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Review

Genes involved in sex determination and the influence of temperature during the sexual differentiation process in fish: A review

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This review attempts to group the recent hypotheses involved in the complex system of determination and sex differentiation in fish. Based on recent literature, we relate the key genes involved in the genomic cascade as the C*yp19, Dmrt1, Sox9, Foxl2, Esr, Dax1, Sf1 and Amh1,* and still little known action of temperature on them. As the sex reversal is a highly desired process in fish farming aiming at obtaining male mono-sexual populations (due to weight gain of males), several techniques based on direct and indirect manipulation of phenotypic sex are being tested. Recent surveys show the use of temperature as alternative to the process of sex reversal. However, high or low temperatures have limited effect, in addition to there being a window of sex reversal in which temperature acts, varying from species to species. Thus, we draw a parallel with the role of temperature in the process of sex reversal and its effect on genes of the genomic cascade, which has been the subject of several studies that attempt to explain how temperature would be acting in this process. Intracellular receptors, such as those used for steroid hormones, act as transcription factors to regulate target genes moving between the nucleus and cytoplasm and, in the hormone absence, are linked to the complex of heat shock protein 90 kDa (Hsp90). Through this mechanism, it is possible to predict that fluctuations in temperature can influence the action of hormones, the increased transcription of genes involved in steroidogenesis and hence in sexual differentiation, becoming an alternative to explain where the temperature is acting. However, this literature review discusses the correlations between the genomic cascade, the action of intracellular receptors and the influence of temperature within this large system of determination and sex differentiation in fish.

Key words: Fish, gene, sex differentiation, temperature.

INTRODUCTION

Sexual differentiation in fish has been extensively studied, but little is known about the biochemical pathway responsible for sex reversal process and the true role of temperature in this process.

Many studies show that genes involved in sexual differentiation, many already sequenced, involve a genomic cascade: *Cyp19*; *Dmrt1; Sox9; Foxl2; Esr; Dax1; Sf1* and *Amh*, acting in a coordinated manner

(Baroiller et al., 2008, 2009; D'Cotta et al., 2007; Ijiri et al., 2008).

For some species, sex reversal is a highly desired process in fish farming aiming at obtaining monosex male populations, since males have higher growth rate and weight gain than females (Tachibana et al., 2004). In the case of the tilapia (*Oreochromis niloticus*), the fact that this species reach sexual maturity very early, usually between the 4^{th} and 6^{th} month of life, generates overcrowding in culture environments (Kubitza, 2000), and therefore very necessary to use techniques that control this population growth (Phelps and Okoko, 2011). In addition to sex reversal, other methods are used to control the size of the fish population. Jegede (2010) using *Hibiscus rosa sinesis* (4.0 g diet HLM/kg) showed disintegration and necrosis of spermatids in the testes as well as severe atretic follicles in the ovaries, revealing an effective inhibitor of reproduction in tilapia.

Overall, the most widely used and effective method for sex reversal from females to males in many fish species is the use of steroid hormones, which should start before gonadal tissue differentiation (Nakamura et al., 1998; Yamamoto, 1969). However, the use of hormones in animal production has been questioned at any stage of farming in such aspects as dosage and duration of use in animals intended for human consumption. Thus, alternatives influenced by consumer concerns about the residual effects of steroid hormones in the environment and human health are sought (Oliveira et al., 2008).

Some researchers have used variation of temperature as an alternative method (Baras et al., 2001; Tessemura et al., 2006). Many studies indicate the use of different water temperatures for sex reversal in fish; but where, how and when temperature is acting in the cascade of genes is a question not yet answered (Rougeot et al., 2008).

Thus, given the great number of investigations in this field through the study of genes involved in sex differentiation in fish and the use of temperature as a tool for sex reversal, there is the need to draw a picture of the genes involved in sex differentiation and relate the role of temperature in these processes in order to develop new alternatives for future studies. manipulation of the phenotypic sex of fish have been tested. They are aimed at the elimination of females in farm environments through sex reversal achieved using hormone treatments and genetic or environmental manipulation. According to

METHODS OF SEX REVERSAL

Several techniques based on direct and indirect Yamamoto (1969), treatment must begin before the onset of sexual differentiation.

Direct methods of sex reversal

Direct methods of sex reversal act on the physiological processes that determine masculinization, feminization or sterilization. These methods are the most widespread and used, such as the use of androgenic steroid hormones (Beadmores et al., 2001; Popma and Lovshin, 1996) which aims at eliminating or minimizing breeding in tanks, achieving higher production due to better growth of males, and obtaining more uniform lines.

The genotype determines the gene responsible for protein expression, which will act to determine the genotypic and phenotypic sex (Figure 1). There are thus two ways in the determination of females: the gene *Cyp19a1a* encodes a polypeptide and expresses a protein, the aromatase (Cyp19a1a), which catalyzes the denaturation (aromatization) of the A ring of androgens (C19) and converts them to estrogens (C18). In this process, methyl 19 is removed and this enzyme is linked to the membrane located in the endoplasmic reticulum of estrogen-producing cells of the ovaries, placenta, testis and adipose and brain tissues. Aromatase is encoded by the gene *Cyp19a1a* and acts in the formation of the complex NADPH-FERRIHEMOPROTEIN reductase in the cytochrome P-450 system. In males, when the gene *Cyp19a1a* for some reason is not expressed, the protein is not encoded and initiates the testis formation.

In European sea bass, Navarro-Martin et al. (2011) showed that methylation of the sea bass *cyp19a* promoter is the cause of the lower expression of *cyp19a* in temperature-masculinized fish, and may be an essential component of the long-sought-after mechanism connecting environmental temperature and sex ratios in vertebrate species with temperature-dependent sex determination.

The four direct methods of sex reversal can be used in the lots standardization (for both males and females). For reversal of genotypic females to phenotypic males, the commonly used method is the incorporation of steroid hormones in the feed (Phelps and Popma, 2000; Popma and Green, 1990). The hormone most often used in this process, due to its androgenic potential, easy availability and low cost, is the synthetic androgen 17-alpha-methyltestosterone (Phelps and Popma, 2000), which is a methylated derivative of testosterone (Piferrer and Donaldson, 1991).

According to Phelps and Okoko (2011), a dose greater than 240 $mgkg^{-1}$ of the hormone 17 alpha methyltestosterone could inhibit those endogenous hormones. Therefore, there was no efficient masculinization in genetic females. However, the reason for such reduction of efficacy has not been determined yet. The concentration of hormone 17 alpha methyltestosterone that showed the best performance

