The Expression of CYP19a Gene at Two Temperature Levels During the Thermosensitive Period of the Nile Tilapia (Oreochromis niloticus)

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Animal Molecular Breeding, 2015, Vol.5, No.2  doi: 10.5376/amb.2015.05.0002

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Rodrigues et al., 2015, The Expression of CYP19a Gene at Two Temperature Levels During the Thermosensitive Period of the Nile Tilapia (Oreochromis niloticus), Animal Molecular Breeding, Vol.5, No.2, 1-6 (doi: 10.5376/amb.2015.05.0002)

Abstract The gene expression of ovarian aromatase is regulated according to the period of exposure to different post-fertilization temperatures. Previous studies show that high temperatures regulate aromatase gene expression, or transcription factors responsible for gene expression during sex differentiation in fish. The fact that this enzyme is part of the reproductive hormone estrogen synthesis pathway, leads researchers to study how the differential expression of this gene can influence the proportion of males and females in the process of sexual reversion. The objective of this study was to evaluate the effect of temperatures at 25°C and 35°C on the expression of ovarian aromatase during the period of sexual reversion in Supreme strain of the Nile Tilapia (Oreochromis niloticus). We conducted two times the same experiment under the same conditions and at an interval of 12 months between both. We used 1500 larvae three days after hatching, in the final phase of resorption of the yolk sac per experiment maintained in water in a closed recirculatory system. For each experiment we used eight experimental units, each containing 93 larvae that were separated into two groups of four repetitions for each temperature treatment. Treatments lasted a total period of 30 days, with a 12L:12D photoperiod. Total RNA was extracted from a sample of four larvae that was collected every week from each treatment using a Quick-RNA™ MiniPrep (R1054) extraction kit. The synthesis of cDNA was performed using 10ng of RNA with the GoScript™ Reverse Transcription System kit. We were not able to completely block the expression of aromatase in the 35°C treatments in the Supreme strain since we found a variation in the responses to the expression of aromatase. Gene expression was detected during the thermosensitive period (TSP) in both treatments. We suggest that the bands that present low levels of expression in the treatment at 35°C probably belong to male individuals, while the high intensity bands in the 25°C treatment are most probably females.

Keywords Aromatase; Temperature; Sex Reversal; Supreme Strain; RT-PCR

1 Introduction

The phenotypic definition of sex in fish can be influenced by changes in the biosynthetic pathway of estrogen, caused either through the supply of hormones or through the use of temperature with the objective of sex reversal in many species.

In Nile Tilapia (Oreochromis niloticus), the definition and differentiation of sex are respectively controlled by chromosomes (XX and XY) and by environmental factors (Delvin and Nagahama, 2002).

Although the exact mechanisms involved in the natural definition of sex are yet to be clarified, we know that steroid hormones play a fundamental role in gonad development and sexual differentiation of fish (Chang and Lin, 1998; Kitano et al., 1999; Lee et al., 2001; Guiguen et al., 2010).

Aromatase (Cytochrome P-450) is the enzyme that catalyzes the denaturation (aromatization) of the A ring of C19 androgens and converts them into C18 estrogens. Methyl-19 is also removed in this process (Simpson et al., 1994). This enzyme attaches itself to
the membrane, located in the endoplasmic reticulum of estrogen producing cells of ovaria, placenta, testis, adipose and encephalic tissue. It is encoded by the CYP19 gene and it works on the formation of the nadph-ferrihemoprotein reductase complex in the Cytochrome P-450 system (Simpson et al., 1994). In fish, aromatase is predominantly expressed in the gonads and the brain.

This enzyme being a part of the estrogen synthesis pathway, a reproductive hormone, leads researchers to study how the differential expression of this gene can influence the proportion of males and females in the process of sexual reversion.

Teleostean fish express two cDNA subtypes of the CYP19 gene: the ovarian aromatase (CYP19a) and the brain aromatase (CYP19b). In species like the rainbow trout (Oncorhynchus mykiss) (Dalla Valle et al., 2002; Tanaka et al., 1992), the Atlantic halibut (Hippoglossus hippoglossus) (Matsuoka et al., 2006), the Nile tilapia (Oreochromis niloticus) (Tsai et al., 2001), the zebrafish (Danio rerio) (Kishida and Callard, 2001), the Lebranche mullet (Mugil brasilienis) (Ocullado et al., 2007), the channel catfish (Ictalurus punctatus) (Trant, 1994) and the Japanese medaka (Oryzias latipes) (Fukada et al., 1996) the two isoforms of the CYP19 have already been isolated. The ovarian aromatase was obtained through cloning of ovaria and the brain aromatase was isolated from brain tissue. In species like the goldfish (Carassius auratus) (Gelinas et al., 1998) and the rosy red strain of fathead minnow (Pimephales promelas) (Halm et al., 2001) only brain aromatase was isolated.

