Temperature Dependent Sex Determination

In Zebrafish (*Danio rerio*)

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Summary

Despite zebrafish (*Danio rerio*) is an important model for understanding vertebrate development during the last decades, sex determination and differentiation are not clear. To date no sex chromosome has been identified. On the contrary a number of genes have been linked to sex determination process in zebrafish. Environmental factors, such as water temperature, have an effect on sex determination mechanism. Till now only few studies were conducted on temperature dependent sex determination and differentiation in zebrafish.

The targets of the thesis are investigating the influence of elevated rearing temperature at 35^{o}C on sex determination during the early stages (embryogenesis) and later on the sex differentiation during larval development in zebrafish.

In the first experiment, effect of early heat shock (35^{o}C) during the embryogenesis was analyzed onto phenotypic sex in zebrafish. The fertilized eggs were generated by crossing a mitotic gynogenetic male with three golden coloured females and subjected the embryos to heat treatment of 35°C applied from 5-10 hpf, 5-24 hpf and 5-48 hpf, which corresponds to the following developmental stages: gastrula, gastrula to segmentation, and gastrula to pharyngula stage, respectively.

In the second experiment**,** the effect of an elevated temperature at 35^{o}C for 10 day (20-30 dpf) or (25-35 dpd) was analyzed during the larval development onto phenotypic sex in zebrafish. In the first trial, the larvae were generated by matings between four normal golden females and a normal male (NM). In the second trial, the larvae were derived from matings between six normal golden females and a mitotic gynogenetic male (Gyn). All the larvae were reared at 28.5^{o}C until the start of the treatment at 35^{o}C (control at 28.5^{o}C, treatment groups (20-30 dpf and 25-35 dpf) at 35^{o}C.

It could be concluded from this study that:

- water temperature has a strong effect on sex determination of zebrafish during the early embryonic stage (gastrula) and later on sex differentiation during the larval development (25-35 dpf).

- there is a possibility to change the pathway of sexual determination during early embryonic stages in zebrafish by exposure to high water temperature ($35^{o}C$).

- the phenotypic sex of zebrafish can be altered using early heat shock ($35^{o}C$) applied for a very short period in the gastrula stage (5-10 hpf).

- temperature dependent sex in zebrafish is influenced by the male spawner, the female spawner and the interaction of genotype by environment.

- a phenotypic sex of zebrafish seems to be most susceptible towards rearing temperatures during the period from 25-35 dpf, a share of the phenotypic variance is caused by genotype x environment interactions in zebrafish.

Chapter 1

Introduction

1. Introduction

A sex-determination system is a biological system that determines the development of sexual characteristics in an organism. Most sexual organisms have two sexes. In mammals and birds, sex determination is genetic: males and females have different alleles or even different genes that specify their sexual morphology. This is often accompanied by chromosomal differences. In teleost fish, sex determination mechanisms are very complicated events and display an amazing diversity (Volff, 2005). Besides genetic factors, environmental conditions play important roles during the sex determination and/or differentiation in many fish species (Baroiller et al., 2009b). Zebrafish (*Danio rerio*) is now the pre-eminent vertebrate model system for studying the vertebrate's development. Sex determination and differentiation mechanisms are not clear. The relationship between the genetic background and the environmental conditions on sex determination process needs to be investigated. Generally, water temperature seems to be the most effective environmental factor that has an effect on sex determination and differentiation pathways in fish.

1.1. Sex determination

Sex determination is the process deciding the sex of a developing embryo. This is usually determined genetically; however it is a delicate process, which in many cases can be influenced by environmental factors. During the last few decades, scientific attention increased to study sex determination regimes in many species to clarify the relation to some sex-linked diseases depending on the great variation among different species. An example is mammals in which females have two identical sex chromosomes (*XX*) and the males have two distinct sex chromosomes (*XY*). In birds, a *ZW* sex-determination system was found (Smith et al., 2007) and some insects instructs female development from two different sex chromosomes (*ZW*), while males possess two of the same kind of chromosomes (*ZZ*) (Arunkumar et al., 2009). In other species, sex determination could be controlled under polygenic factors which exist in some other vertebrates and a lot of fish species (Volff and Schartl, 2001; Devlin and Nagahama, 2002). Additionally, one or more autosomal factors may contribute to sex determination (minor genes) such as assumed in Tilapia (Tessema et al., 2006). Environmental factors have also an effect on the sexual fate in many other species (alligators, most turtles, and

some fish species) (Baroiller and Guiguen, 2001; Pieau et al., 2001; Godwin et al., 2003).

1.2. Sex determination in fish

In contrast to the stable regularity of sex determination regimes which established in mammals and birds, teleost fish display an amazing diversity of sex-determination systems. Male heterogamety (*XY*, as is generally the rule in mammals) and female heterogamety (*WZ*, the system at work in birds) are different sex determination regimes and were observed sometimes within the same fish genus and even the same fish species (Volff et al., 2007). More complicated systems can involve multiple sex chromosomes and multiple gene loci (influence from autosomal loci on sex determination and polygenic sex determination). Hermaphroditism has been observed in fish; environmental factors can also influence their sex-determination systems. Almost nothing is known about the mechanisms driving the diversity of sex determination in fish, and the evolutionary significance of the various mechanisms remains almost completely obscure (Hayes, 1998). Inequality sex determination mechanisms in fish and poor information need to be investigated to clarify the evolution and development sex determination and differentiation process in fish.

1.2.1. Study sex determination in fish is important for several reasons

There are many reasons for the importance to study sex determination and differentiation in fish, which can broadly help to understand this process (reviewed by Devlin and Nagahama, 2002). In particular, such studies have provided important insight into the plasticity of the sex determination process in vertebrates. The biology and ecology of fish are sufficiently diverse to provide unique examples of sex-determination mechanisms, yet they possess many of the same processes and pathways that are used in other vertebrate systems. Also, fish provide unique opportunities to investigate and test theoretical concepts of sex determination, ranging from evolutionary mechanisms to biochemical processes. Some important farmed species have sex-related growth, for example, the sea bass (*Dicentrarchus labrax*) and the bastard halibut (*Paralichthys olivaceus*). It is significant to reveal the sex determination process and control the sex ratio in these species considering economic aspects of aquaculture. On the other hand, the identification and comparative analysis of sex determination regimes in some famous model organisms, like zebrafish (*Danio rerio*) medaka (*Oryzias latipes*)

and platyfish (*Xiphophorus maculates*) are useful in evolution studies and also helpful for understanding the sex determination system in cultured fish (Zhang et al., 2009).

1.2.2. Diversity of sex determination mechanisms in fish

Sex determination in fish is characterized by extraordinary variation, including genetic and/or environmental sex determination, male or female heterogamety, single gene and polygenic systems, protandry, protogyny and simultaneous hermaphroditism, social influence on sexual determination, and many combinations and variations between and within these systems (Devlin and Nagahama 2002; Conover, 2004; Ospina-Alvarez and Piferrer, 2008; Baroiller et al., 2009b). (Figure 1)

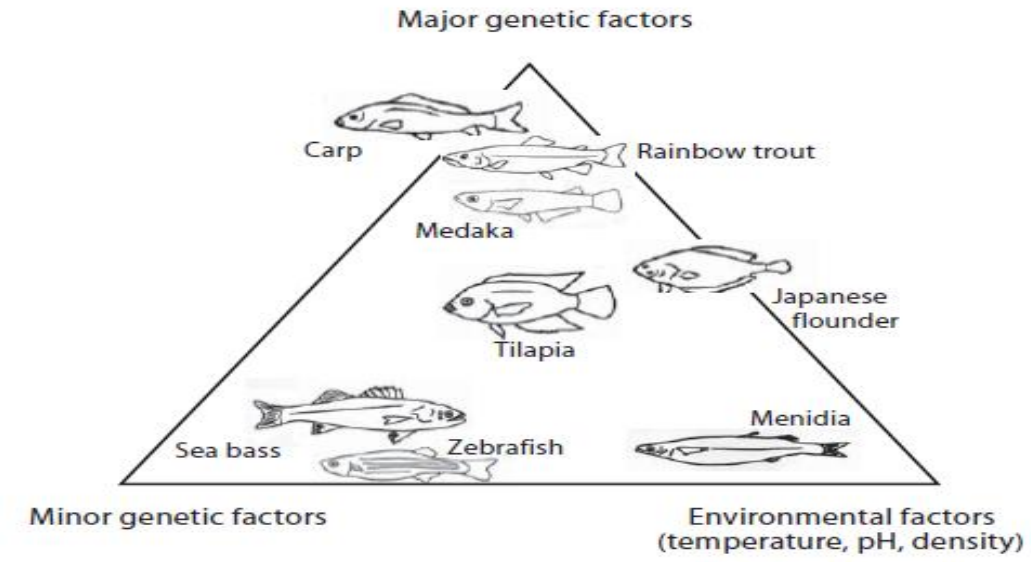

Figure 1: Schematic triangle representing the three factors influencing sex in fish: major genetic factors, minor genetic factors and the environmental factors (Baroiller et al., 2009b).

In recent decades, major breakthroughs have been made in studying sexual development in fish. This has attracted the attention of working in developmental biology, genetics, evolution, ecology, conservation and aquaculture. Generally, the gonads of fish are very labile with respect to sex determination, but once a particular developmental profile has been selected by intrinsic controls, or directed by exogenous factors such as hormones, the state of gonadal differentiation may then be stably perpetuated throughout subsequent development (Devlin and Nagahama, 2002). However, exceptions to this stability exist in fish, exemplified by the ability of some hermaphroditic species to alter the course of sex differentiation. These are specialized cases where sex determination is altered to allow flexible production of gamete types to maximize fitness. The stability of

sex differentiation in gonochoristic species implies that sex-determination events primarily function during early development to set the course of gonadal development. Subsequent maintenance of the differentiated state is accomplished through the stability of gene expression patterns and feedback mechanisms to ensure a consistent profile of cellular and hormonal signals. Several levels of control for gonadal determination seem feasible for fish, including intrinsic cell-autonomous genetic mechanisms, or endocrine, paracrine, behavioral, or environmental signals (Devlin and Nagahama, 2002; Baroiller et al., 2009b).

1.3. Sex determination mechanisms in fish

Sex determination can follow very different evolutionary dynamics depending on the vertebrate lineage considered (Schartl 2004; Graves 2006). In mammals, a system of XY male heterogamety has driven sexual development over the past 180 million years of evolution. The male determining gene is the transcription factor gene *Sry*, present on the Y but not on the X chromosome. In birds, a ZW system of sex determination with female heterogamety is present in species separated by as much as 100 million years of evolution. An excellent candidate for the function of the master sex-determining gene is the Z-chromosomal dmrt1, which encodes a transcription factor also involved in sexual development in mammals and other vertebrates (Nanda et al., 1999, 2008). In contrary to stable regulatory sex-determination mechanisms which are stabilized in mammals and birds, an astonishing diversity of sex-determination mechanisms has been observed in fish (Devlin and Nagahama, 2002; Volff, 2005; Mank et al., 2006; Volff et al., 2007). Besides different forms of hermaphroditism, gonochorism in fish is under the control of various sex determination systems involving genetic and/or environmental factors, such as temperature or social factors. Genetic systems with male or female heterogamety, with or without influence of autosomal factors, as well as polygenic systems have been described. More than two types of sex chromosomes can coexist in the same species (e.g. X, Y and W chromosomes in platyfish (*Xiphophorus maculates*) (Volff and Schartl, 2001) sometimes in the form of two non-homologous pairs of sex chromosomes (ZW and XY pairs in the blue tilapia (*Oreochromis aureus*) (Lee et al. 2004). Related fish species or even different populations from a same species can have different mechanisms of sex determination, indicating a rapid evolutionary turnover of the control of sexual development in fish.

1.3.1. Genotypic sex determination

Most fish species lack distinguishable heteromorphic sex chromosomes, suggesting that they are at an early stage of differentiation. Indirect methods coupled to progeny testing have been commonly used to elucidate the genetic sex determination (GSD) system in fish. Female (XX/XY) and male (ZZ/ZW) homogamety are the most prevalent GSD systems, but sometimes there are losses of either the Y or W chromosome (X0 or Z0 systems), or translocations/ fusions with an autosome (XX/XY1Y2 or X1X2/Y). Possible autosomal influences have been reported in a growing number of species considered to have a monogenic system. More complex systems with multiple sex chromosomes can exist within the same species, e.g. X, W and Y chromosomes in the platyfish, (*Xiphophorus maculates*) (Schartl, 2004). Polygenic determination with multiple factors located throughout the genome has also been reported (Devlin and Nagahama, 2002; Vandeputte et al., 2007). In some closely related species even amongst sister species (i.e. tilapias) both XX/XY and ZZ/ZW systems prevail, indicating frequent shifts during evolution and speciation events (Baroiller et al., 2009b).

1.3.2. Sex related genes

Some genes have been linked to sex determination or differentiation process such as *dmrt1, sox9a, amh, wt1, ftz-f1, gata,* and so on along with the development of molecular genetics (Von Hofsten and Olsson, 2005; Jørgensen et al., 2008). These genes have been found in many species across the animal kingdom besides fish. By estimating evolutionary distances and constructing phylogenetic trees of their proteins, the evolutionary history of these genes can be indicated. For example, molecular phylogenies were determined for vertebrate *DMRT1* proteins in order to reconstruct the evolutionary history of *DMRT1* and *DMRT1Y*. It shows that *DMRT1Y* from medaka was more closely related to the *DMRT* from the same fish rather than to the *DMRT* from platyfish, fugu, tilapia and trout. Thus, the formation of *DMRT1Y* by duplication of *DMRT1* in medaka arose after the separation from platyfish (Veith et al., 2003).

