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Differential expression analysis of genes involved in high-temperature induced sex differentiation in Nile tilapia

Chun Ge Li [a,1], Hui Wang [a,1], Hong Ju Chen [a], Yan Zhao [a], Pei Sheng Fu [b], Xiang Shan Ji [a,*]

[a] College of Animal Science and Technology, Shandong Agricultural University, Taian 271018, China
[b] Shandong Institute of Freshwater Fisheries, Jinan 250117, China

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ABSTRACT

Nowadays, high temperature effects on the molecular pathways during sex differentiation in teleosts need to be deciphered. In this study, a systematic differential expression analysis of genes involved in high temperature-induced sex differentiation was done in the Nile tilapia gonad and brain. Our results showed that high temperature caused significant down-regulation of *CYP19A1A* in the gonad of both sexes in induction group, and *FOXL2* in the ovary of the induction group. The expressions of *GTHα*, *LHβ* and *ERα* were also significantly down-regulated in the brain of both sexes in the induction and recovery groups. On the contrary, the expression of *CYP11B2* was significantly up-regulated in the ovary, but not in the testis in both groups. Spearman rank correlation analysis showed that there are significant correlations between the expressions of *CYP19A1A*, *FOXL2*, or *DMRT1* in the gonads and the expression of some genes in the brain. Another result in this study showed that high temperature up-regulated the expression level of *DNMT1* in the testis of the induction group, and *DNMT1* and *DNMT3A* in the female brain of both groups. The expression and correlation analysis of HSPs showed that high temperature action on tilapia HSPs might indirectly induce the expression changes of sex differentiation genes in the gonads. These findings provide new insights on TSD and suggest that sex differentiation related genes, heat shock proteins, and DNA methylation genes are new candidates for studying TSD in fish species.

1. Introduction

Although susceptibility to environmental influences on sex is seen in some species of fish, this is also common in reptiles (Conover and Heins, 1987; Baroiller et al., 1995; Godwin et al., 2003; Blázquez and Somoza, 2010). In fish displaying environmental sex determination, the main environmental factor influencing sex seems to be temperature (temperature-dependent sex determination, TSD) (Baroiller and D'Cotta, 2001; Ospina-Alvarez and Piferrer, 2008). In Nile tilapia, the genetic sex is generally determined by a mixture of major (XX/XY) and minor genetic factors (Baroiller et al., 1999; Devlin and Nagahama, 2002; Cnaani et al., 2008). However, high temperature can override this and switch the mechanism when the gonad is undifferentiated (Tessema et al., 2006). It is well known that high temperature treatment applied after hatching (around 10 days post-fertilization) and lasting from 10 to 28 days significantly skewed sex ratios towards males (Baroiller et al., 1995, 2009; Abucay et al., 1999; Baras et al., 2001; Tessema et al., 2006; Rougeot et al., 2008; Dang et al., 2011). In the masculinization process of tilapia, high temperature acts on the cascade of sex differentiation, somehow impeding ovarian differentiation and redirecting the pathway towards testis development (D'Cotta et al., 2001a, 2001b). High temperature may be activating or repressing sexual differentiating genes by following a common pathway with normal testicular differentiation in Nile tilapia (D'Cotta et al., 2007, 2008; Poonlaphdecha et al., 2013; Vernetti et al., 2013).

Administration of androgens to fish larvae at gonad undifferentiated stages can generate partial or complete masculinization in a number of fish species (Leet et al., 2011). Likewise, functional female phenotypes can also be induced at this same period with estrogens (Baroiller and D'Cotta, 2001). From this, it is evident that the sex steroids play a pivotal role in fish sex determination and differentiation. It is well known that the aromatase enzyme (=*CYP19* gene) catalyzes the conversion from testosterone to 17β-estradiol and generally from androgens to estrogens (Baroiller et al., 1995) and if inhibited, blocks estrogen production causing a female to male sex reversal (Guiguen et al., 1999; Uchida et al., 2004). Furthermore, the transcription factor *FOXL2* has been characterized as an ovarian specific upstream regulator of a *CYP19A1A* promoter that would co-activate *CYP19A1A* expression, along with some additional partners such as NR5A1 (SF1) or cAMP (Wang et al., 2007). The changes in transcription of genes involved in steroidogenesis and hence in sexual differentiation, become an alternative to explain where the temperature is acting (D'Cotta et al., 2001a, 2001b, 2007, 2008; Poonlaphdecha et al., 2013; Vernetti et al., 2013). There are many genes associating with

* Corresponding author at: 61, Daizong Street, College of Animal Science and Technology, Shandong Agricultural University, Taian 271018, Shandong, China.
E-mail address: xsji@sdau.edu.cn (X.S. Ji).
[1] Contributed equally.