The gene expression of ovarian aromatase is regulated according to the period of exposure to different post-fertilization temperatures. This expression also changes throughout the period of sex differentiation. Previous studies show that high temperatures regulate aromatase gene expression, or transcription factors responsible for gene expression during sex differentiation in fish (Baroiller and D’cotta, 2001; D’cotta et al., 2001; Tsai et al., 2003; Ospina-Álvarez and Piferrer, 2008). In the Nile tilapia, exposure to high temperatures (±35°C) drives sexual differentiation towards phenotypic males, while at environmental temperatures (±25°C) sexual differentiation is more balanced, with half of the individuals becoming females and the other half males. The first patterns of gene expression of CYP19a can be detected around 30 DPF (days post fertilization) in the rainbow trout (Vizziano et al., 2007). In the case of the Nile tilapia, high levels of gene expression are present in females while they remain low in males, according to some authors (Trant et al., 2001; Chang et al., 2005; Ospina-Álvarez and Piferrer, 2008).

Aiming to improve our knowledge on the action of temperature in the process of sexual differentiation of the Nile tilapia, the objective of this study was to evaluate the effect of temperatures at 25°C and at 35°C on the expression of ovarian aromatase during the period of sexual reversion in this species.

2 Results and Discussion

The expression levels of the CYP19a gene are directly related to the differentiation of somatic cells of ovaria (Guiguen et al., 2010). Studying the expression of the gene related to the process of sex differentiation we can infer on its participation and role during the sexual reversal process in tilapia.

In this study, we detected the expression of the CYP19a gene during sex differentiation using RT-PCR in tilapia larvae that were collected weekly from mid April to mid May 2010 and from May to June 2011, three days post hatching, in the T25 and T35 treatments. There was a clear variation along the sampling period and within treatments (Figure 1 and Figure 2 to first and replication time respectively). In comparison, the control gene (β-actin) was kept stable throughout each experiment.

The results of both experiments were basically the same (Figure 1 and Figure 2) and similar expressions were observed during sex differentiation period.

During the 3-16 days post hatching stages, the intensity of bands in the T35 treatment was similar in all samples. In T25 there was a different response, with a high expression after 3 days of hatching and a subsequent reduction between the 9th, 16th and 23rd days.

In the Supreme strain of tilapia, results show that the expression of the CYP19a gene was different between
Figure 1 – First experiment conducted from the 15th of April to the 19th of May 2010. CYP19a gene expression analyzed through RT-PCR. Agarose gel at 1% stained with GelGreen (Biotium, USA), with samples of tilapia larvae kept in different temperature treatments during the sex differentiation period (03-30 days post hatching): 03 – Samples collected before treatment was started (time zero); 09 – samples collected after one week of treatment; 16 – samples collected after two weeks of treatment; 23 – samples collected after three weeks of treatment; 30 – samples collected after four weeks of treatment; The β-actin gene was used as a control for each period of analysis.

Figure 2 – Second experiment conducted from 11th of May to 9th of June 2011. CYP19a gene expression analyzed through RT-PCR. Agarose gel at 1% stained with GelGreen (Biotium, USA), with samples of tilapia larvae kept in different temperature treatments during the sex differentiation period (03-30 days post hatching): 03 – Samples collected before treatment was started (time zero); 09 – samples collected after one week of treatment; 16 – samples collected after two weeks of treatment; 23 – samples collected after three weeks of treatment; 30 – samples collected after four weeks of treatment; The β-actin gene was used as a control for each period of analysis.

The two treatments during the tested time periods. Differences between samples of T25 and T35 were significant until the 23rd day post hatching, a finding that was also reported in other species like sea turtles (Merchant-Larios et al., 1997). Assuming that the level of CYP19a expression is correlated with the production of estrogens (Wang et al., 2010), changes in gene expression will be evidence of changes in the phenotypic sex differentiation process.

(Kwon et al., 2001) obtained similar results in a previous study and found that the expression of the CYP19a gene, during the sex differentiation period in female tilapia was very high, and that in males, between 15 and 27 days post hatching, gene expression was low. (Guiguen et al., 2010) observed that before the sex differentiation period started the expression of the CYP19a gene in ovarian tissue of the rainbow trout was 100 times higher than that in tilapia.

On the 30th day post hatching gene expression was high in both treatments (T25 and T35), similar to other studies with tilapia (D'Cotta et al., 2008; Baroiller et al., 2009); but we were not able to completely block the expression of aromatase at 35°C.

Based on our results and the background information outlined, we suggest that the bands that present low levels of expression in the treatment at 35°C probably belong to male individuals, while the high intensity bands in the 25°C treatment are most probably females.

Although we did not analyze the importance of transcription factors in the process of sex determination in this study, there is information that supports that their bonding to CYP19a gene promoters has a strong effect on its expression (Wang et al., 2007, 2010; Guiguen et al., 2010).