The identification and comparative analysis of sex determination genes are also useful for understanding the fish sex determination system. It has been suspected that there might be not only one master gene in the sex determination system initially but also other sex-related genes. The sex-related genes striving for stronger influence over the sex determination process during evolution. When one gene overrides the effects of

other genes, sex will be determined by a single-locus system and sex chromosomes may develop (Devlin and Nagahama, 2002). Thus, fish sex determination systems show less strict genetics, more gonad plasticity and more variation than mammals.

1.3.3. Environmental sex determination in fish.

Environmental sex determination (ESD) is more widespread than it was previously expected (reviewed by Baroiller et al., 1999). Until the first evidence that temperature had effects on sex differentiation in Atlantic silverside (*Menidia menidia*) by Conover and Kynard (1981), most of the studies had been focused on reptile and amphibian models. Hayes (1998) described sex determination as 'the mechanisms directing sex differentiation whereas sex differentiation is the development of testis or ovaries from the undifferentiated gonads. In vertebrates displaying an environmental sensitivity, the genetic sex determination takes place during fertilization by the combination of genetic factors brought by the male and female breeders. Environment factor alterations (temperature, hormone treatment, hypoxia, population, density, and pH) are known to influence phenotypic sex in several fish species (Zhang et al., 2009). They can either determine the sex or influence the sex differentiation (Baroiller et al., 2009b). The most common environmental cue affecting sex determination in fish is water temperature (Baroiller et al., 2009b). Other environmental conditions such as hormonal treatments, fish density, pH and hypoxia have also been shown to influence the sex ratio of fish species from very divergent orders. Hormonal treatments such as exposure to 17α-methyltestosterone have already been used to produce all males in in the rainbow halibut (*Paralichthys olivaceus*) (Yamamoto, 1998). Population density has been suggested to determine the sex ratioin some fish like lampreys (Beamish, 1993) and eels (Krueger and Oliveira, 1999). Also, pH imposes a significant influence on sex determination in some species, like the cichlid genus *Apistogramm* (Romer and Beisenherz, 1996). Besides the various environmental conditions influencing the sex differentiation in numerous of fish species, hypoxia has an effect on sex differentiation. Treatments (0.8 mg O_2/l) performed in the zebrafish gave a male-biased population (74.4 ± 1.7% males) compared to the control (5.8 mg O_2 /l) group (61.9 ± 1.6% males) (Shang et al., 2006). Generally, environmental factors often overlay or modify the outcomes of the genetic background in many species.

Effect of environmental conditions on the sex determination differs among different species according to the sex determination regimes, for example, in Nile tilapia

(*Oreochromis niloticus)* Medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). These species have different sex determination mechanisms. Nile tilapia, (*Oreochromis niloticus*) and Medaka, (*Oryzias latipe*) have a XY sex determination system (Baroiller et al., 2009b; Matsuda, 2005), whereas zebrafish (*Danio rerio*) seems to have a polygenic sex determination (Orban et al., 2009). In each of these species, however, environmental influences and autosomal modifier genes can also dictate sex determination (Siegfried. 2010) (Table 1).

Table 1: Sex determination mechanisms in tilapia, *Oreochromis niloticus*, medaka, *Oryzias latipes*, and zebrafish, *Danio rerio* (Siegfried, 2010).

Species	Sex determination system	Sex determination genes	Modifiers	Recent reviews
Tilapia	XY	Not known. Major sex determination locus on chromosome 1	Temperature; autosomal genes	Baroiller et al., 2009b
Medaka	XY	*Dmy*	Temperature; autosomal genes	Matsuda, 2005
Zebrafish	Polygenic	Not known	Hypoxia; temperature; food availability	Orban et al., 2009

1.3.4. Temperature-dependent sex determination (TDS)

Water temperature is one of the most important physical parameters to consider fish culture operations because of the profound effect it exerts on biological and chemical processes in living systems (Baroiller et al., 2009b). TDS is one of several existing types of environmental sex determination among animals but the only one so far is described reliably in reptiles. The first study, which discovered sex determination under the control of both genotype and temperature, was in Atlantic silverside (*Menidia menidia*; Conover and Kynard, 1981). TSD has been claimed in different other species such as Japanese flounder (*Paralichthys olivaceus*), Nile tilapia (*Oreochromis niloticus*), and Rainbow trout (*Oncorhynchus mykiss*) (D'Cotta et al., 2001; Kitano et al., 2000; Magerhans et al., 2009). The underlying mechanism of TSD has been related to a suppression of aromatase (cyp19) expression at male promoting temperatures resulting in masculinization, and an increased expression in of the aromatase in ovaries at feminizing temperatures (Baroiller and D'Cotta, 2001; Karube et al., 2007). This further supports the importance of this enzyme and its product (estrogen) in ovarian

differentiation in teleosts. Sex ratios under TSD can be biased and the prevalence of this mechanism over time is somewhat surprising, given the drastic environmental changes in temperatures that the earth has undergone. The earliest ontogenetic difference between sexes is an environmental one because the ambient temperature during sensitive periods of early development irreversibly determines phenotypic sex and, therefore, the sex ratio (Bull, 1983; Valenzuela et al., 2003). Studies on the mechanism of sex determination in many important commercially species such as in Nile tilapia (*Oreochromis niloticus*) have demonstrated that this species exhibits a predominantly monofactorial genotypic system with male heterogamety (*XY*) and female homogamety (*XX*). Exposure to high temperature at 36^{o}C can increase the male proportion compared to controls (27^{o}C) (Baroiller et al., 2009b). Effect of temperature on sex determination and /or differentiation is variable among species. Numerous species, which have not passed throughout a clear distinct chromosomal sex, show strong temperature dependent sex determination/differentiation (TSD) (Conover and Kynard, 1981; Strüssmann et al., 1996; Piferrer et al., 2005; Ospina-Alvarez and Piferrer, 2008) (Figure 2).

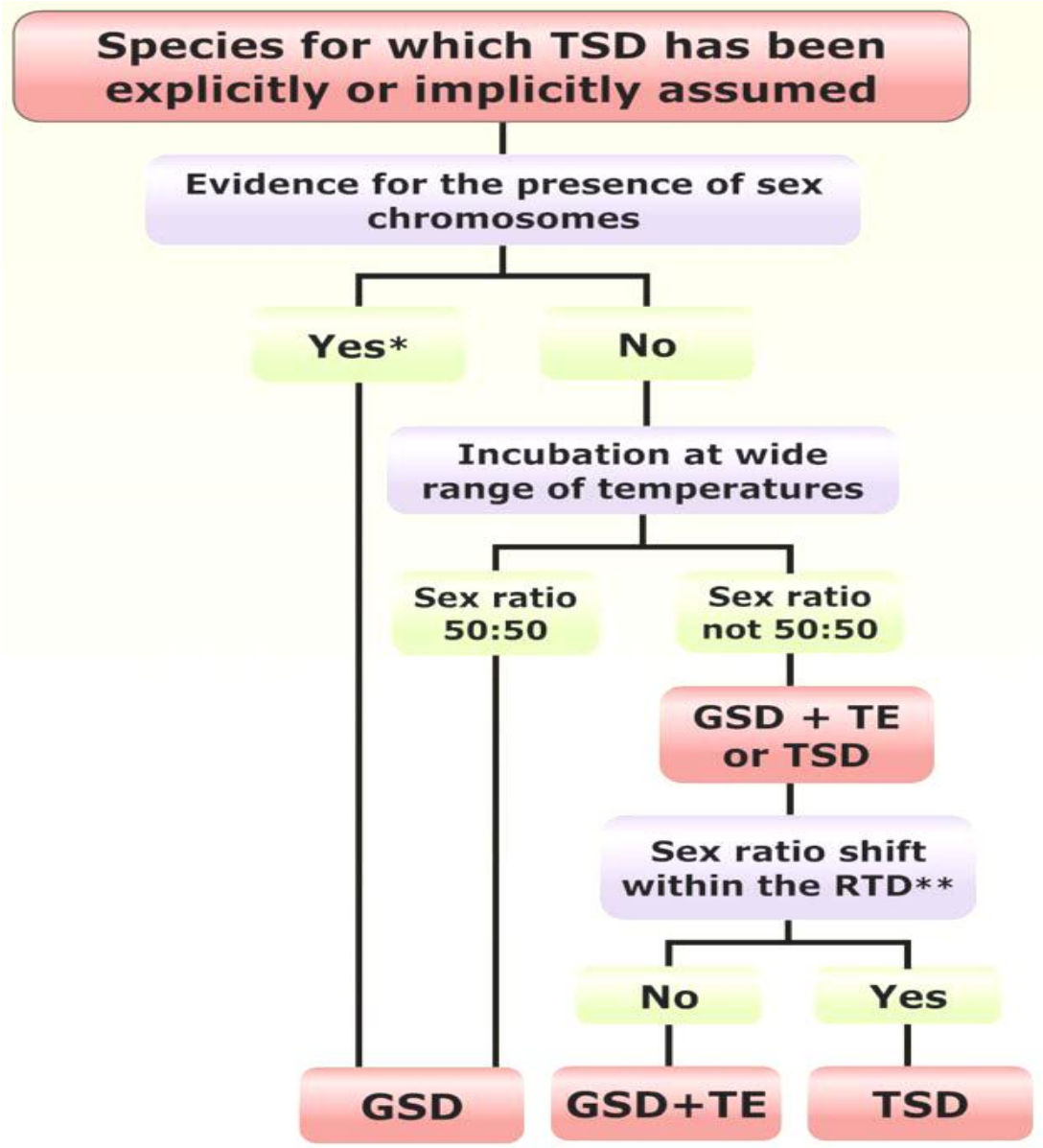

Figure 2: Set of criteria used to determine the presence of temperature-dependent sex determination (TSD) as opposed to genotypic sex determination (GSD), and to distinguish TSD from thermal effects on GSD (GSD+TE)(Ospina-Alvarez and Piferrer, 2008).

*Indicates that the evidence for a sex chromosomal system may come from direct (karyotyping, banding) or indirect methods (e.g., progeny analysis of sex-linked traits, mating experiments or crosses with sex-reversed fish). **Indicates that the sex ratio shift must occur within the range of developmental temperatures during development that includes the thermo sensitive period (RTD) regardless of whether there is response within the range of natural temperatures where the species lives.

1.3.5. The relationship between the environmental factors and sex related genes in fish.

Alterations of environment factors, such as water temperature and hormone treatment, have been applied for controlling sex in commercial production in many fish species. These factors act through effects on some sex-related genes. Water temperature can influence the sex ratio by altering the activity of genes like aromatase (*cyp19*) whereas the actions of hormones (androgen or estrogen) are mediated through specific nuclear receptors (estrogen receptor (*ER*) or androgen receptor (*AR*)) and other genes. The expression profiles suggested that the anti-androgen and the estrogen largely operate via distinct molecular mechanisms. For instance, in liver, *EE2* (the model synthetic estrogen) exposure up-regulated *ERα mRNA* while flutamide (the model anti-androgen) exposure increased *Erβ* and *ERγ mRNAs* in males and decreased *AR mRNA* in females. There were also some commonalities between flutamide and *EE2* action mechanisms. They both decreased gonadal sex steroid receptor expression (gonadal *AR* and ovarian *ERα*), increased expression of *CYP19A* and *CYP19B* that code for estrogen-producing enzymes, decreased expression of *AMH* and *DMRT* which were involved in testis differentiation, and decreased expression of hepatic genes which mediate wider physiological processes (Filby et al., 2007). In another species, such as rainbow trout, the expression of sex-related genes during ovary-to-testis trans-differentiation has been analyzed. It is revealed that masculinization with androgens acts firstly by repressing granulosa cell-related genes, including genes involved in ovarian differentiation (like *cyp19a1a*), and subsequently by repressing genes for early oogenesis (like *Sox 23* and *Sox 24*) (Baron et al., 2008). Sex determination mechanism in the fish remains largely unknown so far (Zhang et al., 2009)

1.4. Zebrafish (*Danio rerio*) characteristics.

During the last 20 years zebrafish (*Danio rerio*) was gaining increasing popularity as a vertebrate model for various biological studies and used as an important model organism in developmental biology and genomic research. Zebrafish is a free spawning minnow native to South Asia and has a wide tropical and sub-tropical geographic distribution that extends over eastern India, north to Nepal and across Bangladesh into northern Burma (Laale, 1977). Recent habitat surveys in Bangladesh revealed that zebrafish are typically found in floodplain areas in open, shallow lakes and waterlogged rice fields (Spence et al., 2006).

During the last few decades zebrafish is the favorite bioassay organism, due to its small size, robustness, short life cycle and the fact that under laboratory conditions it can be induced to breed all year round. Development from the fertilized egg to full reproductive maturity takes only 3–4 months. This relatively short generation interval makes zebrafish suitable for partial and full life cycle tests to evaluate the effects of chemicals on ontogenetic differentiation and reproduction of fishes. Little information were provide about sex determination mechanism in zebrafish (Jørgensen et al., 2008; López-Olmeda and Sánchez-Vázquez, 2011).