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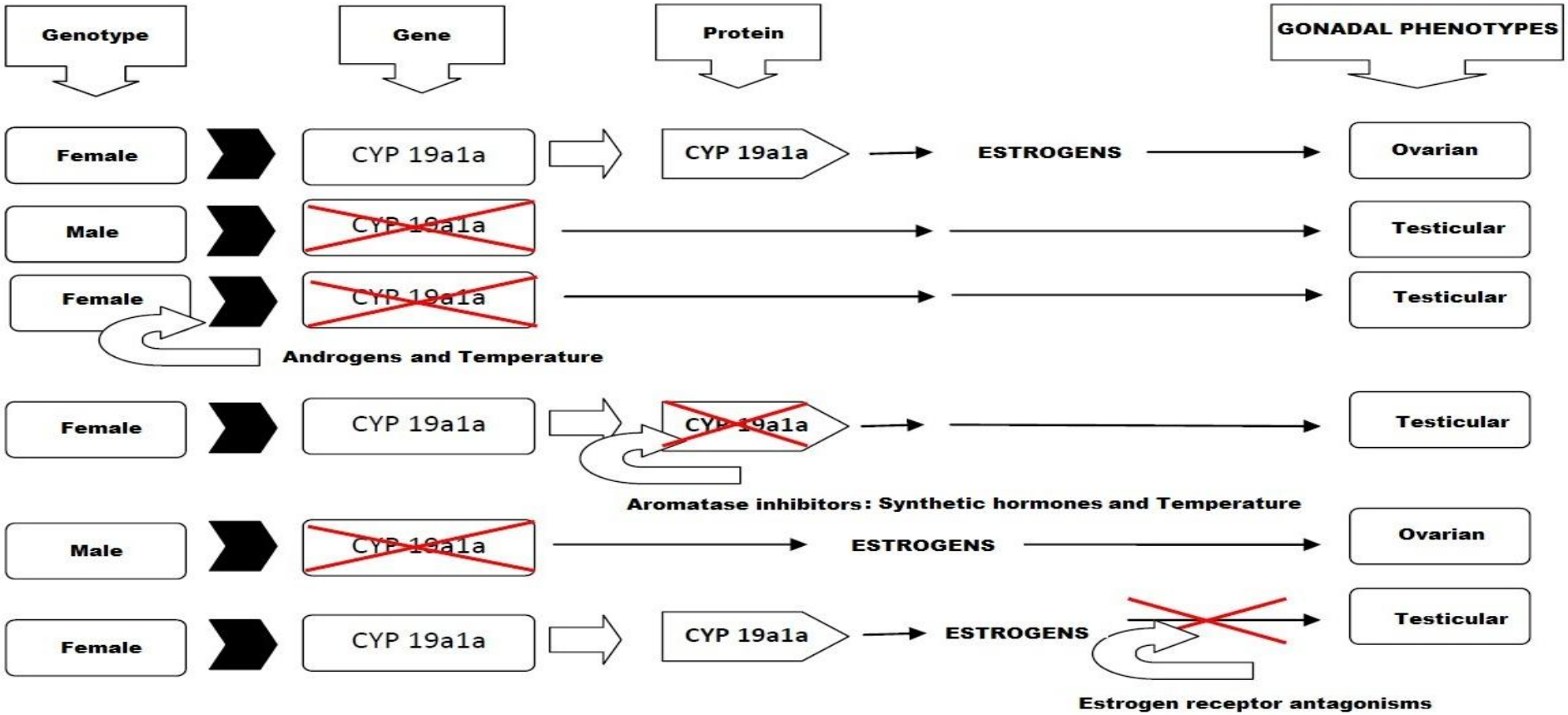

Figure 1. Sexual determination and direct treatment of sex reversal scheme adapted from Guiguen et al. (2010). The normal rout for ovarian development involves the *Cyp19a1* gene transcription and aromatase protein production that will act in synthesis of estrogens and ovarian development. In genotypic males, the blockage of *Cyp19a1* gene transcription leads to testicular development. In females, the use of androgens inhibits *Cyp19a1* gene synthesis and lead to testicular development, the same occurring when aromatase inhibitors are used. In males the blockade of *Cyp19a1* gene synthesis and use of estrogens leads to ovarian development. In females, even occurring the aromatase synthesis and estrogens production, the use of estrogens receptors inhibitors (antagonists) leads to testicular development.

in obtaining phenotypic males is between 15 and 60 mg kg^{-1} with percentage > 97% (Okoko and Ronald, 2011).

Enzyme inhibitors (Figure 1) are non-steroidal compounds that can interfere with the metabolismof endogenous steroid hormones before or during the period of sexual differentiation. They are similar to specific substrates of aromatase (testosterone and androstenedione) and generate competition for the enzyme active site, for example,

Fadrozole, Letrozole, Anastrozole and Vorozole (Bombardella et al., 2004). The antagonist estrogen-receptors (Figure 1) are compounds that prevent estrogens production. They are used for sex reversal, since they bind to specific intranuclear receptors of steroid hormones, preventing the production of estrogen and

leading to the development of testes (Lehningher et al., 1995).

The normal route for ovarian development involves the *Cyp19a1* gene transcription and aromatase protein production that will act on the synthesis of estrogens and ovarian development. In genotypic teleost males, the block of *Cyp19a1* gene transcription leads to testicular development. In females, the use of androgens inhibits the synthesis of *Cyp19a1* gene and leads to testicular development, the same occurring when aromatase inhibitors are used. In males the blockade of *Cyp19a1* gene synthesis and use of estrogen leads to ovarian development. In females, even during the synthesis of aromatase and estrogen, the use of inhibitors of estrogen receptors (antagonists) leads to testicular development (Guiguen et al., 2009).

Indirect methods of sex reversal

The indirect treatments of sex reversal act directly on genetic processes or on environmental variables, which indirectly influence the physiological processes that determine the phenotypic sex (Donaldson, 1996). According to Baroiller (1995), sex determination is predominantly due to the existence of a large gene located on a pair of sex chromosomes.

Sexual differentiation requires a variety of biochemical pathways that involve many different proteins, for example, transcription factors, enzymes, receptors, second-messenger systems and others (Devlin and Nagahama, 2002). Whereas temperature can greatly influence both the structure and function of these molecules, sex reversal via temperature has been studied by several researchers as alternative to hormonal treatment (Azaza et al., 2008; Baras et al., 2000, 2001; Tsai et al., 2003; Wang and Tsai, 2000). These studies provide evidence that low water temperature (~25°C) favors female sex differentiation while high temperature (~35°C) favor male sex differentiation in most fish species.

GENES OF GENOMIC CASCADE INVOLVED IN SEXUAL DIFFERENTIATION

Cyp19 gene

The *Cyp19* gene encodes enzymatic proteins that have sequences with about 60% homology among each other. The key enzyme to balance the ratio of steroid hormones is the *Cyp19* aromatase (which is the terminal enzyme in the steroidogenesis pathway). The appropriate expression of this enzyme is essential for differentiation of sex and reproduction in vertebrates (Conley and Hinshelwood, 2001; Gelinas et al., 1998).

Using *in situ* hybridization, Harvey et al. (2003) demonstrated that the *Cyp19* gene is located on different chromosomes in the tilapia, *O. niloticus*. Chang et al. (2005) observed that aromatase isoforms have divergent distribution in tissues in response to exogenous estrogen and pattern of expression during gonad ontogeny.

The P450 cytochrome Aromatase (P450arom; *Cyp19*) is a product of the *Cyp19* gene, a terminal enzyme in the biosynthesis of estrogen that catalyzes the conversion of estrogen into androgen (Conley and Hinshelwood, 2001). Estrogen is essential for gonadal development and other diverse physiological processes ranging from normal growth to reproductive behavior. Aromatase, which is mainly expressed in the ovary, was designated *Cyp19a/P450aromA/Cyp19a1* (Harvey et al., 2003).

Two types of aromatase are known in tilapia: cerebral (*tCyp19b*) and ovarian (*tCyp19a*). The comparison of nucleotide sequences between genes *tcyp*19 and their corresponding cDNA clones indicate that *tcyp19a* and *tcyp19b* contain nine exons sequences and that DNA sequences at the boundaries between exon/intron follow the rule described by Breathnach and Chambon (1981). The coding region of exons of *tCyp19b* have 50, 71, 65, 64, 70, 65, 79, 67 and 60% homology with the *tCyp19a* corresponding exons, although there are great differences in nucleotide sequences among introns (Figure 2) (Chang et al., 2005). The similarity in genetic structure between *tcyp19a* and tcyp19b supports the suggestion that these two aromatase genes arose from a single ancestral gene in the early vertebrate evolution (Chiang et al., 2001b).

Binding sites of several transcription factors have been identified in P450arom mammalian and Atlantic Stingray genes (Ijiri et al., 2000), but not in the *Cyp19b* of zebrafish or goldfish. Using Sequence Motif Search (http:// motif.genome.ac.jp), TFSEARCH (http://www.cbrc.jp/researchdb/ TFSEARCH.html) and Dragon ERE Wnder (http://sdmc.Lit.org.sg/ERE-V2), which are promoters analyses tools available on the internet. Ijiri et al. (2000) analyzed the *tCyp19* promoter sequences for several transcription factors: steroidogenic transcription factor -1/Ad4 BP (SF-1/Ad4); GATA 4 transcription factor; Wilm (WT1-KTS), SRY, CRE, ERE (estrogen) and RAR-2-related to connection regions. These differences in the promoter/regulatory structure suggest that the expression of two *tCyp19* genes is regulated by different transcription factors (Ijiri et al., 2000).