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gonadal sex differentiation (Ijiri et al., 2008). A systematic differential expression analysis of genes involved in high temperature-induced sex differentiation in Nile tilapia was very necessary.

In recent years, epigenetic mechanisms such as DNA methylation and histone modifications have been implicated in the complex regulation of *CYP19* gene (Kumar et al., 2009; Monga et al., 2011). These mechanisms exert effects that are essential to the regulation of gene expression. Navarro-Martín et al. (2011) found that exposure of undifferentiated sea bass larvae to high temperature increased the *CYP19A* promoter methylation levels of females and males, indicating that high temperature induced-masculinization involves DNA methylation-mediated control of aromatase gene expression. As a result, it is meaningful to determine the expression changes of DNA methyltransferase genes after high temperature treatment in Nile tilapia.

Heat shock proteins (HSPs) are a class of functionally related proteins involved in the folding and unfolding of other proteins and their expressions are induced by heat and other stresses (Kohno et al., 2010). It is reported that for several steroid receptors, binding to *HSP90* was required for the receptor to be in a native hormone-binding state, and for all of the receptors, hormone binding promoted dissociation of the receptor from *HSP90* and conversion of the receptor to the DNA-binding state (Pratt and Toft, 1997). Furthermore, heat shock protein 27 as an estrogen receptor-β (*ER*β) associated protein could act as a co-repressor of estrogen signaling (Al-Madhoun et al., 2007). As a result, HSPs are interesting candidates to play important roles during the interaction of temperature and estrogen signaling (Kohno et al., 2010).

Considering the possible regulatory role of sex differentiation genes, DNA methylation genes, and HSPs in fish sex differentiation, we hypothesized that high temperature effects on sex differentiation in the Nile tilapia could involve high temperature-regulated expression of them. The objectives of the present study were to determine the mRNA expression changes of sex differentiation genes, DNA methylation genes, HSPs in the Nile tilapia brain and undifferentiated gonads after high temperature treatment.

2. Materials and methods

2.1. Fish culture and sampling

Nine hundred Nile tilapia larvae at 7 days post fertilization (dpf) from 5 families (100–250 larvae each family) were obtained from Shandong Institute of Freshwater Fisheries (Jinan, China). The larvae were randomly divided into three groups and reared in 0.5 m^3 tanks in the experimental base of Shandong agricultural university under natural photoperiod, and fed pelleted tilapia food of appropriate sizes from 9 dpf onwards. The high temperature treatment to induce masculinization in the Nile tilapia was performed as previously described (Baroiller et al., 1995; Tessema et al., 2006; Dang et al., 2011) and temperature treatments were initiated at 10 dpf. Water temperature of group one (high-temperature induction group) was gradually elevated to 36 °C in 4 h and cultured at 36 °C for 12 days. The temperature of group two (recovery group) was also increased to 36 °C in 4 h and the larvae were cultured at 36 °C for 9 days. Then, the temperature decreased to 28 °C and continued to rear at 28 °C for 3 days. Group three (control group) was cultured at 28 °C for 12 days. Fish were simultaneously sampled at 22 dpf (the end of temperature treatment) for the three groups under a stereomicroscope dissecting both the gonad and brain–pituitary. It has been reported that the appropriate masculinization parameters in Nile tilapia were 36 °C treatment of 9–10 dpf larvae for 9–12 days (Tessema et al., 2006; Dang et al., 2011). In order to acquire larvae of same size, the larvae in the induction group were treated for 12 days at 36 °C and the larvae in the recovery group were treated for 9 days at 36 °C. Previous reports determined the expression changes of several sex differentiation genes of high-temperature treated larvae compared with control larvae (D'Cotta et al., 2001b, 2007; Poonlaphdecha et al., 2013). Nowadays, nobody knows whether the expression levels of sex differentiation related genes changed if the high-temperature treated fish were transferred to culture water (28 °C) for several days. In this study, we determined the expression changes of selected genes of larvae in the recovery group compared with control larvae. Because the Nile tilapia gonad at 22 dpf is very small, the gonad and a little peritoneum together were scraped with knife. 30–40 experimental fish for each group were sampled, and the gonad and brain–pituitary were respectively stored in liquid nitrogen for RNA extraction.