1.4.1. Sex determination mechanism in zebrafish

Sex determination process is a complex trait in zebrafish, not employing sex chromosomes till now (Sola and Gornung, 2001; Ueda et al., 2001), number of candidate genes have previously been investigated with roles in sex differentiation in zebrafish such as *dnd*, *fancl*, *cyp19a1a* and *b*, *ff1a - d*, *foxL2*, *sox9a* and *b*, *wt1a* and *b*, *amh*, *Dmrt1* and *Cyp21a2* (Trant et al., 2001; Onichtchouk et al., 2003; Weidinger et al., 2003; Uchida et al., 2004; Kuo et al., 2005; Rodriguez-Mari et al., 2005; Von Hofsten and Olsson, 2005; Schulz et al., 2007; Jørgensen et al, 2008; Siegfried and Nusslein-Volhard, 2008; Rodriguez-Mari et al, 2010; Bradley et al., 2011). On contrary, Wallace and Wallace (2003) assumed that the sex determination in zebrafish is not determined by genetic factors. Zebrafish are undifferentiated gonochorists, as defined by Yamamoto (1969). All individuals first initiate oogenesis, forming an immature non-functional ovary before developing a fully differentiated ovary or testis. In the "juvenile ovary", perinuclear oocytes are present in gonads of all fish (Takahashi, 1974; Maack and Segner, 2003; Wang et al., 2007). Subsequent initiation of testis specification in developing males becomes apparent by an irregular appearance and degeneration of oocytes. Then an increase of somatic stromal cells is seen followed by the initiation of spermatogenesis (Takahashi, 1974; Maack and Segner, 2003). In zebrafish, female develop earlier than males, with the appearance of primary oocytes in the gonad at about three weeks of age and discernable testes at 40 days after hatching (Takahashi, 1977). Expression of both male and female genes became sexually dimorphic only after 25 dpf (Krovel and Olsen, 2004; Rodriguez-Mari et al., 2005; Wang et al., 2007; Jørgensen et al., 2008; Siegfried and Nusslein-Volhard, 2008).

During transformation stage, the gonad in zebrafish goes through a process similar to that of sequential hermaphrodite teleosts that change sex from female to male during

their adulthood (Frisch, 2004). Therefore testis differentiation in zebrafish could serve as a potential model for the protogynous sex changers (Wang et al., 2007). In developing females, progression of oogenesis occurs. This mode of gonad development has been reported in other fish including rainbow trout and the Sumatra barb (Misic, 1923; Takahashi and Shimizu, 1983). According to Wang et al. (2007), the status of ovary differentiation and 'juvenile ovary to testis' transformation is indicated by the dynamic expression changes of EGFP (enhance green fluorescent protein) in vas: using *egfp* transgenic zebrafish. The increasing fluorescence intensity suggests the increase in oocytes number and the growth of ovarian lumen and shape. In contrast, the decreasing fluorescence indicates the degeneration of oocytes and the transformation of ovary into testis. Males were divided into three types based on EGFP expression level during the juvenile ovary stage (Figure 3).

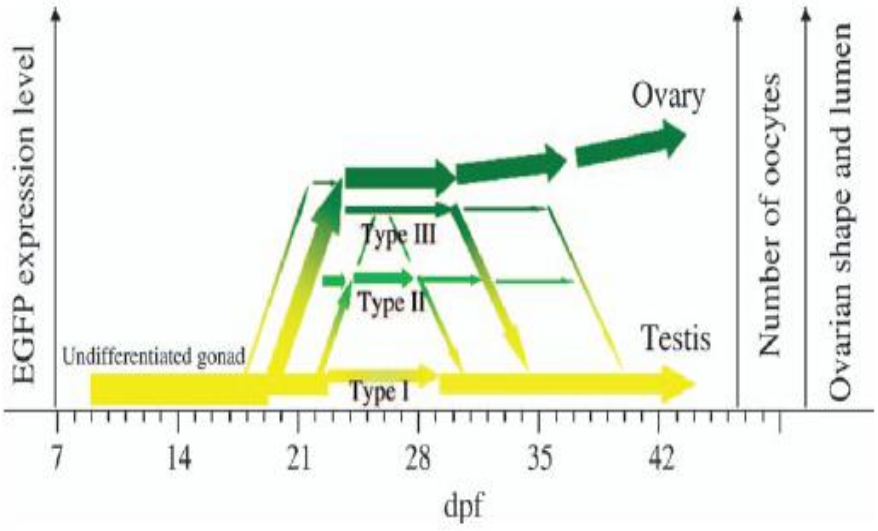

Figure 3: The model for gonad differentiation in zebrafish shows high levels of variation in the intensity, onset and duration of gonad transformation (Wang et al., 2007).

Environmental factors including hormones, temperature and hypoxia are known to perturb sex differentiation in zebrafish (Westerfield, 1995; Hill and Janz, 2003; Uchida et al., 2004; Shang et al., 2006), while germ cells also control female gonad development (Siegfried and Nusslein-Volhard, 2008). Less is known for TSD during the precious stages (embryogenesis) and during the larvae development in zebrafish.

1.4.2. Effect of water temperature on sex determination in zebrafish

A few studies were done on the effect of rearing water temperature on sex determination or differentiation in zebrafish. Elevated water temperatures (15-25 dph) lead to an increase of the phenotypic males from 0% at the control (28.5^{o}C) to 68.8%, 100% at 35^{o}C and 37^{o}C, respectively (Uchida et al., 2004) (Figure 4).

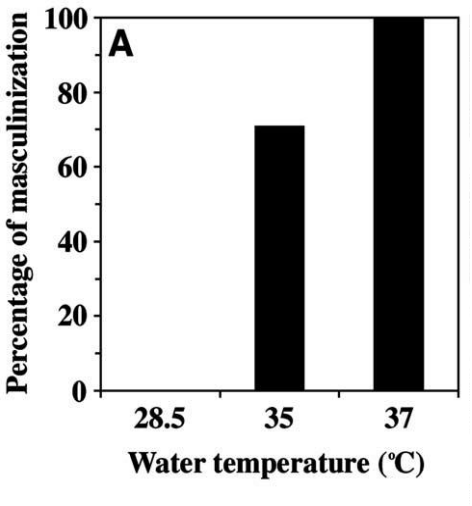

Figure 4: Gonadal masculinization of genetic all-females was induced by high water temperature between 15 and 25 days post-hatching. As a result of histological observation of the gonads at 40 days post-hatching, the percentage of masculinization in genetic all-females at 28.5 ^{o}C (control), 35 ^{o}C and 37 ^{o}C were 0 %, 68 %and 100 %, respectively. (Uchida et al., 2004).

In zebrafish, PGCs are essential for the formation of female gonads, and maintain the initial ovary (Siegfried and Nüsslein-Vollhardt, 2008). The elevated water temperatures have a significant effect on proliferation of PGCs (Lee et al., 2009; Silem et al., 2009). Additionally, the elevated water temperatures have also an effect on the aromatase activity and lead to increase the apoptosis of the primary ovary structure (Uchida et al., 2004) (Figure 5).

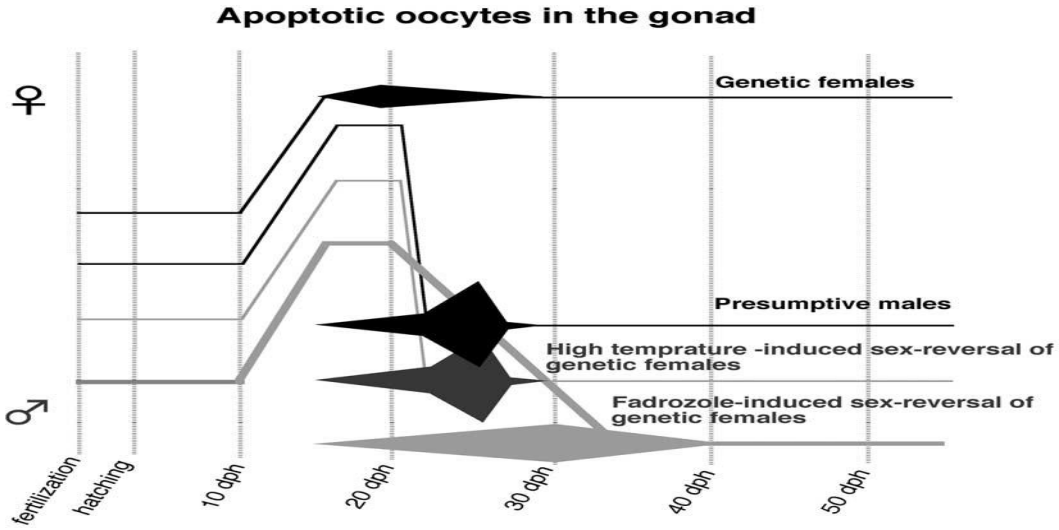

Figure 5: Model for oocyte apoptosis causing periods in the gonads of presumptive males, genetic females, and high temperature-induced sex reversal of genetic females and fadrozole-induced sex-reversal of genetic females. (Uchida et al., 2004)

Analysis of sex determination in zebrafish (*Danio rerio*) is useful in evolution for study and also helpful to understanding the sex determination system in cultured fish. There are numerous species of fish in the animal kingdom in which their reproductive mechanisms are highly variable (Devlin and Nagahama, 2002). While some reproduce asexually, most do sexually (Ward, 2002). Among sexual fish species, sex determination systems are mostly affected by both environmental and genetic factors

1.5. Objectives and goals

1-This study represents part of an effort to establish the zebrafish to investigate the effect of elevated water temperature on sex determination pathway and later on the transformation stage (sex differentiation).

2- To clarify and explain the effect of elevated water at 35^{o}Ceither during the embryogenesis or later during the larval development.

3-To study the interaction between the genetic factors (GSD) and environmental condition (temperature) and it's reflecting on the phenotypic sex ratio.

Chapter 2

Effect of rearing temperatures during embryonic development on the phenotypic sex in zebrafish (*Danio rerio*)

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Abstract

In zebrafish (*Danio rerio*) a polygenic pattern of sex determination or a female heterogamety with possible influences of environmental factors is assumed. The present study focuses on the effects of elevated water temperature (35°C) during the embryonic development on sex determination in zebrafish. Eggs derived from three golden (gol) females were fertilized by the same mitotic gynogenetic male and exposed to a water temperature of 35°C applied from 5-10 hpf, 5-24 hpf and 5-48 hpf, which corresponds to the following developmental stages: gastrula, gastrula to segmentation, and gastrula to pharyngula stage, respectively. Hatching and survival rates decreased with increasing exposure to high water temperatures. Reductions in the hatching and survival rates were not responsible for differences in sex ratios. Accordingly, exposition of the fertilized eggs to high temperature (35°C) lead to an increase of the male proportion from 22.0 % in the controls to a balanced sex ratio (48.3 %, 47.5 %, 52.6 %) in the gastrula, segmentation and pharyngula groups, respectively. These results prove the possibility to change the pathway of sexual determination during early embryonic stages in zebrafish by exposure to high water temperature.

Keywords: Zebrafish; temperature-dependent sex determination; embryogenesis

1. Introduction

Sex determination and differentiation processes in fish are complex and labile mechanisms under the control of genetic (GSD) and/or environmental factors (ESD) (Conover, 2004; Ospina-Alvarez and Piferrer, 2008; Baroiller et al., 2009a). Water temperature is the most relevant environmental factor affecting the sex determination process in fish (Devlin and Nagahama, 2002; Baroiller et al., 2009b). Although the zebrafish is considered the most important model to study developmental genetics and -biology, as well as biotechnology in vertebrates (Kimmel, 1989; Barinaga, 1990; Kahn, 1994; Fishman, 2001; Grunwald and Eisen, 2002; Rubinstein, 2003; Amsterdam and Hopkins, 2006), the mechanism of sex determination in zebrafish is largely unknown. Neither sex chromosomes, nor sex-linked mutations or markers have been observed so far (Sola and Gornung, 2001; Traut and Winking, 2001; Wallace and Wallace, 2003).

Recently, Tong et al, 2010 reported that sex determination in zebrafish is controlled by female-dominant genetic factors (*ZW*). In zebrafish, exposure to high water temperature during the sex differentiation period (15-25 dph) leads to sex-reversal of genotypic females to phenotypic males, proving the prevalence of temperature-dependent sex determination (TSD) (Uchida et al., 2004). In other species, such as (*Oreochromis niloticus*) *or* (*Oreochromis aureus*), the phenotypic sex is under the control of major genetic factors located on *LG1* or *LG3* (*XX/XY* or *ZZ/ZW*), which can be overridden by minor genetic factors (parental effects) and temperature during a critical period (Cnaani et al., 2008; Baroiller et al., 2009a).

Recently, gonadal sex reversal was even induced during embryogenesis by increasing the incubation temperature in Medaka (*Oryzias latipes*) and Nile tilapia (*Oreochromis niloticus*) (Selim et al., 2009; Rougeot et al., 2008). However, little information exists about exposure to high water temperature during the early developmental stages and its effects, later on sex differentiation in zebrafish. Therefore, the aim of our study was to investigate the influence of elevated water temperature (35^{o}C) on the sex determination process during different early developmental stages, the gastrula stage (5-10 hpf), segmentation stage (5-24 hpf) and pharyngula stage (5-48 hpf), of embryonic development in zebrafish.

2. Material and Methods

2.1. Breeders and egg collection

The experimental population of zebrafish (*Danio rerio*) used in the present experiment was a golden (gol) strain, which is defined to lack the melanophore pigmentation resulting in a yellow coloration with faint yellow stripes (Lamason et al., 2005). This strain was used in earlier studies and maintained according to the description of Hörstgen-Schwark (1993). All experiments were conducted at the recirculation system of the Division of Animal Sciences, Goettingen University (Germany).

2.2. Thermal treatment

Fertilized eggs were derived from a mating of a mitotic gynogenetic male to three normal females. Each of the half sib families was generated in duplicate using the same parents. Numbers of fertilized eggs were in the range of 336 – 940 per family. The eggs from each half sib family were divided in equal proportions into four groups, a control group incubated at 28°C and three treatment groups.

The treatment groups were incubated at 35°C from 1) 5-10 hpf, 2) 5-24 hpf, or 3) 5-48 hpf. The treatment durations correspond to the following developmental stages: 1) gastrula stage, 2) gastrula to segmentation stage, and 3) gastrula to pharyngula stage (Kimmel et al., 1995). Thus, the three treatment groups will be referred to as gastrula, segmentation, and pharyngula group throughout the manuscript. The incubation system was equipped with a heating system (Biotherm 2000). Water temperature was checked three times a day during the experimental periods. After finishing the thermal treatments all groups were kept at 28 °C in Petri dishes (diam. 9 cm, max. 100 eggs/ dish) until hatching.