Analysis of the promoter structure of aromatase isoforms points to the decisive role of *Cyp19a1a* gene for sexual differentiation of teleost (Trant, 1994; Tanaka et al., 1995; Fukada et al., 1996; Callard and Tchoudakova, 1997). Studies indicate that generally S*ry, Dmrt1, Sox9, Fgf9, Gata-4, Wt1-kts* and *Sf1* are genes involved in mammalian sex determination and key to the beginning of the sex differentiation cascade among the first days of embryogenesis (Parker et al., 1999). Peichel and Graves (2010) conclude that the homologies in sex determination in fish were due to independent re-use of particularly

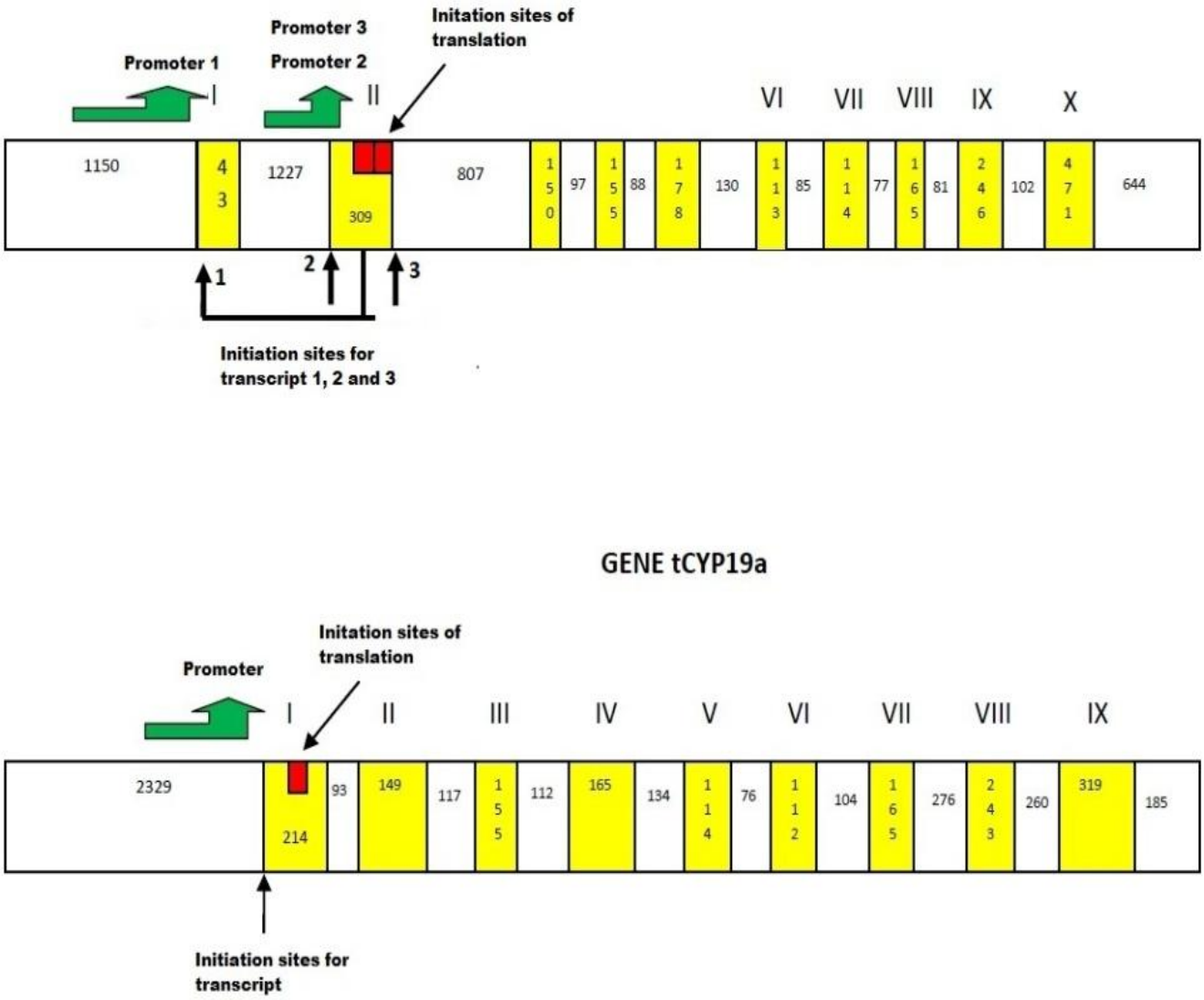

Figure 2. *tCYP19b* and *tCYP19a* gene schematic structure. Exon and intron regions are indicated by open and solid boxes, respectively. Boxes numbers represent fragments sizes. Transcription and translation initiation sites were sign by darts. Adapted from Chang et al. (2005).

handy genes (copies of *DMRT1* and *SOX3*), and of fish chromosomes that have become sex chromosomes multiple times.

Overall, gonadal aromatase *Cyp19a1a* is more abundant than that of cerebral (*Cyp19a1b*). The expression of two *Cyp19* genes have been investigated in the period between 0 and 41 days after fertilization (dpf), which is the expected time of sex determination and differentiation in zebrafish. The expression was higher after the outbreak in the period from 4 to 8 dpf. The *Cyp19a1b* expression pattern was different in two populations, suggesting association with sexual differentiation (Thorgaard, 1977).

Multiple forms of aromatase were found in teleosts including goldfish, zebrafish and sea bass (Blazquez and Piferrer, 2004; Chiang et al., 2001a, b; Gelinas et al., 1998; Kishida and Callard, 2001; Tchoudakova and Callard, 1998; Callard and Tchoudakova, 1997; Tong et al., 2001). According to Chang (2004), the *tCyp19a* was only detected in the ovary of tilapia fingerlings aged at 15, 20, 25 and 35 days.

Several studies confirm that estrogen is necessary for ovarian differentiation (Kobayashi et al., 2003; Kwon et al., 2000; Nakamura et al., 1998). It is possible that the level of *Cyp19a* gene expression control sex determination by regulating estrogen synthesis in teleosts (Fukada et al., 1996; Tanaka et al., 1995; Tchoudakova and Callard, 1998; Trant, 1994). The high level of *Cyp19a* expression in the ovary is consistent with the high *tCyp19a* levels of P450 aromatase activity and the production of 17 beta estradiol, which have been observed in teleost follicles during vitellogenesis (Kagawa

et al., 1984; Tanaka et al., 1995; Trant et al., 2001). In zebrafish, the gene *Cyp19a* (2.1 kb) is expressed mainly in follicular cells during vitellogenesis (Chiang et al., 2001a; Kishida and Callard, 2001a, b; Trant et al., 2001). Similarly, *Cyp19a* was only expressed at high levels in the ovary during vitellogenesis in goldfish and bass (Blazquez and Piferrer, 2004; Gelinas et al., 1998; Tchoudakova and Callard, 1998). Based on the TATA box location, blocking the signals and transcription initiation sites, it is possible to deduce a possible promoter for the *tCyp19a* gene (Chang et al., 2005).

With northern blot, Yoshiura et al. (2003) demonstrated the parallel expression of *tCyp19a* and the *SF-1/Ad4BP* transcription factor during ovarian growth and showed that the HCG levels are simultaneously stimulated by the mRNA levels of *tCyp19a* and *SF-1/Ad4BP* in the follicle growth, suggesting that *Cyp19a* regulates the expression of gonadotropin by modulating the SF -1/Ad4. Kwon et al. (2001) observed a high expression of *tCyp19a* in female tilapia during early sexual differentiation, and reported that expression is greatly reduced in males between 15 and 27 days after hatching. In addition, Guiguen et al. (1999) reported a large increase in aromatase expression in the ovaries of rainbow trout prior to sexual differentiation.