Some studies involving the Nile tilapia confirm that estrogen is needed and is most relevant in the process of ovaria differentiation (Kwon et al., 2000; Kobayashi et al., 2003), thereby revealing that aromatase plays a part in the definition of sex in teleosteans by regulating the synthesis of estrogens (Maldonado et al., 2002).

The inhibition of aromatase results in the development of male gonads (Chang et al., 2005; D'Cotta et al., 2007; Ijiri et al., 2008; Baroiller et al., 2009). In contrast, the high levels of expression of the sexual differentiation gene leads to the synthesis of estrogen and consequently to the formation of ovary.

3 Materials and Methods
Location and Procedure
Having regard to previous studies which show that the
temperature during sex differentiation, in progenies of tilapia, induces down-regulation of the gene $\text{CYP19a}$ (D’Cotta et al., 2001, 2008; Baroiller et al., 2009); we repeat the same experiment twice under the same conditions and at an interval of 12 months between both.

The experiments were conducted in the facilities of the Universidade Federal de Pelotas from the 15th of April to the 19th of May 2010 and from 11th of May to 9th of June 2011 with the approval of the university’s Ethics Committee (CEEA registry number: 1152).

Fry of the Supreme strain were obtained from the commercial pisciculture company located in Rolândia, Paraná state, Brazil. The Supreme strain was introduced in Brazil in the year of 2004 from Malaysia by the Aquabel Larviculture.

We used 1500 larvae three days after hatching, in the final phase of absorption of the yolk sac per experiment. The larvae were maintained in water in a closed recirculatory system comprised 40L polypropylene boxes and a 300L biological filter with constant aeration.

**Thermal Sex Reversal Experiment**

For each experiment we used eight experimental units, each containing 93 larvae, that were separated into two groups of four repetitions for each temperature treatment: at 25°C (T25) and 35°C (T35). Treatments lasted a total period of 30 days, with a 12L:12D photoperiod. Animals were fed short amounts of a protein rich (56%) commercial feed (provided by Aquabel) every hour, during the 12-hour light period. After the 30-day period, water in the tanks was gradually cooled down in T35, and heated up in T25 until both treatments reached the same temperature of 27°C. Water quality was maintained using biological filters and daily siphoning (20-25% of total water volume). The physico-chemical parameters of aquaria were controlled weekly and mortality was checked daily through direct observation of experimental units.

**Total RNA Extraction and RT-PCR of Aromatase**

Every week a sample of four larvae was collected from each treatment (T25 and T35). In total we collected six samples per treatment, including a first sample at time zero (before the temperature treatments were started). Total RNA was extracted from larvae with an average mass of 0.118g using a Quick-RNA™ MiniPrep (R1054) extraction kit, following the instructions of the manufacturer (Zymo Research, USA). Extracted RNA was dissolved in 35µL of DNase/RNase-Free Water and stored at -80°C. The quantification of RNA was performed using a NanoDrop spectrophotometer (NanoDrop Technologies, DE). The synthesis of cDNA was performed using 10ng of RNA with the GoScript™ Reverse Transcription System kit following the instructions of the manufacturer (Promega, USA).

The “Reverse transcriptase” (RT-PCR) amplifications were performed using primers designed from the genome sequence (GenBank ID: AF472620) (Chang et al., 2005), with the Vector NTI 8.0 (Invitrogen, USA) software for the amplification of fragments in the codifying region of the $\text{CYP19a}$ gene of Tilapia. The sequence of the obtained primers was the following: forward 5’TGCAGGCTGTTCTACATCA TCACCC 3’ and reverse 5’GGCAACCTGAACCACCTGGGCAC 3’.

To determine the relative amplification we used the $\beta$-actin gene (Overbergh et al., 2003), using the following primers: forward 5’AGAGGGAAATCGTG CGTGAC 3’ and reverse 5’CAATAGTGTGACCTG GCCGT 3’. RT-PCR reactions were performed in a final volume of 25µl, containing 30ng of cDNA, 2pmoles of each primer, 1X of PCR buffer [10mM Tris HCl (pH 9.0), 1.5mM MgCl2 and 50mM KCl], 200mM of each dNTP and 0.5U of Taq DNA polymerase (Fermentas, USA). PCR reactions were performed using an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Alemanha). We used the following program for amplification: an initial step of 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 3 minutes, annealing at 56.8°C for 45 seconds, an extension at 72°C for 45 seconds, and a final extension at 72°C for 8 minutes. Amplifications were checked with 10µl of the RT-PCR products in 1% agarose gel stained with GelGreen (Biotium, USA).

**4 Conclusions**

We were not able to completely block the expression of aromatase in the 35°C treatments in the Supreme
strain of tilapia since we found a variation in the response to the expression of aromatase. Gene expression was detected during the thermosensitive period (TSP) in both treatments at 25°C and 35°C.

Expressions were presented during sex differentiation period, further developments of this work with RT Quantitative Reverse Transcription PCR could be future research to understand the expression patterns.

Acknowledgements

We want to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for awarding the aid grants and financing that allowed for the realization of this study.

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