Dead eggs were removed after 1, 5, 10, 24 and 48 hpf. The hatching (72 hpf) and survival rates (90 dpf) were determined by counting the number of fish alive in all experimental groups. Thereafter, the hatched fry were transferred to 5-l containers.

2.2.1. Rearing of juveniles

Hatching and rearing of experimental fish were carried out according to standard procedures developed by Von Hertell et al. (1990). Oxygen was maintained around 6 ppm (minimum), the pH value of water was 8.0 ± 0.2, ammonia and nitrite concentrations were 0.02 mg/l ± 0.01 and 0.01mg/l ± 0.01, respectively. In the recirculation system a 12 h day/night rhythm was applied. First feeding started 6 days post fertilization with artemia and artificial food (Tetramin, ad lib.) until the fish reached sexual maturation (90 dpf).

All fish were sacrificed using a lethal dose of anaesthetic (2-phenoxyethanol). The phenotypic sex of all fish was determined by microscopical examination of the gonads at 90 dpf. Therefore, gonad tissue was removed and squashed with a cover slide and the gonad samples were prepared for microscopical observation with an optical light microscope at 40 magnification. Sex identification was based upon the existence of oocytes in the females and upon the lobular morphology of the testis in the males.

2.2.2. Statistical analysis

The sex ratios were analyzed by fitting a generalized linear mixed model using the GLIMMIX macro (binominal error distribution, logit function) in SAS version 9.1, with sex coded as a binary trait (0 = male, 1 = female) (McLean et al., 1991). As fix factors the treatment, and as random factors the family and the replicate were included in the model. Effects on hatching and survival rates were analyzed using a generalized linear

model (Proc GLM) in SAS. Differences between sex ratios of treatment and control groups were tested for significance using a 2 x 2 contingency table with a $\chi 2$ test for balanced groups or Fisher's exact test for unbalanced groups and male or female frequencies below five.

To account for the variable number of offspring per treatment group and replicate, mean sex ratios were weighted according to the number of offspring. In order to test for differential mortality in the treatment groups, the observed frequencies of males (n males) and females (n females) were multiplied with the survival rate (% survival) in the corresponding full sib control to obtain the theoretical number of surviving fry (n_{theor}). Subsequently the difference between the theoretical number of fish (n_{theor}) and the observed number of males (n males) was calculated to obtain the theoretical number of females, if all dead fish were females ($n_{theor.}$- n males = $n_{theor.}$ females). Accordingly, the theoretical number of males (n_{theor} males) was calculated, if all dead fish were assumed to be males ($n_{theor.}$- n females = $n_{theor.}$ males). Fisher's exact test was applied to test the observed against the theoretical frequencies in two scenarios, 1) all dead fish were males, 2) all dead fish were females (Table 3).

3. Results

3.1. Effect of early temperature treatment on hatching and survival rates in zebrafish

The hatching and survival rates tended to decrease with increasing exposure to high water temperature (35^{o}C) in the gastrula (5-10 hpf), segmentation (5-24 hpf) and pharyngula group (5-48 hpf) (Table 1). The control groups had the highest mean values for hatching and survival rates with 27.14 % and 22.18 %, respectively. The mean hatching and survival rates (6.95 % and 4.83 %) in the pharyngula group (5-48 hpf) were significantly ($P< 0.05$) different from the control, the gastrula (5-10 hpf), and the segmentation (5-24 hpf) group.

No significant differences could be observed between the control, gastrula (5-10 hpf) and segmentation (5-24 hpf) groups. Furthermore, repeated matings of the same parents resulted in highly repeatable hatching and survival rates in the control and treatment groups of family number two and three. Only the second replicate of the family one showed a decrease of the hatching and the survival rate (45.3 % and 37.4 %), which might be attributed to low egg quality during spawning (Table 1).

3.2. Effect of early temperature treatment on sex ratios in zebrafish

The overall sex ratio in the control group was 22.0 ± 6.6 % of males. The sex ratio in the control group differed significantly from a 1:1 distribution ($P< 0.001$). However, exposition of eggs to high water temperature (35^{o}C) during the embryonic stages lead to a significant increase of the male proportion from 22.0 % in the control groups to balanced sex ratios (50.0 %, 47.5 % and 52.6 %) in the gastrula (5-10 hpf), segmentation (5-24 hpf) and pharyngula group (5-48 hpf), respectively. Increasing the time of exposure from 5-10 hpf, to 5-24 hpf or to 5-48 hpf did not lead to significant differences of sex ratios among the treatment groups.

Weighting the mean sex ratios by the number of sexed individuals gave values comparable to the observation sex ratios (Table 2). Repeated matings of the same parents gave almost equal sex ratios both in the control and the treatment groups. No differential mortalities could be observed in the gastrula (5-10 hpf) and segmentation (5-24 hpf) group, when the observed and theoretical frequencies of males and females were compared (see supplemental table 1). In the pharyngula group (5-48 hpf) both scenarios, 1) if all dead fish were assumed to be male and 2) if all dead fish were assumed to be female, resulted in significant differences between the observed and the theoretical frequencies of males and females in one replicate of two families (family 1 and 2, replicate 1). In the second replicate of family 2, a significant difference could only be detected, if the dead fish were assumed to be female (Table 2).

4. Discussion

The sex determining system in zebrafish shows a high plasticity and no sex chromosomes have been identified (Traut and Winking, 2001; Wallace and Wallace, 2003; Orban et al., 2009). Recently, a female heterogametic system (*ZZ*/*ZW*) was reported in zebrafish (Tong et al., 2010). Before, the sex determination mechanism in zebrafish has been described as multigenic with possible weak secondary influences from environmental factors (Orban et al., 2009).

The extent of interaction between the major genetic, minor genetic and environmental factors is still unknown in zebrafish, but it might differ within and between populations, as observed in Nile tilapia (*Oreochromis niloticus*) (Tessema et al., 2006).

Elevated water temperature during sex differentiation (15-25 dpf) leads to significant effects on the phenotypic sex of genetic all-female progenies (Uchida et al., 2004).

According to Shang et al. (2006) hypoxia can also affect the sex differentiation and sexual development of zebrafish.

The timing and duration of the water temperature treatment is of critical importance to induce phenotypic sex reversal in fishes (Piferrer, 2001). Generally in fish, little information is provided about the effects of water temperature on the sex determination process before hatching (Rougeot et al., 2008). Therefore, the present study focused on the effects of an elevated water temperature (35^{o}C) during the embryonic development on the phenotypic sex in zebrafish.

Elevated water temperatures during the embryonic development are known to cause abnormalities in early development and increase the physical stress during these stages (Heugens et al., 2001). In the present investigation, extending the exposure period of embryos to high water temperature (35^{o}C) from 5-10 hpf or 5-24 hpf to 5-48 hpf significantly reduced the hatching and survival rate (Table 1). Irrespective of the hatching and survival rates, the present study proves that it is possible to change the sex ratio during zebrafish embryogenesis through exposure of eggs to elevated water temperature (35^{o}C). Differential mortalities of females or males could not be observed in the gastrula (5-10 hpf) and segmentation (5-24 hpf) groups. In the pharyngula group, only one out of six replicates differed significantly for scenario two only (scenario 2 = all dead fish were females), thus indicating that differences between the observed and the theoretical frequencies of males and females might rather be attributed to the overall low number of individuals. However, all sex ratios in the pharyngula group were pooled and weighted according to the number of sexed fish. The weighted sex ratios did not differ from the simple means, indicating that mean sex ratio was not negatively affected by the number of observations.

The mating between normal females (*XX*) and a sex reversed gynogenetic male, as applied in the present study, would give 100% females in the F1-generation according to Uchida et al. (2004). In contrast, Tong et al. (2010) assumed that sex determination in zebrafish is controlled by female-dominant genetic factors (*ZW*). Theoretically, the mitotic gynogenetic males would be *ZZ* or sex reversed males *WW*. The female proportion in the F1-generation would be 100% female (in case using *ZZ* as a father) or 50% female (in case using *WW* as a father). Actually, in the present study the female proportion in the F1-generation was 78 % in the control groups. Neither of the assumed

system, *XX*/*XY* nor *ZW*/*WW*, is sufficient to explain the observed sex ratios obtained in our experiments.

Moreover, the sex ratios in replicates of the control and the treatment groups were highly repeatable, indicating that the sex ratio at both ambient and early elevated temperatures might be under genetic control. Sex reversal during the incubation periods, gastrula (5-10 hpf), segmentation (5-24 hpf) and pharyngula (5-48 hpf), leads to an increase of the male proportion from 22 % in control groups to balanced sex ratios (47.37 %, 47.85 % and 52.63 %) in gastrula (5-10 hpf), segmentation (5-24 hpf) and pharyngula stage (5-48 hpf), respectively. Extending the thermal treatment starting from 5-10 hpf to 5-24 hpf and 5-48 hpf did not lead to a further increase of the male proportion (see table 2), indicating that beside the window determined by Uchida et al. (2004) from 15-25 dph there is a temperature sensitive window from 5-10 hpf.

According to Kimmel et al. (1995) the first appearance of the germ ring is at 5.7 hours post fertilization (at 28.5°C). Generally the effect of precocious temperature treatments on phenotypic sex has been studied only in a few fish species such as Nile tilapia, *Oreochromis niloticus* (Rougeot et al., 2008) and medaka (*Oryzias latipes*) (Selim et al., 2009). However, both groups found that heat treatment during the embryogenesis can have an effect on the sex determination process in those species. In medaka (*Oryzias latipes*) and hynnan ricefish (*Oryzias curvinotus*) a DMY-controlled XX/XY sex chromosomalhas been identified (Matsuda et al. 2003; Nanda et al. 2002). Elevated water temperatures may affect proliferation and development of germ cells (Selim et al., 2009) as well as the development of oocytes during later stages (Uchida et al., 2004).

Earlier studies deal with two scenarios to explain the effect of water temperature on sex determination in zebrafish (*Danio rerio*), the first scenario assumes an effect of the high water temperatures on the survival and apoptosis of PGCs (Baroiller et al., 2009b), which are essential for the formation of female gonads in zebrafish, and maintain the initial ovary (Siegfried and Nüsslein-Vollhardt, 2008). The second scenario assumes an effect of high water temperature during the sex differentiation period (15-25 dph). During this stage the inhibition of the aromatase activity might lead to apoptosis of the primary ovary structure, which in turn leads to sex-reversal of genotypic females to phenotypic males (Uchida et al., 2004).

However, Selim et al. (2009) found that a temperature increase from 27°C to 32°C starting from stage 25 (beginning of sex differentiation) until stage 36 (hatching at 6 days post fertilization) led to a functional masculinization of *XX*-females, indicating that high temperatures inhibited the proliferation and development of germ cells. Lee et al. (2009) found that in the Pufferfish (*Takifugu rubripes*) early high temperature treatments (32°C) induced a degeneration of the gonads, shown by a complete lack of germ cells. However, morphologically high-temperature treatments had no effect on sexual differentiation and female ovary characteristics were maintained. Despite, the male-specific gene *dmrt1* was expressed in ovarian tissues with degenerated germ cells, indicating a masculinizing effect of early high temperatures. In zebrafish (*Danio rerio*) Siegfried and Nüsslein-Vollhardt (2008) showed that the germ line is required for the ovary versus testis fate. When the germ line was absent, the gonad underwent testis fate and normal somatic structures were developed. As the primordial germ cells are sensitive to high water temperature (Lee et al., 2009; Selim et al., 2009), apoptosis might lead to the development of male somatic tissues (Baroiller et al., 2009b).

The fact that in the present study the major trigger on the progeny sex ratios could be seen already during the period from 5-10 hpf, might emphasize the susceptibility of germ cells to high water temperatures and their importance for the sex differentiation process in zebrafish. As suggested by Baroiller et al. (2009b), the early number of germ cells (present between 5-10 hpf) might be a critical value for the commitment to testes versus ovary fate in some fish species. At ambient temperatures, all individuals are committed to female oocyte development resulting first in the development of non-functional ovaries, which then either remain ovaries or undergo testes development through regression and apoptosis of female somatic structures (Maack and Segner, 2003). As shown by Siegfried and Nüsslein-Vollhard (2008) the absence of the germ-line will directly lead to the development of male somatic tissue. In the present study, it is not known whether the gonads of the sex reversed fish underwent the "normal" transition from a proto-ovary to testes, nor if the sex reversed males were fertile (because they were scarified for sexing) but an effect of high early rearing temperatures on the number and survival of germ cells might have influenced the transition from proto-ovaries to testes which has to be examined, however, in further experiments.

Conclusion

The present study showed that the phenotypic sex of zebrafish can be altered using early elevated temperatures applied for a very short period in the gastrula stage from 5-10 hpf. These findings indicate that two windows of thermal responsiveness exist in zebrafish, the first during embryonic development (5-10 hpf) and the second during the sex differentiation period Uchida et al. (2004). Moreover the present study proved that sex ratios in progenies derived from repeated matings of the same parents kept at ambient temperatures or treated at elevated temperatures during embryonic development are highly repeatable and thus seem to be under genetic control. Furthermore the role of high temperature applied during embryonic development emphasizes the need of research related to the expression of the phenotypic sex and germ cell survival. Further investigations are needed to study the role of temperature treatments during embryonic development on the proliferation of germ cells in zebrafish.