Cyp19a inhibition in male teleost individuals can lead to bi-potential gonad to turn into a testis. In contrast, the down-regulation of genes responsible for sex determination in females deregulates the *Cyp19a* expression, which leads to high estrogen production and thus triggers the development of ovaries (Callard et al., 2001; Chang et al., 2005; Sawyer et al., 2006).

In addition, some studies have reported that estrogen may conversely inhibit the expression of sex-determining genes (Crews et al., 2001). With American turtle and trout, Crews et al. (2001) observed that treatment with estrogen, either directly or indirectly, represses the SF-1 leading to the development of female animals, and that inhibition of estrogen production caused by up-regulation of SF-1 leads to the development of males.

In Chang et al. (1997), cerebral aromatase designated *CypP450aromB/Cyp19a2* was described through a cDNA obtained from ovarian tissue of tilapia. The enzyme is expressed at high levels in brain tissue, strongly induced by estrogen and highly homologous to *Cyp19b* of goldfish and zebrafish, being designated in tilapia as *tCyp19b*. According to Chang (2004) part of the *tCyp19b* sequence shows 63.8% global homology with *tCyp19a* (Figure 2) and on average 60% homology with the aromatase of catfish, *Cyp19b* of goldfish, *Cyp19a* of goldfish, aromatase of medaka, *Cyp19b* and *Cyp19a* of zebrafish, *Cyp19b* of bass (Blazquez and Piferrer, 2004; Chang et al., 1997, 2005 Gelinas et al., 1998; Kishida and Callard, 2001; Tanaka et al., 1995; Tchoudakova and Callard, 1998; Callard and Tchoudakova, 1998; Trant, 1994).

The sequence of *tCyp19b* also shares homology with aromatase of frogs, chicken, rat, human placenta, swine placenta and ovary (Corbin et al., 1988; Harada, 1988; McPhaul et al., 1988; Simpson et al., 1987; Terashima et al., 1991).

Through real-time polymerase chain reaction (RT-PCR) with tilapia tissue, Chang (2005) detected the expression of two isoforms of P450arom in *tCyp19b* during sexual differentiation in all brain tissues collected from 1, 10, 15, 20, 25 and 35 days old and in gonads aged at 15, 20, 25 and 35 days. This study found no significant differences between the sexes and, therefore, the results suggest that *tCyp19b* is expressed at high levels in the brain and at low levels in other tissues. In studies with goldfish and sea bass, brain aromatase expression was detected at high levels in brain tissue (Blazquez and Piferrer, 2004; Gelinas et al., 1998; Tchoudakova and Callard, 1998), it was observed in zebrafish with predominant expression of brain aromatase occurring in the brain (Chiang et al., 2001a; Kishida and Callard, 2001; Trant et al., 2001).

According to Trant et al. (2001), the individual expression of *Cyp19a1b* during determination and sex differentiation distributed between the two groups (male and female) indicates that this gene may be associated with sexual differentiation in zebrafish. A high mRNA level detected of Cyp19b in brain tissue of the teleost suggests that the gene is involved in the production of neural-estrogen in the brain (Callard et al., 2001; Chang et al., 2005; Sawyer et al., 2006).

According to studies of Chang et al. (2005), analyzing the TATA box location, blocking the signals and transcription initiation sites, it is possible to deduce three possible promoters for gene *tCyp19b*. Prosecutors 1 and 2 of *tCyp19b* are located at the 5' end of exons I and II, respectively, suggesting that they are involved in regulating expression of transcription 1 and 2 respectively. The promoter 3 is located in the middle of exon II, suggesting that it is involved in the initiation of transcription 3. It is also possible that promoter 2 regulates the transcription of both 2 and 3, with alternative splicing, producing different transcriptions (Figure 2). Gelinas et al. (1998) also concluded that *in vivo* administration of estrogen and androgen in goldfish there were high mRNA levels of *tCyp19b* in the brain.

Evidence indicates that the differing responses of the two aromatase genes for estrogen are due to the presence or absence of the transcription factor ERE (estrogen responsive element) in their promoters.

According to Gelinas et al. (1998) working on tilapia, it is possible to suggest that the *tCyp19b* transcription is regulated by estrogen and preferentially expressed during neural estrogen biosynthesis, as consequence of estrogen binding to the ERE transcription factor in the promoter 1.

However, when investigating the aromatase expression in embryos and juveniles of zebrafish, Kishida and Callard (2001) and Trant et al. (2001) emphasized the important role of *Cyp19b* for sexual differentiation of gonads. Two groups have reported that estradiol, ethinyl

estradiol and 17-methyltestosterone could increase the production of mRNA of *Cyp19b* in zebrafish embryos.

Blazquez and Piferrer (2004) also reported that *Cyp19b* expression was increased in brains of male sea bass after sexual differentiation (200 to 250 days after fertilization). These results suggest that *Cyp19b* is deeply involved in sexual differentiation of species.

On the other hand, the presence of ERE in the promoter region of *Cyp19b* gene suggests that the exogenous estrogen effect on the gonad development, at least partially, occurs through up-regulation of *Cyp19a* expression. Thus, Kishida and Callard (2001) and Trant et al. (2001) observed the increased expression of *Cyp19b* in zebrafish embryos after treatment with estrogen. The elevated expression of *Cyp19b* by exogenous estrogen has the same effect as the high natural expression of *Cyp19a* under physiological conditions on the ovary ontogenesis. Besides the high expression of *Cyp19b* in the period of sexual differentiation and later period of sex differentiation, which was observed in zebrafish and sea bass, it indicates that the *Cyp19b* gene activation is a consequence of sex determination and its expression is involved in gonadal development after differentiation (Blazquez and Piferrer, 2004; Kishida and Callard, 2001; Trant et al., 2001).

Kishida and Callard (2001), Trant et al. (2001) and Blazquez and Piferrer (2004) also reported that the mRNA levels of *Cyp19a* are much higher than that of *Cyp19b* in the early sexual differentiation of zebrafish and sea bass. In contrast, Gato-Kazeto et al. (2004) investigated the high and low expression of *Cyp19b* and found no correlation with sex in adulthood in zebrafish. In addition, a recent study on the expression of *Cyp19b* in the brain of adult zebrafish showed that levels of expression were the same in males and females (Resko et al., 2000).

***Dmrt1* gene**

Dmrt1 (Doublesex-and mab-3-related transcription factor-1) is a transcription factor expressed in XY gonads and Sertoli cell lineage before any sexual differentiation and it is essential for its occurrence (Raymond et al., 1998). Dmrt1 is a member of a specific family of transcriptional regulators - those that contain the DM domain DNA binding motif (Matson and Zarkower, 2012).

The gene *Dmrt1* has been cloned in a variety of invertebrates and vertebrates, including fish. In each one, the embryonic expression of Dmrt1 occurred in a sexually different way, where high levels were observed in the developing male gonad before sexual differentiation (Lei and Heckert, 2004).

In several species, such as frog, alligator, trout, birds and rats, *Dmrt1* gene is expressed at high levels in males compared to females, suggesting that high expression is required for testicular differentiation while low expression is compatible with the ovary differentiation (Raymond et al., 2000; Nanda et al., 2000).

The gene *Dmrt1* probably have an indispensable role in the event of sex determination and may be involved in testis development of teleost fish, which is analogous to its supposed role in some mammalian species (Loffler and Koopman, 2003). Works on Medaka indicate that *Dmrt1* regulates the spermatogonia differentiation (Kobayashi et al., 2004).

The *Dmrt1* expression at the beginning of the sexual differentiation period may indicate that the gene is involved in the signaling cascade in the early determination and sex differentiation in zebrafish (Hofsten and Olsson, 2005). In a genome-wide linkage analysis in zebrafish has implicated a locus containing *Dmrt1* as a major determinant of sex (Bradley et al., 2011).

In a recent model of gonadal development in medaka, *Dmrt1* expression was detected from 10 dph (post-hatching days), thus being the first gene to be differentially expressed in males and females (Kurokawa et al., 2007).