Six one-year-old adult fish were purchased from Shandong Institute of Freshwater Fisheries (Jinan, China). The identification of sex was confirmed by optical microscopy following dissection and the gonads were collected and stored in liquid nitrogen for RNA extraction.

2.2. RNA extraction and cDNA synthesis

The RNA was respectively extracted from the gonads and brain–pituitary using a TRIzol reagent (Tiangen, Beijing) according to the manufacturer's instruction. Reverse transcription (RT) contained two steps. (1) Reaction of the genomic DNA removal was performed at 42 °C for 2 min and then 4 °C in a total volume of 10 μL consisting of 1 μg total RNA, 5 ×gDNA Eraser buffer, and 1 μL gDNA Eraser. (2) - Reaction to reverse transcription was performed at 37 °C for 15 min, 85 °C for 5 s and then 4 °C in a total volume of 20 μL consisting of 10 μL reaction solution of step 1, 4 μL 5× PrimeScript buffer, 4 μL PrimeScript RT Enzyme MixI, and 1 μL RT Primer Mix (Takara, Dalian, China).

2.3. Sex identification of Nile tilapia larvae

The RNA was extracted from the gonads of adults and larvae. The real time PCR, the *CYP19A1A* and *ER*β as primer (Table 1) and the gonad cDNA as template were utilized to determine the Ct (cycle threshold) value for *CYP19A1A* and *ER*β in adult or larvae (Ijiri et al., 2008; Blázquez et al., 2009). According to the obtained Ct values from adults, we can set the fold change threshold value of *CYP19A1A* between females and males. Similarly, the phenotypic sex of Nile tilapia larvae or high-temperature treated larvae was determined according to the set *CYP19A1A* Ct threshold value (Poonlaphdecha et al., 2013). qRT-PCR of *ER*β was done to further verify the phenotypic sex identification result of *CYP19A1A*.

2.4. Gene selection and cloning

A total of 18 genes (Table 1) were selected based on their possible role in fish sex differentiation. The 18 genes included 4 HSPs, 12 sex differentiation genes, and 2 DNA methylation genes. The 4 HSPs were heat shock protein 27, *HSP27*; *DNAJB1*, also named as heat shock protein 40; heat shock protein 70, *HSP70*; and heat shock protein 90, *HSP90*. 12 sex differentiation genes were *CYP19A1A* (cytochrome P450, family 19; ovarian type of aromatase), *FOXL2* (forkhead box L2), *CYP19A1B* (cytochrome P450, family 19; brain type of aromatase), *ER*α (estrogen receptor α), *ER*β (estrogen receptor β), *CYP11B2* (cytochrome P450, family 11, steroid 11β-hydroxylase), *DMRT1* (doublesex and mab-3 related transcription factor 1), *SOX9A* (SRY-box containing gene 9a), *GTH*α (gonadotropin α), *FSH*β (follicle-stimulating hormone β), *LH*β (luteotropic hormone β), *AR*α (androgen receptor α). 2 DNA methylation genes were *DNMT1* (DNA-methyltransferase 1-like) and *DNMT3A* (DNA-methyltransferase 3a). In addition, elongation factor 1α was selected as internal quantitative control (Du et al., 2008). 14 genes (*CYP19A1A*, *CYP19A1B*, *CYP11B2*, *DMRT1*, *FOXL2*, *ER*α, *ER*β, *SOX9A*, *HSP27*, *DNAJB1*, *HSP70*, *HSP90*, *DNMT1* and *DNMT3A*) were used for