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Tables

Table 1: Effect of high water temperature (35°C) during the embryonic development from 5-10 hpf, 5-24 hpf and 5-48 hpf on hatching rates and survival rates (90 dpf) of zebrafish (fertilized eggs derived from repeated matings between a mitotic gynogenetic male and three different females)

			Hatching rates % (0 hpf - 72 hpf)				Survival rates % (0 hpf - 90 dpf)			
			Treatment				Treatment			
Family	Rep	N	Control	5-10 hpf	5-24 dpf	5-48 hpf	Control	5-10 hpf	5-24 hpf	5-48 hpf
1	1	366	56.82	49.41	30.16	10.45	46.59	45.88	30.16	8.96
1	2	524	11.45	6.87	6.87	3.05	9.16	5.34	6.11	1.53
2	1	940	19.57	16.17	14.47	7.66	18.72	14.47	11.06	6.81
2	2	740	21.08	15.68	15.68	2.70	17.30	15.68	13.51	1.62
3	1	364	24.18	18.68	17.58	14.29	18.68	14.29	10.99	7.69
3	2	336	29.76	17.86	13.10	3.57	22.62	16.67	11.90	2.38
$\bar{x}$ ± SD		545	27.14^{a} ± 15.7	20.78^{a} ± 14.7	16.31^{ab} ± 7.7	6.95^{b} ± 4.7	22.18^{a} ± 12.8	18.72^{a} ± 13.9	13.96^{ab} ± 8.3	4.83^{b} ± 3.4

Mean values with a different superscript letter were significantly different at a level of $P< 0.05$, df = 1; hpf = hours post fertilization; dpf = day post fertilization; Rep = replication; N = initial number; SD standard deviation

Table 2: Effect of high water temperature (35°C) during the embryonic development from 5-10 hpf, 5-24 hpf and 5-48 hpf on sex ratios of zebrafish (fertilized eggs derived from repeated matings between a mitotic gynogenetic male and three different females)

Treatment	Family	Replicate	N sexed	Males %	Chi-square	Males % weighted
Control	1	1	41	21.95	12.90**	20.75
	1	2	12	16.67	5.33*	
	2	1	44	27.27	9.09**	27.63
	2	2	32	28.13	6.12*	
	3	1	17	11.76	9.94**	19.44
	3	2	19	26.32	4.20*	
	$\bar{x}$ ± SD			22.01 ± 6.59	31.32**	25.32
5-10 hpf	1	1	29	38.46	0.31 ns	42.09
	1	2	7	57.14	0.14 ns	
	2	1	34	52.94	0.12 ns	49.21
	2	2	29	44.83	0.31 ns	
	3	1	13	46.15	0.08 ns	48.15
	3	2	14	50.00	0.00 ns	
	$\bar{x}$ ± SD			48.25 ± 6.57	0.12 ns	46.94
5-24 hpf	1	1	38	47.37	0.11 ns	47.83
	1	2	8	50.00	0.00 ns	
	2	1	26	53.85	0.15 ns	49.02
	2	2	25	44.00	0.36 ns	
	3	1	10	50.00	0.00 ns	45.00
	3	2	10	40.00	0.40 ns	
	$\bar{x}$ ± SD			47.54 ± 4.92	0.24 ns	47.86
5-48 hpf	1	1	6	50.00	0.00 ns	50.00
	1	2	2	50.00	0.00 ns	
	2	1	16	56.25	0.25 ns	57.90
	2	2	3	66.67	0.33 ns	
	3	1	7	42.86	0.14 ns	44.45
	3	2	2	50.00	0.00 ns	
	$\bar{x}$ ± SD			52.63 ±8 .07	0.27 ns	52.77

Fertilized eggs were derived from repeated matings between a mitotic gynogenetic male and 3 different females.

*P < 0.05, df = 1; **P < 0.001 df = 1, ns = not significant; hpf = hours post fertilization; SD = standard deviation; chi-square = chi-square value for the deviation from a 1:1 distribution; Males % weighted = Σ (n males sexed (replicate 1) * male % (replicate 1) + n males sexed (replicate 2) * male % (replicate 2))/ n sexed per family)

Supplemental table 1: Comparison of observed frequencies of male and female zebrafish in different treatment groups (5-10 hpf, 5-24 hpf, and 5-48 hpf) with theoretical male and female frequencies if all dead fish are assumed to be male (scenario 1) or all dead fish are assumed to be female (scenario 2).

Family	Treatment	Final number	Survival (%)	Theor. final number	Sex	n	n_theor. males	Two sided Pr.<= P Scenario 1	n_theor. female	Two sided Pr.<= P Scenario 2
1	1	41	46.6	41	0	9	9	n.t.	9	n.t.
1	1	41	46.6	41	1	32	32		32	
1	2	29	34.1	40	0	13	24	0.2321	13	0.2346
1	2	29	34.1	40	1	16	16		27	
1	3	38	30.2	59	0	18	39	0.0911	18	0.1315
1	3	38	30.2	59	1	20	20		41	
1	4	6	9.0	31	0	3	28	0.0418*	3	0.0418*
1	4	6	9.0	31	1	3	3		28	
1	1	12	9.2	12	0	2	2	n.t.	2	n.t.
1	1	12	9.2	12	1	10	10		10	
1	2	7	5.3	12	0	4	9	0.6169	4	0.3765
1	2	7	5.3	12	1	3	3		8	
1	3	8	6.1	12	0	4	8	0.6479	4	0.6479
1	3	8	6.1	12	1	4	4		8	
1	4	2	1.5	12	0	1	11	0.2747	1	0.2747
1	4	2	1.5	12	1	1	1		11	
2	1	44	18.7	44	0	12	12	n.t.	12	n.t.
2	1	44	18.7	44	1	32	32		32	
2	2	34	14.5	44	0	18	28	0.3633	18	0.3616
2	2	34	14.5	44	1	16	16		26	
2	3	26	11.1	44	0	14	32	0.1251	14	0.0822
2	3	26	11.1	44	1	12	12		30	
2	4	16	6.8	44	0	9	37	0.0379*	9	0.0116*
2	4	16	6.8	44	1	7	7		35	
2	1	32	17.3	32	0	9	9	n.t.	9	n.t.
2	1	32	17.3	32	1	23	23		23	
2	2	29	15.7	32	0	13	16	0.7989	13	0.7991
2	2	29	15.7	32	1	16	16		19	
2	3	25	13.5	32	0	11	18	0.4287	11	0.5851
2	3	25	13.5	32	1	14	14		21	
2	4	3	1.6	32	0	2	31	0.1664	2	0.029*
2	4	3	1.6	32	1	1	1		30	
3	1	17	18.7	17	0	2	2	n.t.	2	n.t.
3	1	17	18.7	17	1	15	15		15	
3	2	13	14.3	17	0	6	10	0.7131	6	0.7106
3	2	13	14.3	17	1	7	7		11	
3	3	10	11.0	17	0	5	12	0.4153	5	0.4153
3	3	10	11.0	17	1	5	5		12	

3	4	7	7.7	17	0	3	13	0.167	3	0.3068
3	4	7	7.7	17	1	4	4		14	
3	1	19	22.6	19	0	5	5	n.t.	5	n.t.
3	1	19	22.6	19	1	14	14		14	
3	2	14	16.7	19	0	7	12	0.4969	7	0.4969
3	2	14	16.7	19	1	7	7		12	
3	3	10	11.9	19	0	4	13	0.2359	4	0.3904
3	3	10	11.9	19	1	6	6		15	
3	4	2	2.4	19	0	1	18	0.1857	1	0.1857
3	4	2	2.4	19	1	1	1		18	

(Treatments: 1 = control, 2 = 5-10 hpf, 3 = 5-24 hpf, and 4 = 5-48 hpf; theor. final number = observed frequencies of males and females multiplied with the survival rate in the corresponding full sib control; n = the observed frequencies of males and females; sex: 0 = male, 1 = female; n_theor. males = theoretical final number - n females; n_theor. females = theoretical final number - n males; scenario 1 = all dead fish are assumed to be male; scenario 2 = all dead fish were assumed to be female; Pr.<= P = derived from Fisher's exact test; n.t = not tested; * = significantly theoretical frequencies differ sgnificantly from the observed frequencies of males and females at a level of $P < 0.05$ at 1 degree of freedom)

Chapter 3

Elevated temperature applied during gonadal transformation leads to male bias in zebrafish (*Danio rerio*)

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Abstract

Temperature effects on sex determination or differentiation exist in many fish species, with high temperatures predominantly producing more males. The present study aimed at elucidating the genetic background of temperature effects on sex differentiation in zebrafish. Experimental fish were generated by matings between four or six golden females and a normal or a mitotic gynogenetic male, respectively. All the larvae were reared at 28.5^{o}C until they were divided into three groups per full sib family, a control raised at 28.5^{o}C, two treatment groups reared at 35^{o}C, from 20 to 30 dpf or 25 to 35 dpf, respectively. Back cross progenies, reared at 28.5°C, were derived from F1 temperature-treated sons (35°C, 25-35 dpf), sired by a mitotic gynogenetic male and their corresponding mothers. No significant differences were observed regarding the survival rate between the control and treatment groups. Significant differences in the phenotypic male proportions from the controls were observed in groups treated at 35^{o}C.The sex ratio in zebrafish was influenced by the male spawner, the female spawner, and a significant interaction of genotype by temperature. Back cross experiments point to a continuum of major genetic, minor genetic and environmental factors in the expression of the phenotypic sex in zebrafish.

Keywords: Zebrafish; temperature; sex determination; sex differentiation; sex ratio

1. Introduction

Sex determination mechanisms in most fish species are not clear, at present, and have a broad diversity. Between different species, such mechanisms range from genetic to environmental, from monogenic to polygenic, and from hermaphroditism to gonochorism (Devlin and Nagahama, 2002; Conover, 2004; Ospina-Alvarez and Piferrer, 2008; Baroiller et al., 2009a). Environmental factors affect the sex determination in many gonochoristic fish species, including zebrafish (Uchida et al., 2004; Shang et al., 2006; Baroiller et al., 2009a). Generally, water temperature seems to be the most prevalent environmental factor influencing sex in fish (Baroiller et al., 1999; Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Conover, 2004; Ospina-Alvarez and Piferrer, 2008).

The variations of water temperature within the normal range govern the speed of chemical reactions and provide important cues to control the timing of reproductive

cycles (Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002; Conover, 2004; Ospina-Alvarez and Piferrer, 2008; Penman and Piferrer, 2008; Baroiller et al., 2009a; Luckenbach et al., 2009). Temperature regulates the expression of the ovarian aromatase cyp19a1, which is consistently inhibited in temperature masculinized phenotypic males (Baroiller et al., 2009b). Additionally, water temperatures serve to control the proliferation of germs cells, which also could be a critical threshold for male or female sex differentiation (Selim et al., 2008; Baroiller et al., 2009b).

Zebrafish has received increased attention in last decades by the scientific community (genetics, neuroscience, development, physiology, toxicology and biomedicine) (Kahn, 1994; Vascotto et al., 1997; Fishman, 2001; Grunwald and Eisen, 2002; Rubinstein, 2003; Amsterdam and Hopkins, 2006; López-Olmeda and Sánchez-Vázquez, 2011). Yet, it is still difficult to classify the type of sex determination that occurs in zebrafish (Jørgensen et al., 2008; López-Olmeda and Sánchez-Vázquez, 2011). Morphological differences in the chromosomes of the two sexes have not been identified by classical karyotyping, implicating polygenetic or environmental signals in sex determination (Amores and Postlethwait, 1999; Sola and Gornung, 2001; Traut and Winking, 2001; Wallace and Wallace, 2003).

With regard to previous studies, sex determination in zebrafish might be controlled by a combination of genetic and environmental factors (Siegfried and Nüsslein-Vollhardt, 2008; Orban et al., 2009; Abozaid et al., 2011; Bradley et al., 2011; López-Olmeda and Sánchez-Vázquez, 2011). Tong et al. (2010) assumed that the phenotypic sex of zebrafish is determined by female-dominant genetic factors (*ZW*). A similar female-dominant system has been reported in some birds, amphibians, and fish (Ezaz et al., 2006; Santi-Rampazzo et al., 2007; Venere et al., 2008; Yoshimoto et al., 2008; Salvadori et al., 2009; Smith et al., 2009).

Additionally, elevated water temperature has been shown to strongly influence the phenotypic sex of zebrafish during sex differentiation (Uchida et al., 2004). Furthermore, it has been shown that elevated temperatures have an effect on the phenotypic sex of zebrafish during the embryonic development (Abozaid et al., 2011). Recently, two QTL for sex determination have been detected on chromosome 5 and 16, explaining 16% of the variance in the phenotypic sex (Bradley et al., 2011). However,

the authors clearly state that the complex genetic factors and environmental cues are likely to interact in zebrafish.

Therefore the goal of the present study was to clarify the effects of elevated water temperature (35^{o}C) during different stages of sex differentiation (20-30 dpf and 25-35 dpf) on representative numbers of zebrafish. The study especially aimed at exploring the effect of different mating partners in order to investigate the genetic background of interactions between genetic sex determination (GSD) and temperature effects on the expression of the phenotypic sex.

2. Material and methods

2.1. Brood stock

All experiments were performed with a golden laboratory zebrafish strain (go1) (*Danio rerio*) used already in earlier studies for the development of mitotic gynogenetic fish (Hörstgen-Schwark, 1993). This strain is characterized by a recessive colour mutant (a lack of melanophore pigmentation) in homozygous condition; thus, featuring a yellow coloration with faint yellow stripes (Lamason et al., 2005).The larvae were either derived from matings between a normal golden male and four golden females or from matings between a mitotic gynogenetic male and six golden females in the first generation. Back crosses were generated using four temperature-treated sons from four matings between a mitotic gynogenetic male and golden female with their respective mothers. All experiments were carried out in the warm water recirculation system for zebrafish of the Division of Aquaculture and Fresh Water Ecology of the Department of Animal Sciences, Goettingen University (Germany).