According to studies by Guan et al. (2000), *Dmrt1* was expressed in the testis of normal XY males and XX males in tilapia, indicating that this is a gene not connected to Y whose expression is correlated with testicular formation. These authors hypothesized that a Y chromosome signal (regulatory genes) acts in *Dmrt1* in males to promote the testis. In XX males, hormonal or environmental influence seem to compensated for the absence of a signal from the Y chromosome, which results in sex reversal of XX and up-regulation of *Dmrt1* expression.

Despite the studies performed to date, it is still unknown the major target genes of this important transcriptional factor. According to Wang et al. (2010), an over transgenic expression of *Dmrt1* in XX fish resulted in decreased expression of the aromatase gene; reduced serum levels of 17 beta estradiol; delayed development of ovarian cavity, varying the degrees of follicular degeneration, and even caused a partial to complete sex reversal. These results indicate that the aromatase *Cyp19* is one of the *Dmrt1* targets. It suppresses the feminization pathway by transcription repression of aromatase gene and estrogen production in the tilapia gonads and possibly other vertebrates.

***Sox9* gene**

The Sox gene family (SRY-related genes containing HMG) encodes an important group of regulators involved in development and sex determination. The HMGbox (high mobility group) that characterizes the Sox proteins is a binding domain with the DNA, and proteins encoded by Sox genes act as transcription factors. Using a comparative genomics approach, Bagheri-Fam et al.

(2010) showed that testis-specific enhancer within *Sox9* gene (TESCO) contains an evolutionarily conserved region (ECR) of 180 bp which is present in all vertebrates except fish.

In zebrafish, two genes were identified: *Sox9a* and *Sox9b*. Both contain the HMG and are able to bind to the recognition site AACAAAG in a similar way to murine *Sox9* (Chiang et al., 2001a).

The expression patterns of *Sox9a and b* are different in adult zebrafish. *Sox9a* shows a broad expression pattern and it is found in the brain, kidney, muscle, testis and pectoral fin, while *Sox9b* is only found in the ovary (Chiang et al., 2001a; Hofsten and Olsson, 2005).

Both, *Sox9a and b* are expressed during embryogenesis in cells involved in craniofacial and brain development (Chiang et al., 2001a). Furthermore, *Sox9a* appears as essential for chondrogenic development (Yan et al., 2002) and *Sox9b* is involved in the neural crest development (Li et al., 2002). The *SRY* gene is the member of the Sox-gene family. It is the determinant of Y male chromosome in most mammals (Kobayashi et al., 2004; Lei et al., 2007).

According to Berta et al. (1990) and Hacker (1996), *SRY* is a little conserved gene that seems to be unique in mammals. In contrast, *Sox9* is a conserved gene, present in all types of vertebrates. Like *SRY*, *Sox9* gene is required for testicular development in mammals, and its deficiency can result in sex reversal in human males. The *Sox9* expression is seen immediately after the SRY gene and may be a downstream effector. In mice, *Sox9* mediates the onset of *Amh* expression (anti-Mullerian hormone) in Sertoli cells (Yao and Capel, 2005). According to Chiang (2001a), *Sox9* expression during gonadal differentiation is up-regulated in the testes and down-regulated in the ovaries of mammals, birds and turtles.

The organization and function of Sox-family genes is poorly understood in other kinds of vertebrates and, despite the wide distribution of the *Sox9* genes in fish, the gene has been investigated only in a few ones (Chiang, 2001a; Zhou et al., 2002).

The expression levels of *Sox9* were similar from 9 to 29 dpf in XX and XY gonads of tilapia, getting stronger shortly after in males XY (Ijiri et al., 2008). In medaka, Nakamoto et al. (2005) found high levels of *Sox9a and b* in somatic cells surrounding the germinative cells during early sexual differentiation of gonads. Subsequently, during the early stages of development of testicular tubules (seminiferous tubules), the expression of *Sox9a* and *Sox9b* is maintained only in XY gonads, and it is markedly reduced in XX gonads. Some studies suggest that *Sox9* is not involved in the initial determination and sexual differentiation, but it is necessary for testicular tubules development (Nakamoto et al., 2005). Using both transgenic and chimeric *sox9b* medaka mutants, Nakamura et al. (2012) showed that medaka *Sox9b* is required for germ cell proliferation and also to survival, but not for testis determination or subsequent process of early testis differentiation.

Foxl2 gene

Foxl2 is a transcription factor involved in the gonad differentiation and ovarian function in various vertebrates (Wang et al., 2004). The *Foxl2* gene has a specific ovarian expression in mammals, chicken and rainbow trout (Loffler et al., 2003; Baron et al., 2004).

In tilapia, the *Foxl2* gene is expressed on the ninth day after fertilization in XX gonads at levels only slightly higher than in XY; subsequently, levels increase linearly in the XX ovaries (Ijiri et al., 2008). In the Medaka, *Foxl2* was expressed primarily in somatic cells that surround the germ cells in XX gonads immediately after the initiation of ovarian differentiation and is maintained in granules cells throughout the ovary development (Nakamoto et al., 2006).

Wang et al. (2007) through *in vitro* studies identified that *Foxl2* binds to the *Cyp19a* promoter and activates its transcription (Figure 3). Many studies have reported the involvement of *Foxl2* gene in the differentiation of females holding cell lineages, granule cells and the formation and/or maintenance of ovarian follicles in various vertebrates (Govoroun et al., 2004; Loffler et al., 2003; Nakamoto et al., 2006; Wang et al., 2004), thus confirming the *Foxl2* correlation with *Cyp19a* expression (Ijiri et al., 2008; Baroiller et al., 2008).

Estrogen receptors (*Esr*) gene

In vertebrates, estrogens (especially 17-beta-estradiol) play a critical regulatory role in many physiological processes, including growth, differentiation and homeostasis of the reproductive organs of males and females, in addition to influencing the functioning of skeletal, cardiovascular, immune and central nervous systems (Korach, 1994).

The first Er (*Er alpha*) was isolated from humans (Green et al., 1986). The presence of a second subtype, *Er beta*, has been found in mammals, birds, fish and amphibians. More recently, a third subtype, *Er beta2*, was discovered in teleost fish; and it is closely related to *Er beta* suggesting that it reflects in total or partial duplication of this gene in fish (Filby and Tyler, 2005).

Currently, the zebrafish nomenclature (Zebrafish Nomenclature Official Guideline; http://zfin.org) was followed to standardization; *Er alpha* has been termed *Esr1*, the subtype *Esr beta1* became *Esr2b* and the subtype *Er beta2* became *Esr2a.*

The biological significance of multiple *Esr* is far from being completely understood. *Esr1* and *Esr2* are structurally similar (Beato, 1989; Pakdel et al., 1989). They show different binding capabilities (Pakdel et al.,

