao & Capel 2005). Moreover, notable sex-/developmental stage-specific differences during gonadal development have been observed in expression pattern of such major sex determining genes in the few studies on different TSD species, which further suggests proper characterization of the transcriptional diversity and expression analysis of these genes in TSD species is necessary (Yao & Capel 2005; Schmahl et al., 2003; Western et al., 2000; Ramsey et al., 2007).

In contrast, to the apparent differences in expression patterns of the major sex-candidate genes, effect(s)/interplay of hormonal environment on early gonadal differentiation is revealed to be more conserved and universal among TSD species. A number of studies have shown that embryonic sex can rather be easily manipulated by modifying the hormonal regimen, e.g., estrogens treatment can lead to female development at male promoting temperature (MPT), while male embryos can be induced at female promoting temperature (FPT) by treatment with Aromatase inhibitors. In this hormonal cascade, Aromatase (CYP19) is the core important enzyme that regulates the conversion of androgens to estrogens (Lance 1997), and thus can override the effect of temperature on sex-determination. Now, there is a general consensus that temperature exerts its influence in species with TSD by acting upon the genetic mechanisms that govern steroidogenic enzymes or steroid hormone receptors (Sarre et al., 2004). Therefore, Aromatase, one core regulator of hormonal cascade, may be one important target of close scrutiny in our pursuit of unraveling the molecular machinery underlying the TSD which is strongly influenced by the hormones (Valenzuela & Lance 2004).

To get insight into the molecular basis of TSD, C. palustris (Indian mugger) provides a model system. The temperature sensitive period in this species has been broadly defined corresponding to the developmental stages 21st to 25th (comprising ~22 - 30 days of embryonic development in male and female embryos, respectively) by egg shift incubation studies (Lang & Anderson 1994). Understanding molecular basis of TSD using Indian mugger is one major program in the area of developmental biology at CCMB. To address this interesting but challenging problem multiple approaches are being pursued that broadly include: isolation and characterization of homologues of known sex-determination related candidate gene(s), search for new genes with putative role in TSD, and creating large biological resources for future functional genomic studies. In this background, the present study was planned to identify/isolate crocodilian homologues of three important candidate genes namely, Dmrt1, Wt1 and Aromatase from C. palustris and to examine their spatio-temporal expression during gonad
development through TSP. The characterization of these candidate genes from Indian mugger was expected to unravel their functional role (if any) in TSD, and also to help ascertain the extent of their conservation between corresponding genetic elements involved in TSD/GSD. In addition, it was planned to explore their sex-specificity and potential use as biomarker for embryo sexing to delineate the determinate/indeterminate phases of TSD thus helping in narrowing down the long TSP which is an important need to undertake effective functional genomic studies. Simultaneously, the study also proposed to attempt search for novel differentially expressed gene(s) during early TSP that may be: involved in the developmentally important decision of sex determination and differentiation in case of TSD and/or immediate targets of the machinery that senses the temperature and regulates gene expression.

In specific, the main objectives of the study were:

- Isolation, characterization and expression analysis of crocodilian homologues of candidate genes: Aromatase, Dmrt1 and Wt1 from Crocodylus palustris.

- Search for the gene(s) differentially expressed during early temperature sensitive period (TSP) in developing gonads of putative male and female embryos, and their characterization.