2.2. General husbandry of experimental fish and water parameters

The experimental fish were maintained according to standard procedures (Von Hertell et al., 1990; Westerfield, 1995). Briefly, the larvae were maintained on a 12 h day/night rhythm, which was applied in 4-l fresh water tanks from 4 days post-fertilization with a diet of Artemia salina nauplii and commercial feed (Tetramine baby; Tetra, Melle, Germany) until the fish developed sexual maturation at 90 dpf. The pH value of water was 8.0 ± 0.2, while ammonia and nitrite concentrations were 0.02 mg/l ± 0.01 and 0.01mg/l ± 0.01, respectively.

2.3. Thermal treatment

The larvae were maintained at 28.5°C until starting the thermal treatment and then divided into three groups. The control group was kept at 28.5^{o}C, whereas the two thermal treatment groups were exposed to 35^{o}C for ten days from 20-30 dpf or 25-35 dpf. After finishing the thermal treatment, all groups were kept at 28.5^{o}C again until the larvae developed sexual maturation at 90 dpf. The larvae from the back-crosses were continuously kept at 28.5^{o}C until 90 dpf. The recirculation system was equipped with a heating system (Biotherm 2000) to keep the temperature at the experimental values (± 0.2°C). Water temperature was monitored three times each day during the experimental periods.

2.4. Experimental design

2.4.1. First generation (F1)

In the present study two different mating schemes were applied. In the first mating scheme, the eggs were obtained from four golden females (a, b, e, f) and fertilised using sperm from a normal male. In the second mating scheme, the eggs were collected from six golden females (a, b, c, d, e, f), four of which (a, b, e, f) were the same females as in the first mating scheme. The eggs of these six golden females were fertilised with sperm from a mitotic gynogenetic male. The hatched larvae of each half sib family were reared at 28.5^{o}C until the start of the thermal treatment at 20 or 25 dpf. The larvae were divided into three groups per full sib family, a control which was kept at 28.5^{o}C and two thermal treatment groups which were subjected to high water temperature (35^{o}C) from 20-30 dpf or 25-35 dpf. After the thermal treatment periods all larvae were kept at 28.5^{o}C again, until the end of the experimental period around 90 dpf.

2.4.2. Backcrossing

A backcrossing trial was carried out between F1 temperature-treated sons, treated at 35°C from 25-35 dpf, sired by a mitotic gynogenetic male and their corresponding mothers (a, b, e, f) (Figure 1). The backcross offspring were constantly kept at 28.5^{o}C until the fish reached to the sexual maturity (90 dpf), when all fish were sacrificed and sexed.

2.5. Determination of gonadal sex

The sex ratio in the present study was determined in each individual group. Except for the temperature-treated males which were used to generate the back cross, all remaining

fish were sacrificed at 90 dpf by using a lethal dose of anaesthetic (2-phenoxyethanol). Subsequently, the phenotypic sex of all fish was determined by microscopical examination of the gonads.

Therefore, gonad tissue was removed and squashed with a cover slide and the gonad samples were prepared for microscopical observation with an optical light microscope at 40 x magnification. Sex identification was based upon the existence of oocytes in the females and upon the lobular morphology of the testis in the males.

2.6. Statistical analysis

Effects of the thermal treatments on the survival rates were analyzed using a generalized linear model (Proc GLM) in SAS. The fixed effects female spawner, male spawner, and treatment on the male proportion were analysed by fitting a generalized linear mixed model using the GLIMMIX macro (binominal error distribution, logit function) in SAS version 9.1, with sex coded as a binary trait (0=female, 1=male) (McLean et al., 1991). Generalized linear mixed models using the GLIMMIX macro were also used to compare the male proportion in the control of the first generation and the male proportion in the back cross for the same females.

3. Results

3.1. Effect of rearing temperature on survival rates in zebrafish

In table 1, the mean survival rates from 20-90 dpf are shown for matings between a normal male with four normal females (a, b, e, f), matings between a mitotic gynogenetic male with six normal females (a, b, c, d, e, f), and the respective backcrosses of temperature-treated sons with their mothers. Regardless of whether a normal male or a mitotic gynogenetic male was used as a sire in the first generation (F1), no significant differences were found for the mean survival rates between the controls (F1: normal male; mitotic gynogenetic male) and thermal treatment groups kept at 35°C either from 20-30 dpf or from 25-35 dpf.

Also, no significant differences could be detected in the mean survival rate between the controls (F1) and the back-crosses.

3.2. Effect of rearing temperature on phenotypic sex ratios in zebrafish

The male proportions in the temperature treated groups (35°C; 20-30 or 25-35 dpf) increased significantly when compared to the control at 28.5^{o}C (table 2). Furthermore,

both the male (normal vs. mitotic gynogenetic male) and the female parent exhibited a significant effect on the progeny sex ratios (see supplemental table 1). However, in the controls, the sex ratios differed significantly, with respect to the sire, giving 63% of males when a normal and 17% when a mitotic gynogenetic sire was used. For the sire a significant interaction between genotype and treatment was found, indicating genotype-environment interactions. However, for the dam the interaction between genotype and environment lacked significance. Moreover, due to the specific differences in the male genotypes, families did not rank similarly in each of the different environments (see figure 3). Table 2 shows that when a normal male (NM) was used, a significant increase in the male proportion from 63% in the control to 84% and 91% was observed when the fry were treated at 35^{o}C from 20-30 dpf or 25-35 dpf, respectively.

When a gynogenetic male was used as sire, the increase in the male proportion in the temperature treated groups was even more pronounced, giving 17% males in the control and 70% or 81% in the groups treated from 20-30 dpf or 25-35 dpf (35^{o}C), respectively. Differences between the two thermal treatment groups were significant, irrespective of the male used ($P< 0.01$). Thus, certain families tended to produce variable sex ratios in different environments leading to a significant interaction term of genotype and environment (family x treatment; see supplemental table 2).

3.3. Back-crosses

In the F1-generation male proportions among the four families sired by a mitotic gynogenetic male where comparable (see table 2). In contrast, three out of four back cross families, sired by temperature-treated sons and their corresponding mothers, exhibited male proportions significantly different from the expected (F1) sex ratios.

Compared to the F1 sex ratios, backcrosses showed generally higher male percentages, falling into two classes. Family a and e gave equally distributed sex ratios (55%; 52%), whereas family *b* and *f* gave sex ratios close to a 3:1 ratio (26.3%; 21.4%). In order to test if the observed sex ratios might be explained by a female heterogametic system, the back cross sex ratios were thus tested against a theoretical distribution of 1:1 or 3:1. Two of which were not significantly different from a 1:1 distribution ($P< 0.62$, $P< 0.82$) (see figure 2) and two of which were consistent with a distribution of 3:1 ($P< 0.87$; $P< 0.54$).

4. Discussion

4.1. Effect of elevated rearing temperatures on the survival rates in zebrafish

Generally, zebrafish tolerate a wide range of temperatures from 24.6^{o}C to 38.6^{o}C according to Engeszer et al. (2007). As limit temperatures of 38.6°C were not approached in the present study, survival rates were comparable among control (28.5°C) and temperature treated (35°C) groups, like observed by Uchida et al. (2004). In contrast, in Uchida et al. (2004), rearing zebrafish at a water temperature of 37°C or 39°C from 15 to 25 dph, reduced the survival rate drastically from 100% at 28.5°C and 35°C to 54.5% and 0%, respectively. Further, in the present study no differences in survival were found between groups treated from 20-30 dpf or later from 25-35 dpf. The sire (normal male or mitotic gynogenetic male) also did not influence the survival in control or treatment groups (see table 1).

4.2. Effect of elevated rearing temperature on the phenotypic sex in zebrafish

There are many evidences showing that in numbers of fish species such as zebrafish (*Danio rerio*) (as is also the case with many reptiles exhibiting temperature-dependent sex determination) exposure to high water temperatures during larval development leads to masculinisation of gonads, i.e., that some genotypic females fail to differentiate as phenotypic females (Kitano et al., 1999; Uchida et al., 2004).

Conversely, in some other species such as Channel catfish (*Ictalurus punctatus*) Black rockfish (*Sebastes schlegeli*) elevated water temperatures lead to inclines in sex ratio towards females (Patino et al., 1996; Omoto et al., 2010). However, in the present study, elevation of the water temperature (35^{o}C) had a significant effect on the phenotypic male ratios of offspring derived from matings between a mitotic gynogenetic male and six females (17.5% at 28°C;70.4% and 81.2% at 35°C from 20-30 or 25-35dpf).

Moreover, a thermal responsiveness was also detected in offspring derived from matings with a normal male. Compared to matings with a mitotic gynogenetic sire, offspring from the same mothers (4 out of 6) mated to a normal male exhibited even higher male proportions in the treated groups (control 28.5°C: 62.8%; 35°C from 20 to 30 dpf: 83.9%; 35°C from 25 to 35 dpf; 91.2%). Using a gynogenetic male as sire, Uchida et al. (2004), obtained similar increases in the male proportion in temperature treated groups (0% in the control, 68.8% at 35°C from 15-25 dph). However, the

authors could not confirm these results in normal outbred populations. At more elevated temperatures (37°C) this group obtained completely masculinised progenies.

However, mortalities were higher than at lower temperature groups In contrast to the present study, the gynogenetic male used by Uchida et al. (2004), sired 100% female offspring at ambient temperatures (28.5^{o}C). The mitotic gynogenetic male used in the present study gave average male proportions of 17% in the first generation. In a previous study, including the same mitotic gynogenetic male, comparable mean male ratios (22%) were obtained (Abozaid et al., 2011). Uchida et al. (2004), anticipated a male heterogametic system (XX/XY) leading to all-female offspring, when using a gynogenetic male as a sire. Pelegri and Schulte-Merker (1999) have proposed a polygenic system of sex determination in zebrafish, based on the existence of mixed sex ratios in gynogenetically produced broods and highly variable sex ratios in broods derived from natural matings.

Tong et al. (2010), recently provided evidence that even a female heterogamety (*ZZ/ZW*) might apply to explain sex ratios at ambient temperatures in zebrafish. Orban et al. (2009) postulated that the mode of sex determination in zebrafish is polygenic and is further (weakly) influenced by environmental factors. However, the influences of the different axis (major genetic factors, minor genetic factors, and environment) involved in zebrafish sex determination are still unknown (Abozaid et al., 2011).

Therefore it might be assumed, that under ambient temperatures (~28°C) two or more autosomal factors can lead to variable sex ratios in offspring of gynogenetic males. Moreover, previous studies have shown that these sex ratios are highly repeatable and seem to be heritable (Abozaid et al., 2011).

The timing and extent of the 'juvenile ovary' phase are highly variable during zebrafish sex determination (Wang et al., 2007) and ovaries begin to be transformed into testes 23-25 dph (Takahashi, 1977). The mechanism of elevated rearing temperatures on the transformation of juvenile ovaries into testes is not clear so far. Uchida et al. (2004), found that exposure to water temperatures of 35^{o}C and 37°C from 15 to 25 dph leads to phenotypic male proportions of 68.8% and 100%, respectively (0% males in the control). Increasing the rearing temperature during the period from 15-25 dph, directly, leads to massive reduction in the number of oocytes and masculinisation of the gonad.

Indirectly, elevated rearing temperatures, might also induce ovarian apoptosis by the inhibition of the aromatase activity in zebrafish (Uchida et al., 2004).

Similar observations, a decreased aromatase activity at elevated water temperatures, have been made in a variety of fish species such as Japanese flounder (*Paralichthys olivaceus*) Nile tilapia (*Oreochromis niloticus*) Medaka (*Oryzias latipes*) Atlantic halibut (*Hippoglossus hippoglossus*) Pejerry (*Odontesthes bonariensis)* (Kitano et al., 1999; D'Cotta et al., 2001; Sato et al., 2005; Van Nes and Andersen, 2006; Karube et al., 2007). Indeed, *P450* aromatase (*Cyp19a1*) is the most important steroidogenic enzyme for ovarian differentiation due to its essential role in the production of estradiol (E2) (Devlin and Nagahama, 2002; Guiguen et al., 2010) and maintaining ovarian development in fish (Yamamoto, 1969; Kitano et al., 1999; Baroiller et al., 2009b).

The significant differences in male proportions between the control and treatment groups, in the present study and the study of Uchida et al. (2004), seem to be directly related to the inhibition of aromatase activity. This is emphasized by the fact that the second window of thermal treatment (20-30 dpf vs. 25-35 dpf), applied in the present study, leads to higher male proportions. In zebrafish, the expression of *cyp19a1a* reaches a peak at 30 dpf in the females (Jørgensen et al., 2008). On the other hand, the massive degeneration of oocytes begins at 25 dpf in the future males (Krovel and Olsen, 2004; Rodríguez-Marí et al., 2005; Wang et al., 2007; Jørgensen et al., 2008; Siegfried and Nüsslein-Volhard, 2008; Rodríguez-Marí et al., 2010).

The massive degeneration of oocytes is accompanied by an abnormal increase with regard to apoptosis of germ cells, which provides a cellular mechanism for the female-to-male sex reversal in zebrafish males (Uchida et al., 2004; Rodríguez-Marí et al., 2010). Thus, the second window (25-35 dpf) would cover the period of the highest aromatase expression (30 dpf) where sexual differentiation of gonads is more or less completed (Takahashi, 1977) and massive oocyte degeneration starts at 25 dpf. The first window (20-30 dpf) would start before oocytes become largely apoptotic (Uchida et al., 2002) and stop before the peak of aromatase expression would be reached (Jørgensen et al., 2008).

The present study shows that the major window of thermal responsiveness in zebrafish is between 25-35 dpf, and that aromatase is likely to play an important role. Probably other genes such as *dmrt1, sox9a, amh, wt1, ftz-f1, gata*, might be conjointly acting, as

they are involved in sex differentiation in zebrafish (Von Hofsten and Olsson, 2005; Jørgensen et al., 2008).