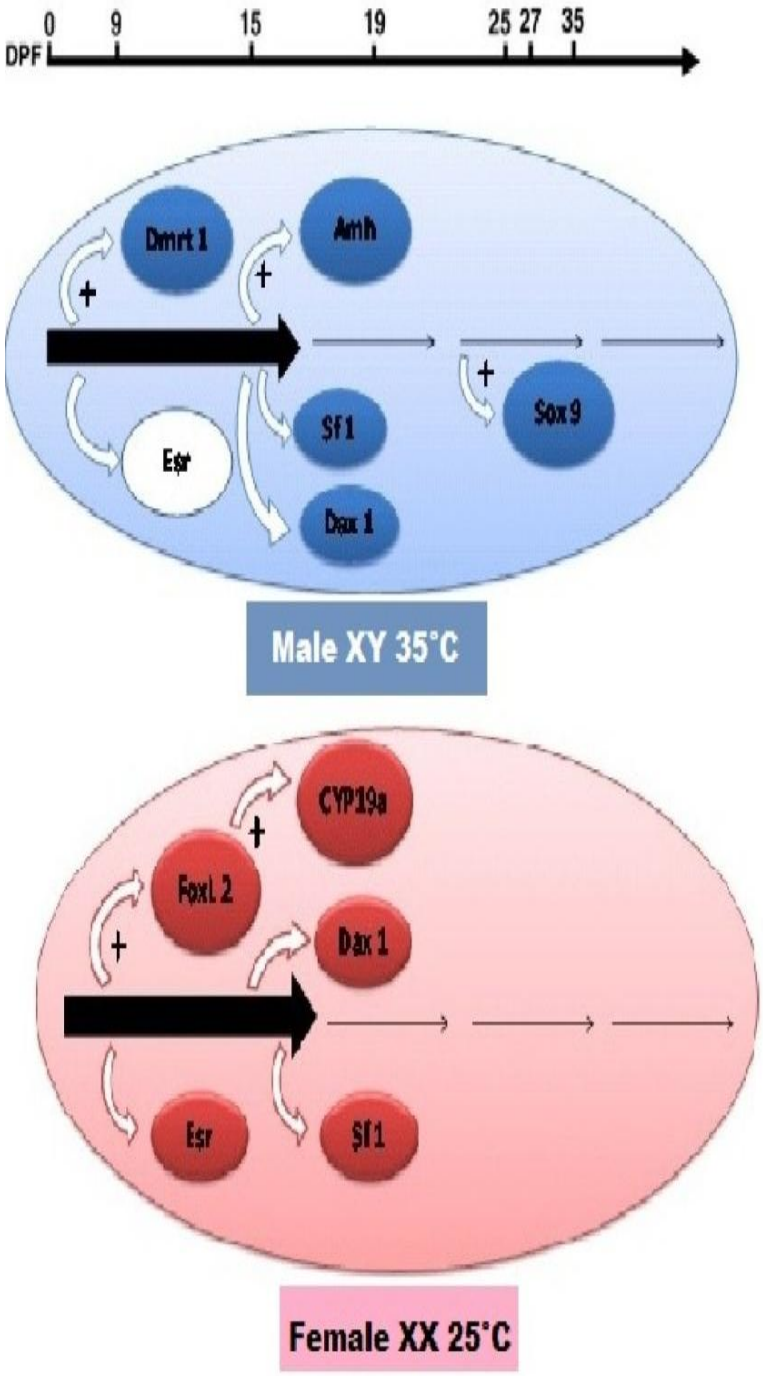

Figure 3. Scheme of genes involved in sexual differentiation and sex determination. Adapted from D'Cotta et al. (2007); Ijiri et al. (2008); Baroiller et al. (2008, 2009).

1989) and transcriptional properties (Filby and Tyler, 2005).

Esr levels are influenced by estrogen (Mommsem and Lazier, 1986), mediated by its effects on gene expression of estrogen receptor (Mackay et al., 1996; Pakdel et al., 1989). This positive control of *Esr* genes by estrogen ensures enough available receiver, stimulating cell types in response to the full presentation of estrogen (Devlin and Nagahama, 2002).

Distinct expression patterns of *Esr1* and *Esr2* have been documented in fish; seeming to vary among species. They exhibit a broad tissue expression, but greatly concentrated in the liver, gonads and with considerably lower levels of expression in the brain,

pituitary, intestine and muscle (Filby and Tyler, 2005).

In tilapia, *Esr1* and *Esr2* genes are expressed in early male and female gonads between 10 and 15 days after hatching (Watanabe, 1999) showing different expression patterns that suggest an important role in the regulation of early sexual differentiation (Devlin and Nagahama, 2002). The results of Tsai et al. (2003) indicated that the brain aromatase, *Esr1* and *Esr2* are differentially regulated according to the environmental water temperature during the developmental period in tilapia.

Huang et al. (2008), studying the expression of three types of *Esr* in male tilapia, have shown that environmental estrogens affect the *Esr* expression and induce reproductive abnormalities in males. These authors also reported that the three types were maximally expressed during the early stage of recurrence, indicating that they play a role in testicular function and spermatogenesis mediated by *Esr.* Clearly, further studies are required to better understand the role of *Esr* in fish and its relationship with temperature.

Anti-Mullerian hormone (*Amh*) gene

In mammals, the anti-Mullerian hormone (*Amh*) is involved in the regulation of gonadal steroidogenesis by inhibiting the aromatase expression in developing gonads (Clemente et al., 1992) also presenting a complex regulation, which involves several factors, including the FTZ-F1 related to genes *Sf1, gata4, Sox9* and *WT1* (Santa Barbara et al., 1998; Rey et al., 2003). In addition, it negatively modulates the differentiation and function of Leydig cells (Racine et al., 1998) through the regulation of several enzymes involved in the steroidogenic pathway.

The anti-Mullerian hormone's (*Amh*) gene, responsible for Mullerian ducts regression in males, was found in fish that do not have Mullerian ducts, and its role is still unknown (Hofsten and Olsson, 2005). According to D'Cotta et al. (2007), the increased expression of the *Amh* gene in male gonads of tilapia is evident between 10 and 15 dpf and after 19 dpf (Ijiri et al., 2008). Hofsten et al. (2005) cloned the *Amh*'s cDNA of zebrafish and noted that this gene was expressed exclusively in the gonads.

By *in situ* hybridization, the *Amh* expression was found predominantly in Sertoli cells, testis and ovarian follicular layer. The regulation of *Amh* transcription in zebrafish has not been elucidated so far.

Sf1 gene

In mammals, two homologous genes to FTZ-F1 were found: *NR5A1* and *NR5A2*. Ad4BP/SF-1 proteins, members of the family of orphan nuclear receptors, have been identified as a specific steroidogenic transcription factor found in tissues (Morohashi et al., 1992, Morohashi and Omura 1990) such as pancreas, liver, intestine; in addition to being involved in cholesterol metabolism, which involves the synthesis of steroids and consequently in sex determination. The *ad4bp/Sf1* gene deletion resulted in lack of reproductive organs in mammals, suggesting that Ad4BP/SF-1 functions as a transcription factor involved in the regulation of enzymes involved in steroidogenesis as well as in tissue differentiation by regulating the *Dax1* gene (Kawabe et al., 1999; Luo et al., 1994). Such genes are regulators that have a key role in steroidogenesis and are involved in testicle determination during the course of sex determination (Ito et al., 1998; Ikeda et al., 1993; Lala et al., 1992; Sadovsky et al., 1995).

In zebrafish, four genes for FTZ-F1 have been identified (*ff1a*, *b*, *c* and *d*) (Hofsten and Olsson, 2005). According to Kuo et al. (2005), the genes *ff1b* and *ff1d* are of the same origin, emerged from the ancestral genes duplication. This hypothesis was supported by the expression patterns, overlap found in embryos of kidney and pituitary cells (Hofsten and Olsson, 2005).

Studies on fish that explain the relationship between P450arom and Ad4BP/SF-1 expression in response to stimulation of gonadotropins on the ovary growth and their relationships with temperature sensitivity are still scarce.

Dax1 gene

Dax1, member of the nuclear receptors family, is encoded and expressed in embryonic gonads of both sexes, but levels of expression have not been determined (Guiguen et al., 2010). According to Zanaria et al. (1994), the *Dax1* gene encodes an atypical protein that is part of the nuclear receptors family.

In rats, the *Dax1* expression was detected during the early stages of gonadal and adrenal development; its expression persisted in ovarian development but decreased in the testis coinciding with the *SRY* gene activation and testis differentiation (Swain et al., 1996). *Dax1* gene homologues were found in chickens (Smith et al., 1999; Smith and Sinclair, 2001) and in the American alligator, species with sex determination by temperature (TSD) (Western et al., 2000). Even though the *Dax1* gene expression has been verified in embryonic gonads of these species, the levels have not been determined, nor their relationship with temperature sensitivity.

THE TEMPERATURE IN THE PROCESS OF REVERSAL AND SEXUAL DIFFERENTIATION

So far, few studies have been performed to evaluate the effect of water temperature on sex ratio in fish.