4.3. The interaction between GSD and temperature effects during expression of the phenotypic sex in zebrafish

Major genetic (GSD), minor genetic and external factors like hormones, density,temperature, and hypoxia are implicated in the sex determination and/or gonad differentiation process of zebrafish (Corley-Smith et al., 1996; Nüsslein-Volhard and Dahm, 2002; Hill and Janz, 2003; Uchida et al., 2004; Shang et al., 2006). The extent of interaction between the different factors still remains unknown (Abozaid et al., 2011).

Here, we provide first evidence for a genotype and temperature interaction during sex differentiation in zebrafish. Due to the specific differences in the male genotypes, a mitotic gynogenetic male and a normal male were used, families did not rank similarly in each of the different environments (see figure 3).

Although the average male proportion in temperature treated groups were higher when a normal male (NM) was used, the relative increase in the male proportions between control and treatment group was higher in groups sired by the gynogenetic male (Gyn) (21% and 29% vs. 53 and 64%). Furthermore, some families tended to produce variable sex ratios in different environments (Figure 3), resulting in a significant genotype x temperature interaction term (see supplemental table 2, F= 2.28, $P< 0017$).

Due to the fact that a significant interaction of the family and the treatment was detected, it might be concluded that partially different sets of genes are acting under different thermal regimes, suggesting certain differences in thermal responsiveness which might be attributed to the specific male genotypes (NM vs. Gyn) used in the present study.

This is further supported by half-sib families ranking the same within sire, showing that G x E interactions might be caused by differences in the genetic make up of the sires. Beside the effect of the male spawner, the female spawner also exhibited an effect on the phenotypic sex ratio at elevated temperature. According to the results of the present study, sex ratio in zebrafish seems to be influenced by both, male spawner and female spawner, and an interaction of genotype x environment.

The genetic component in temperature dependent sex determination in zebrafish remains to be elucidated, but one share of the variance seems to be attributed to G x E interactions, meaning that not all variance of the trait is heritable.

This theory would fit well with Bradley et al. (2011), who describe the sex determination in zebrafish as complex trait, after discovering two major QTL on chromosome 5 and 16. These two QTL for sex determination account for 16% of the phenotypic variance, thus beside other genes, the authors conclude that environmental factors might be another important component in zebrafish sex determination.

The present study confirms the interaction of genotype and temperature. Moreover, the results might indicate that genetic and environmental factors are at least partially not mutually exclusive, although it is assumed that the mode of their control might have evolved distinctly (Rodríguez-Marí et al., 2010; Bradley et al., 2011).

4.4. Back-crosses

The present study aimed at tracing back the sexual genotype of the mitotic gynogenetic male through a series of back crosses. Therefore, temperature treated sons from the F1 were mated to their corresponding mothers and then kept at ambient temperature (28.5°C). The result showed that significant differences in the male proportion persisted between three of the back cross families (mothers a, e, f) and the corresponding F1-families (Figure 2). The deviations from expected sex ratios might further point to the role of two or more of the autosomal factors (minor genetic factor) playing an important, yet unknown, role in sex determination in zebrafish. Opposed to the observations of Uchida et al. (2004), Tong et al. (2010), postulated a possible female heterogamtic system (*ZZ/ZW*) in zebrafish. If, in the present study, the genotype of the mitotic gynogenetic male would be *ZZ* (*WW* would be the other possibility), and mated to a normal *WZ* female it should sire a mixed sex progeny (F1, ♂:♀, 1:1, see figure 2). The observed sex ratio in the control group of the F1 differed significantly (16% males) from the hypothetically equilibrated (1:1) sex ratio. Subsequently, the temperature treated (35°C/ 25-35 dpf) sons derived from the F1-generation were back crossed to their mothers, in order to explain the sexual genotype. The theoretical genotype of the F1-males could be either *ZZ* for the normal males or *WZ*, if sex reversed. Mating a normal male (*ZZ*) to its mother the resulting sex ratio would be 1:1, this applied in the 2 of the 4 back cross families. If mating a temperature sex reversed male (*WZ*) to its

mother, would thus give a theoretical male proportion of 25%. Such a ratio was found in the other two families. The sex ratios obtained in the back cross generation would support the hypothesis of Tong et al. (2010), pointing to the existence of a female heterogametic system. Sex ratios in the F1, however, cannot be explained using a simple model. Clearly, various factors (major genetic, minor genetic, and environmental) are required to explain sex determination in zebrafish, strengthening the results of Bradley et al. (2011), who postulate this mixture of GSD with additional environmental cues.

Conclusion

In conclusion, water temperature has a strong effect on sex determination of zebrafish. The phenotypic sex of zebrafish seems to be most susceptible towards rearing temperatures during the period from 25-35 dpf, when the expression of aromatase is peaking. The male proportion in temperature treated groups seems to be influenced by both, male and female parent. However, a share of the phenotypic variance is caused by genotype x environment interactions, indicating that partially different genes might be acting during GSD or temperature dependent sex expression. Sex ratios in the back cross progenies might point to the possible existence of a female major sex factors (ZZ/ZW), but additional autosomal and environmental factors are clearly needed to explain all observed sex ratios.

Acknowledgement

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Tables and figures

Table 1: Number of sexed individuals and survival rates in F1 zebrafish families derived from matings between a normal male (NM) or a mitotic gynogenetic male (Gyn) and normal females (a-f) reared at 28.5°C or 35°C from 20-30 or 25-35 dpf, as well as in back cross progenies sired by temperature-treated males ($MF1^{temp}$) and corresponding mothers from the F1 constantly kept at 28.5°C.

Generation	Male	Female	N	% Survival		
				28.5°C	35°C 20-30 dpf	35°C 25-35 dpf
F1	NM	a	76	78.9	78.9	80.0
F1	NM	b	62	85.5	79.0	82.3
F1	NM	e	79	89.9	62.0	65.8
F1	NM	f	46	95.7	89.1	78.3
$\bar{x}$ ± SD			65.8 ± 15.1	87.5 ± 7.0	77.3 ± 11.2	76.9 ± 7.4
F1	Gyn	a	43	81.4	67.4	74.4
F1	Gyn	b	51	90.2	96.1	88.2
F1	Gyn	c	31	77.4	80.6	87.1
F1	Gyn	d	59	94.9	100.0	88.1
F1	Gyn	e	81	88.9	95.1	88.9
F1	Gyn	f	82	82.9	79.3	74.4
$\bar{x}$ ± SD			57.8 ± 20.5	85.9 ± 6.4	86.4 ± 12.6	83.5 ± 7.0
BC	$MF1^{tempa}$	a	57	77.2		
BC	$MF1^{tempb}$	b	55	69.1		
BC	$MF1^{tempe}$	e	61	81.9		
BC	$MF1^{tempf}$	f	114	90.4		
$\bar{x}$ ± SD			71.8 ± 28.3	79.7 ± 8.8		

NM = normal male; Gyn = mitotic gynogenetic male; a - f = normal females; N = initial number of fish; SD = standard deviation; $MF1^{tempa}$, $MF1^{tempb}$, $MF1^{tempe}$, $MF1^{tempf}$ = males from full-sib heat treatment (35°C, 25-35 dpf) sired by a mitotic gynogenetic male in F1; BC = backcrossing

Table 2: Initial larvae number, number of sexed individuals and male proportions (90 dpf) in F1 zebrafish families derived from matings between a normal male (NM) or a mitotic gynogenetic male (Gyn) and normal females reared at 28.5°C or 35°C from 20-30 or from 25-35 dpf

Sire	dam	Initial larvae number	28.5°C		35°C		35°C	
					20-30 dpf		25-35 dpf	
			n	% males	n	% males	n	% males
NM	a	228	60	63.3	60	85.0	62	89.5
NM	b	186	53	56.6	49	83.7	51	90.2
NM	e	237	71	78.9	49	91.8	52	96.2
NM	f	138	44	52.3	41	75.6	36	88.9
$\bar{x}$ ± SD		197.2± 45.0		62.8^{a} ± 11		83.9^{b} ± 6.2		91.2^{c} ± 3.2
Gyn	a	129	35	17.1	29	89.7	32	90.6
Gyn	b	153	46	19.6	49	51.2	45	73.3
Gyn	c	93	24	25.0	25	92.0	27	96.3
Gyn	d	177	56	16.1	59	47.5	52	63.5
Gyn	e	243	72	16.7	77	72.7	72	83.3
Gyn	f	246	68	10.3	65	69.2	61	80.3
$\bar{x}$ ± SD		173.5 ±61.1		17.5^{a} ± 5.2***		70.4^{b} ± 19.1**		81.2^{c} ± 11.3*

Overall male ratios with different superscript letters were significantly different among treatments (same row). Significant differences among males (same column) at a level of $P < 0.05$, $P< 0.01$, or $P< 0.0001$ are indicated by *, **, or ***, respectively; NM = normal male; Gyn = mitotic gynogenetic male; n = sexed number; a - f = normal females; SD = standard deviation

Supplemental table1: Analysis of variance for main fix effects with phenotypic sex of zebrafish as dependent variable

Effect	Numerator df	Denominator df	F-value	*P*
Male	1	1487	83.47	<.0001
Female	5	1487	8.57	<.0001
Treatment	2	1487	122.25	<.0001
Male x treatment	2	1487	12.92	<.0001

Supplemental table2: Analysis of variance of phenotypic sex ratios in zebrafish incubated at three different thermal regimes (28.5°C, 35°C from 20-30 dpf, and 35°C from 25-35 dpf) with fix effects family, treatment and their interaction.

Effect	Numerator df	Denominator df	F-value	*P*
Family	9	1468	15.9	<.0001
Treatment	2	1468	132.94	<.0001
Family x treatment	18	1468	2.28	<.0017

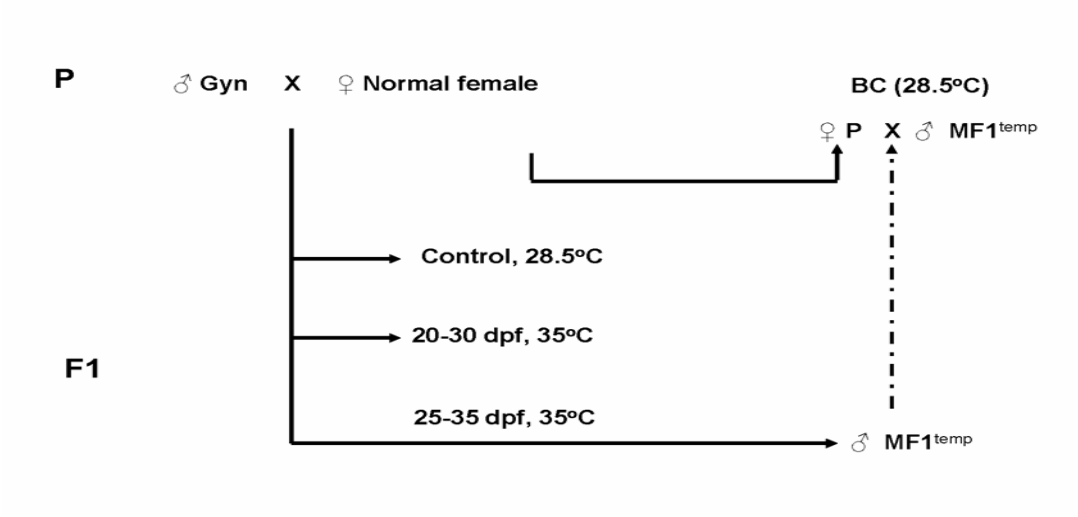

Figure 1: Mating design for the production of zebrafish backcrosses, using temperature treated males ($MF1^{temp}$: 25-35 dpf, 35°C) mated to their respective mothers.

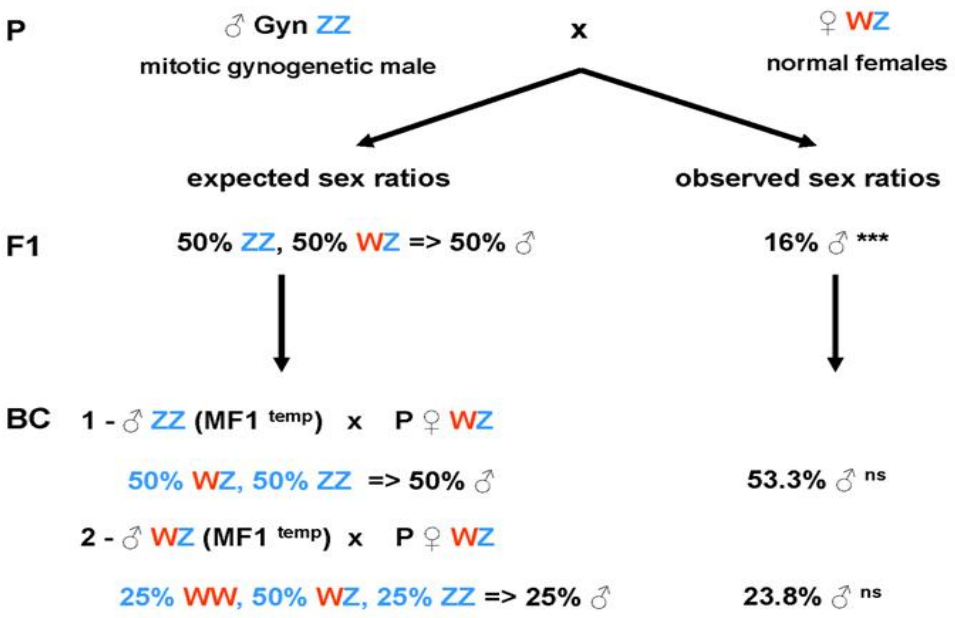

Figure 2: Scheme to explain the theoretical sexual genotype of temperature treated males derived from matings between a mitotic gynogenetic male and normal females in a backcross. The genotype of the mitotic gynogenetic male was assumed to be *ZZ*. If mated to a normal *WZ* female, it would give rise to mixed sex offspring (♂:♀, 50:50). The observed control sex ratio differed significantly from the hypothetically equilibrated sex ratio. Temperature treated sons ($MF1^{temp}$) derived from the F1-generation were back-crossed to their mothers, in order to explain the sexual genotype. The hypothetical genotype of F1-males would be *ZZ*, if a normal male was chosen or *WZ* if sex reversed ($MF1^{temp}$). In case using a normal male (*ZZ*) the resulting sex ratio would be 1:1, this applied in 2 families (see 1). In case a temperature sex reversed male ($MF1^{temp}$, *WZ*) was chosen to be mated to its mother, a theoretical male proportion of 25% should be found. This was the case in 2 of the 4 backcross families (see 2). Sex ratios in the F1 were not conform to the assumed female heterogamete and differed significantly from a 1:1.