In rainbow trout, experiments performed by Van den

Hurk and Lambert (1982) and Baroiller et al. (1999) found no effect of temperature on sex ratio. In salmon, Azuma et al. (2004) performed sex reversal of genetic females to functional males under 18°C (between 42 and 151 dpf).

Craig et al. (1996) however, found a strong feminization (62 to 84% female) in different salmon populations when eggs were exposed to higher temperatures (10.4 to 12.0°C and control: 8.3 to 9.7°C) during embryonic development (from 360 dpf). These results showed that, depending on the genetic background of the sockeye salmon population, temperature manipulation during incubation can change sex ratio. This is similar to the results of Magerhans et al. (2009), which also showed the partial ratios in breeding progenies when exposed to higher post-hatching temperature, producing females. However, the temperature regime varied between studies as in the distortion range in females' percentage: 20% (in the study of Magerhans et al., 2009) versus 28% of females (compared to control, in Craig et al., 1996).

The temperature is generally able to increase or decrease the proportion of females. In both studies (Craig et al., 1996; Magerhans et al., 2009) have proven that the sex ratios of offspring in treatments with temperature could not be attributed to the differential of high mortality of females or males.

High response repeatability to sex reversal with temperature treatments was proven in tilapia by Tessema et al. (2006). In addition, the study of Magerhans et al. (2009) provides evidence that both males and females contribute to the different sex ratios after treatment with temperature.

These studies show a clear paternal and maternal influence on the sex differentiation after treatment with temperature. Taken together, these results suggest that these species are heat sensitive and temperature can affect gonadal differentiation of the sex of individuals during the early stages of development.

The sensitivity of Nile tilapia (*O. niloticus*) to temperature during sexual differentiation is not seen in all progenies. Some breeders, males and females, produce offspring that exhibit high sensitivity to temperature thereby producing high proportion of males in their sexual rate, while others have produced non-sensitive and balanced sex rate.

The mode of temperature action on sexual differentiation during embryogenesis is a process that is still not understood. One hypothesis is that high temperature (35°C) can act directly on the brain aromatase gene (*Cyp19b*) to modify the path of sexual differentiation during embryogenesis (Morrison et al., 2001).

This hypothesis of "brain sexualization" is consistent with the presence of a rudiment of the brain 31 h after fertilization, as seen in Nile tilapia (Morrison et al., 2001), consistent with the idea that sexual differentiation occurs in the brain before occurring in the gonads (Arnold, 2004; Francis, 1992).

In Atlantic halibut (*Hippoglossus hippoglossus*), Matsuoka et al. (2006) also observed higher levels of aromatase gene expression in the brain at an earlier stage of development of the gonads, suggesting that sexual differentiation may begin in the brain before gonadal differentiation.

Another hypothesis is that the high temperature, applied during embryonic development, acts directly on somatic cells of future gonads and/or on the germ cells, which were already present (46 hpf post-fertilization hours) in embryos raised at 27 and 29°C (Morrison et al., 2001).

Sex differentiation by temperature

Numerous cases showed large deviations in experimental sex ratio of Nile tilapia, which cannot be predicted by a simple single-factor model. The segregation of DNA markers linked to sex (Cnaani et al., 2008; Lee et al., 2003, Shirak et al., 2006) confirmed what has been previously postulated on the existence of a single or main multi-allelic sexual determinant as well as an additional epistatic locus (or perhaps multiple loci) probably autosomal (Baroiller et al., 1995, 1996; Baroiller, 1997; D'Cotta, 2001; Hammerman and Avtalion, 1979; Mair et al., 1991, 1997).

Some studies show the sex determination system behavior as reported in zebrafish (Uchida et al., 2004), sole marble (Goto et al., 2000), gold (Kazeto-Goto et al., 2006) Japanese flounder (Yamamoto, 1999) and tilapia (Baroiller et al., 1995; Ovidio et al., 2002). These species also have a XX-XY sex determination system and the high temperature induces the XX fish masculinization. Despite the strong genetic basis for sex determination in tilapia, it is clear that other factors are responsible (Wang and Tsai, 2000) and the temperature seems to be a important factor in sex differentiation of species. On the other hand, in gold fish, zebrafish (Uchida et al., 2004), sole marble (Goto et al., 2000), Japanese flounder (Yamamoto, 1999), Loach (Nomura et al., 1998) and tilapia (Baroiller et al., 1995), TSD can occurs when extreme temperature override genetic sex determination (GSD). In gold fish, genetically female gonads developed into ovaries when fish were reared under 20°C and at 30°C almost all the gonads differentiated into testes. However, the sex ratio varied among the different groups even when reared at the same temperature (90.5 and 72.7% at 23°C). The production of a population in the same sex is useful for research on GSD/TSD as it is easy to judge the genotypic and phenotypic sex of the surviving individuals. However, high or low temperatures have a limited effect. Ospina-Ƃlavarez and Piferrer (2008), classify these results as being most likely due to thermal effects on GSD (GSD + TE) than for a real TSD.

An effect of temperature on sexual differentiation has also been observed in some flatfishes (Luckenbach et al.,

2003; Goto et al., 1999; Yamamoto, 1999). In eight families evaluated for TSD (Haffray et al., 2009), both for higher and lower temperatures (~ 35 and ~ 24°C, respectively), there was increased proportion of females. This interaction of temperature with the sex ratio has been reported in sea bass by Saillant et al. (2002).

An inter-family variation was found by Bezault et al. (2007), pointing to an important parental effect and genotype-environment interactions, as suggested by other studies (Abucay et al., 1999; Baroiller et al., 1995b; Tessema et al., 2006). Such parental effects were also found in other heat-sensitive species such as *Menidia menidia* (Conover and Heins, 1987) and *Odonthestes bonariensis* (Strussmann et al., 1996). Moreover, other experiments showed that high temperatures can have both feminizing and masculinizing effects, depending on the sex genotype (Abucay et al., 1999; Baroiller and D'Cotta, 2001; Kwon et al., 2002).

Wessels and Horstgen-Schwark (2007) provided evidence that an excess of males in groups treated by temperature can be selected for a quantitative trait. They were the first to determine the heritability for sensitivity to temperature in Nile tilapia. The heritability was 0.69, obtained from a selection experiment of two generations of high sensitivity to temperature.

A reproductive scheme of diallel crossing (5 Ч 5) showed that the males' percentage in tilapia was very different at inter-individual level, indicating that there was an important parental effect (Baroiller and D'Cotta, 2001). This was confirmed by an exhaustive investigation in two populations of tilapia by Tessema et al. (2006). Both, male and female, contribute to these parental genetic effects.

Within the Bouaké strain of Nile tilapia, the progeny test in females originating from classical treatment by temperature revealed no XY female by sex reversal (Baroiller, unpublished data), while other studies have demonstrated the presence of XX males in offspring treated with temperature (Baroiller et al., 1995). This suggests that the supposed feminization effect with high temperatures is probably restricted and peculiar to the genotype (eg, YY instead of XY) and/or population/strain/species.

In the work on goldfish of Goto-Kazeto et al. (2006) on the temperature-dependent sex differentiation, sex ratios of females were 94.6 to 100% at 15 to 20°C and 7% of females at 30°C, while in the latter**,** percentage of males increased. The masculinized fish were functional males. When using genotypic XX females, male fish emerged at high temperatures, being evidence of XX fish masculinization and not the result of a skewed sex ratio of fish survival.

In 1995, Baroiller et al. demonstrated that tilapia were sensitive to temperature during the critical period of sexual differentiation. It was possible to masculinize the XX offspring (100% females) with increasing temperature above 32°C, producing functional male phenotype. The use of mono-sex female populations, as well as the progeny testing for males treated by temperature, showed definitely the existence of distortions in the sex ratio of males, corresponding to a sexual inversion of genetic females (XX) for phenotypically functional males. These new males by temperature produce female or almost exclusively female progenies depending on the cross (Baroiller et al., 1995).