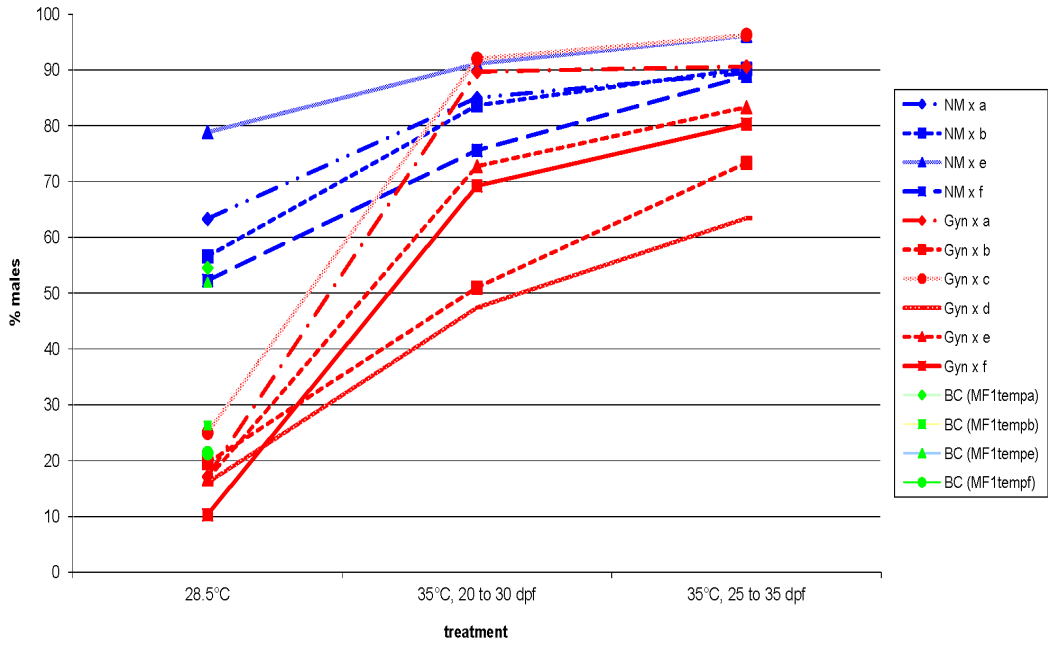

Figure 3: Among family variation in the phenotypic sex ratio of zebrafish incubated at three different thermal regimes (28.5°C; 35°C from 20 to 30 dpf; 35°C from 25 to 35 dpf). Each line represents the reaction norm of one family, parallel lines indicate genetic variation and crossing lines indicate an interaction of genotype and environmental effects.

Chapter 4

General discussion

General discussion

Rearing water temperature has a strong effect on sex determination and differentiation of zebrafish. Such phenomenon (TSD) has been reported in many other reptiles' species (Crews, 1996; Pieau, 1996), amphibians (Hayes, 1998) and many fish species (Atlantic silverside (*Menidia menidia*) Japanese flounder (*Paralichthys olivaceus*) and Nile tilapia (*Oreochromis niloticus*) Rainbow trout *(Oncorhynchus mykiss*) (Conover and Kynard,1981; D'Cotta et al., 2001; Kitano et al., 2000; Magerhans et al., 2009). These studies suggested that the influenced of water temperatures on sex ratio could be more widespread than expected and stimulated various studies on more than 60 species, for either basic or applied research (Baroiller and D'Cotta, 2001; Ospina-Alvarez and Piferrer, 2008). Besides Atlantic silverside, tilapias, pejerrey and rainbow trout, the Japanese hirame (*Paralichthys olivaceus*) and the European sea bass (*Dicentrarchus labrax*) have also become major models to study the mechanisms of thermal influences on sex ratios.

In zebrafish, few studies referred to the effect of elevated water temperature on the sex determination and /or differentiation. According to previous studies on Pufferfish (*Takifugu rubripes*) and medaka (*Oryzias latipes*) the elevated water temperatures during the embryogenesis development have an effect on the proliferation of germ cells and development of oocytes (Lee et al., 2009; Silem et al., 2009) as concluded in the second chapter. On the other hand, elevated water temperatures have also effects on the expression of steroidogenesis during the larval development by modulation of aromatase gene expression which leads to masculinization of genetic females in zebrafish, Japanese flounderand Nile tilapia (Uchida et al., 2004; Kitano et al., 1999; D'Cotta et al., 2001; Kwon et al., 2000).

Interestingly, Kwon et al. (2000) also described the paradoxical feminization of genotypic males (YY genotype) of Nile tilapia by high temperature and its prevention by simultaneous Fadrozole-induced aromatase inhibition. Nevertheless, several other species or strains also show more females at high temperatures and/or more males at low temperatures (reviewed by Strüssmann and Patiño, 1999).

However, the results from chapter two and three are shortly summarized in this chapter in order to get a comprehensive overview about the research results and the integration into the literature context about effect of water temperature on sex determination and differentiation in zebrafish.

The elevated water temperature of 35^{o}C has a significant effect on phenotypic sex ratio either during the precious stage (gastrula, 5-10 hpf) or later during the larval development (transformation stage, 25-35 dpf). Gastrula stage is represented as the earliest thermo-sensitive window for sex determination in zebrafish. Elevated water temperature during the gastrula stage (5-10 dpf) at 35^{o}C might lead to negative effects on the proliferation of germ cells and developing oocytes which later leads to a significant change of the phenotypic sex ratio towards males. However, the presence of germ cells is essential for the formation of female gonads in zebrafish, as in their absence, males without functional gonads are formed (Siegfried and Nusslein-Volhard, 2008; Slanchev et al., 2005). Their gonads are empty testicular shell comprising male somatic cells only (Siegfried and Nusslein-Volhard, 2008). Notably, individuals with reduced germ cells tend to become males (Houwing et al., 2007; Saito et al., 2008). High sensitivity of germ cells to high water temperatures were observed in many other species such as Pufferfish (*Takifugu rubripes*) and Medaka (*Oryzias latipes*) (Lee et al., 2009; Selim et al., 2009). The elevated water temperature of 35^{o}C leads to a significant change of the phenotypic sex ratio towards males and increases the male proportion from 22 % at ambient temperature (28.5^{o}C) to 48.25 % at (35 ^{o}C) in the gastrula group, 5-10 hpf, (Table 2, Chapter 2). The effect of elevated water temperature on the proliferation of germ cells and the development of oocytes' number leads to an increased apoptosis (Uchida et al., 2004; Baroiller et al., 2009b). Extending the thermal treatment (starting from gastrula stage (5-10 hpf) to include the segmentation stage (10-24 hpf) and pharyngula stage (24-48 hpf)) did not lead to a further change in the phenotypic sex ratio and showed a balanced male ratio of 48.25 %, 47.54% and 52.63%, respectively. The number of germ cells during the gastrula stage (5-10 hpf) is a critical value for the commitment to testes versus ovary fate in zebrafish.

The later thermosensitive window in zebrafish during the larval development is 25-35 dpf. An elevated water temperature of 35^{o}C during this stage leads to a masculinization of gonads and significantly increases the phenotypic male proportion. The effect of an elevated water temperature on the phenotypic sex ratio might be explained by two scenarios. First scenario: an exposure to high temperature leads to massive degeneration of oocytes begins at 25 dpf in the future males and skews the phenotypic sex ratio towards phenotypic male (Krovel and Olsen, 2004; Rodríguez-Marí et al., 2005; Wang et al., 2007; Jørgensen et al., 2008; Siegfried and Nüsslein-Volhard, 2008; Rodríguez-Marí et al., 2010). Second scenario: elevated rearing temperatures might also induce

ovarian apoptosis by the inhibition of the aromatase activity in zebrafish (Uchida et al., 2004). Expressions of cyp19a1a reach a peak at 31dpf in zebrafish (Figure 1) (Jørgensen et al 2008). The elevated water temperature during the peak of the aromatase expression leads to the masculinization of the gonad and skews the phenotypic sex ratio towards male.

Thus, rearing water temperatures seem to be differently affecting the expression of some other genes related to sex differentiation and/or steroidogenesis such as those for steroid receptors, other steroidogenic enzymes, and non-steroid hormones in the gonads as well as perhaps in the brain and pituitary (Crews, 1996; Baroiller et al., 1999; D'Cotta et al., 2001; Trant et al., 2001).

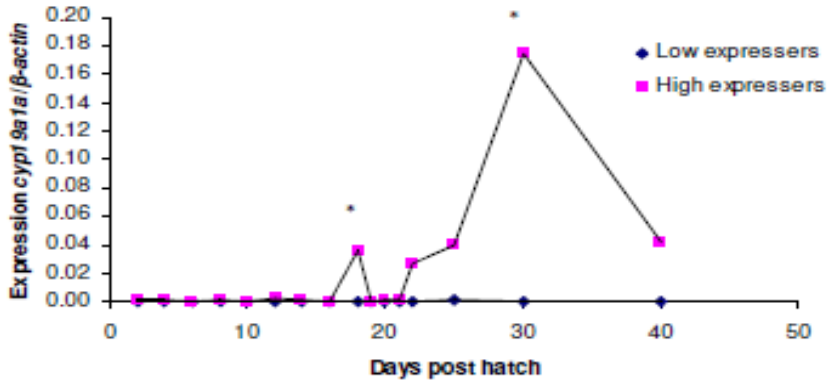

Figure 1: Expression of cyp19a1a in whole juvenile zebrafish homogenate during sex determination and differentiation (Jørgensen et al 2008).

The previous studies referred to that the sex determination regime in zebrafish seems to be female heterogamety (ZZ/ZW) (Hörstgen-Schwark, 1993; Devlin and Nagahama., 2002; Tong et al., 2010) in addition to minor genetic factors (Von Hofsten and Olsson, 2005; Jørgensen et al., 2008; Rodríguez-Marí et al., 2010; Bradley et al., 2011) and environmental conditions at least partially (Uchida et al., 2004; Shang et al., 2006; Orban et al., 2009) (Figure 2).

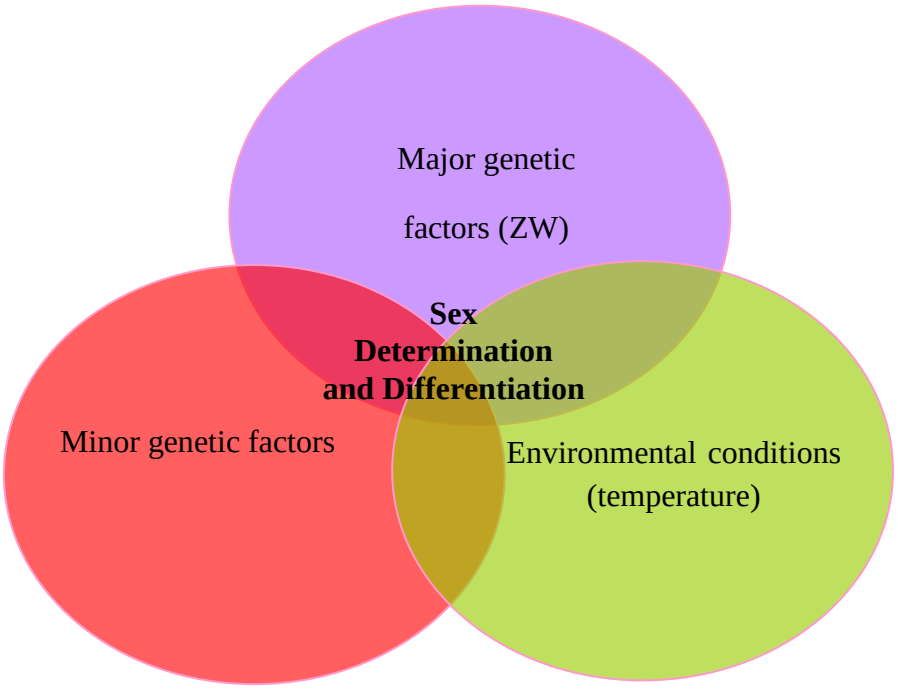

Figure 2: Sex determination and differentiation in zebrafish is a complex trait, but it seems to be controlled by three factors: the major genetic factors, the minor genetic factors and the environmental conditions.

Concluding remarks

1) Sex determination in zebrafish seems to be under interaction between the GSD and TSD.

2) Elevated the water temperature of 35^{o}C has a strong effect on sex determination during the gastrula (5-10 hpf) and later on sex differentiation during transformation stage (25-35 dpf).

3) The sex ratio in control and temperature treated groups seems to be influenced by both, male and female parent.

4) The mechanism of germ cell death in this case till now is unknown and needs more investigations

Thus, further studies should examine the role of apoptosis in gonad and effect water temperature on PGCs proliferation and the transcription of the P450 aroma A, and ER.

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