The limited extent of the temperature effect can be associated with the observation made by Yamamoto (1999) in Hiram, which was not possible to reverse genetic males into phenotypic females using temperature treatment. The small effect of temperature observed by Haffray et al. (2009) also suggests that an early evaluation of the effect of these treatments are made, since other external factors other than temperature could be involved in sex differentiation, but there is also an unknown factor of autosomal influence.

Following these results, the influence of environmental factors on sexual differentiation has been reported in over 60 different species of teleosts (Baroiller et al., 1999; Baroiller and D'Cotta, 2001; Conover, 2004; Godwin et al., 2003). Most of these studies concluded that the temperature sensitivity is impaired because the mechanisms of sex determination of these species could not be well characterized (e.g., Zebrafish, *Danio rerio, Tetraodon*) and physiological, genetic and ecological studies could not be performed to better understand the components of environmental sensitivity.

Periods of sensitivity to temperature

It has long been known that gonadal estrogens act as natural inducers of ovarian development in lower vertebrates (Nakamura et al., 1998; Yamamoto, 1969).

The enzyme aromatase, as mentioned earlier, catalyzes the conversion of androgens into 17-beta-estradiol (Baroiller et al., 1999) and if inhibited, it blocks the estrogen production so that there are sex reversals of females into males (Guiguen et al., 1999, 2010; Kwon et al., 2000).

In XX fish developed ovaries, the *Cyp19a* gene (ovarian aromatase) is up-regulated (D'Cotta et al., 2001; Ijiri et al., 2008; Kwon et al., 2001) such that Ijiri et al. (2008) found elevated *Cyp19a* levels in future ovaries, as early as 9 dpf and increased rapidly until 19 dpf.

The temperature when applied during the period of sexual differentiation induced down-regulation of *Cyp19a* in XX tilapia progenies (D'Cotta et al., 2001) that was observed at 17 dpf (Baroiller et al., 2008). Moreover, the expression levels of *Cyp19a* were correlated with the masculinization proportion by temperature (TM) in XX individuals (D'Cotta et al., 2008). On the other hand, studies on goldfish reported that reversal window is between 7 and 12 dpf (Kazeto-Goto et al., 2006). Thus, in order to achieve an effective sex control by temperature

manipulation, treatments should begin within the sensitive period.

High temperatures could effectively masculinize some progenies if started about 10 days after fertilization (dpf) and maintained for 10 days (Baroiller et al., 1995; Wessels and Horstgen-Schwark, 2007; Tessema et al., 2006). However, if the treatment was applied for a period of 10 days, but started at 7 dpf, it had no effect on the sex ratio (Baroiller et al., 1995). This window to temperature sensitivity coincides with the gonadal sensitivity in relation to other external factors, especially hormones.

Data from Rouget et al. (2008) suggest a small window of sensitivity term in tilapia after fertilization (between 12 and 52 h post fertilization = 4 dpf), before the development of presumptive gonads. Interestingly, two similar windows were also identified for hormone treatments as temperature, hormone treatments or the use of aromatase inhibitors during sexual differentiation can overlap the sex genetic determination, producing sex reversal and functional phenotypes (Baroiller et al., 1999; Guiguen et al., 1999; Nakamura, 1975).

In tilapia (*Oreochromis mossambicus*), a treatment at 20°C for five days starting before 14 dpf induced feminization, while treatments of high temperature for five days at 32°C, initiated after 14 dpf, induced the masculinization (Wang and Tsai, 2000). However, it has not observed feminization with low temperatures ranging from 18 to 23°C in tilapia (*O. niloticus and Oreochromis aureus*) (Abucay et al., 1999; Baroiller et al., 1995; Desprez and Melard, 1998; Tessema et al., 2006).

Recently, Rougeot et al. (2008), studying the process of sexual differentiation in tilapia, demonstrated that even early treatment with elevated temperature applied 12 h after fertilization (hpf) and maintained for 52 ± 2 h (until hatching), can also induce significantly distorted sex ratios among males (8 to 27%, n = 4) in a true offspring of females (100% females in the control group). According to Rougeot et al. (2008), even the high mortality associated with early treatment for high temperature (35 to 36°C) must be considered, since these data suggest that there is a very short thermo-sensible window after fertilization (hpf between 24:52 (dpf = 4), long before gonads development. At this stage (12 hpf), tilapia larvae continue to have a rudimentary brain (31 hpf), as well as primordial germ cells (46 hpf) (Morrison et al., 2001).

Although the induction of deviations in sex ratios by environmental factors has been recognized in many species, the environmental sex determination (ESD) mechanisms are still unknown.

INTRACELLULAR RECEPTORS

According to Squires (2003), glucocorticoid (GR), mineralocorticoid (MR) and androgen (AR) receptors are found predominantly in the cytoplasm of cells, while thyroid hormone receptors (TR), estrogen (ER), progesterone (PR), retinoic acid (RAR) 1.25 and D3-hydroxy vitamin (VDR) are predominantly found in the nucleus.

Steroids and thyroid hormones operate via intracellular receptors. Toft and Gorski (1966) identified estrogen receptors, using hormone radiotracer and found that the receptor was only present in target cells. Receptors for steroid hormones act as transcription factors to regulate transcription of target genes (e.g. aromatase gene). These receptors move between the nucleus and cytoplasm and in the absence of hormone are linked to the 90kDa (Hsp90) heat shock protein complex (Figure 4). Hormone binding to the receptor results in Hsp90 complex release and translocation of the hormone-receptor complex to the nucleus. A dimer of the hormone-receptor complex then interacts with hormone response elements in specific genes that affect DNA transcription. This exposes sites on the DNA template, either directly or through influence of pre-existing repressor molecules to increase the initiation sites for RNA polymerase and increase the transcription (Figure 4). This action of steroid hormones occurs during a much longer time (hours) compared with peptide hormones (Squires, 2003).

Through this mechanism, it is possible to predict that fluctuations in temperature can influence the action of hormones, increasing the transcription of genes involved in steroidogenesis and hence in sexual differentiation, both in fish as in other groups.

CONCLUSIONS

Sex can be determined by multiple mechanisms in vertebrates. Despite the fact that the fate of the gonad is determined genetically, temperature can override it and switch the mechanism when the gonad is undifferentiated (Baroiller et al., 2009). Many genes involved in the sex-determining cascade have been identified and the existence of a complex SDS, combining GSD and thermo-sensitivity.

Research shows that the thermo-sensitive period is variable depending on the species, occurring before the onset of the first sex-specific differences, an active mitosis in the ovary (D'Cotta et al., 2001a; Ijiri et al., 2008; Nakamura et al., 1998). Thus, it can be assumed that high temperatures can act early on the brain under development and/or primordial segregation of germ cells (Rougeot et al., 2008).

As stated by Bull (2008), if the coexistence of TSD and GSD is an "accident" (infrequent relic) or adaptation, it remains being an important issue (Warner and Shine, 2008). To explain the coexistence of sex chromosomes and TSD, benefits of both systems must be combined (Bull, 2008). Therefore, it is possible to suggest that intracellular receptors are presented as key mechanism in the sexual differentiation of animals with TSD system;

however, no work demonstrating the action of this mechanism was performed, although many report that temperature is directly linked to sex determination.

TSD and GSD combined show that sex in fish is governed by the interactions of some components such as: a complex system of genetic determination, with some determinant loci and some minor genetic factors, as well as the influence of temperature. We need to understand how temperature and genetics match for sex determination in fish. For this, three important issues arise in relation to temperature effects: (1) does the temperature act in sex determination, sex differentiation or both? (2) are there different heat-sensitive stages in the cascade of sex determination depending on the species? and (3) what is (are) the true target-organ (s) (gonads, central nervous system, both organs) to the temperature effects?

Among so many studies, the role of temperature on sex determination in fish has not been fully elucidated. To the extent that future research will answer these questions, the use of molecular markers associated with temperature, combined with advances in reproductive techniques brings promising prospects for genetic improvement in aquaculture.

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