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Cross-specific Steroidogenic Enzyme Genes Expression during Gonadal Development in *Lepidocephalus thermalis*

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A B S T R A C T

Sex steroids were involved during the process of sex differentiation, gametogenesis and sex reversal in fish and the expression of steroidogenic enzyme genes and their related transcription factors are critical for the development of the gonads and the regulation of steroidogenesis. The early stage of steroidogenesis is important for biosynthesis of testosterone and estradiol. In this study, three steroidogenic enzyme genes *cyp19a1*, *11βhsd* (11β-hydroxysteroid dehydrogenase) and *3βhsd* (3β-hydroxysteroid dehydrogenase) expression in various stages of gonad development has been analysed. As this is the first recordical study of steroidogenic enzyme genes in *L. thermalis*, the primers of Air breathing catfish, *Clarias gariepinus* for the genes *cyp19a1, 11βhsd* and *3βhsd* has been used to find the expression level at various stages. Based on the previous studies in catfish in these genes, *cyp19a1* is an ovarian aromatase found abundant in differentiating female gonads. *11βhsd* has higher expression in the spawning phase of reproductive cycle and transactivates to regulate male reproduction in teleost. *3βhsd* is important in both spermatogenesis and oogenesis. From similar experimental methods performed in *C. gariepinus* the expression level of these genes in *L. thermalis* is observed. From the graph, it is evident that in *L. thermalis*, higher expression level by *cyp19a1, 11βhsd* and *3βhsd* is in medium sized testis than in the other. Hence these genes play a vital role in spermatogenesis in *L. thermalis*.

Introduction

Sexual reproduction is an important event which enables most of the organism in propagation and transfer genetic information from one generation to another (Raghuveer, 2011). Sex determination is the process by which the sex of an individual is established in an organism (Hughes, 2001; Kondo *et al.*, 2009). Sex differentiation refers to the gonadal development after sex determination, when a bipotential or an indifferent gonad

develops into testis or ovary, and is controlled by various factors which may be genes or hormones (Hughes, 2001).The sex of teleost can be completely reversed or manipulated by exogenous sex steroid treatment around the critical period of sex determination or sex differentiation (Nagahama, 2005; Kobayashi *et al.*, 2008; Raghuveer and Senthilkumaran, 2009). There are several transcription factors which are being implicated in gonadal differentiation.

Teleost fishes are excellent model for studying the event of sex determination and sex differentiation. *Lepidocephalus thermalis*, commonly known as Indian spiny loach, is a freshwater fish found in southern part of Tamil Nadu. In the present study, we have trailed the cat fish primers of *cyp19a1*, 11*β*hsd and *3βhsd* and used in loach gonad, Mesonephric gonadal complex and muscle gonadal gene expression by using quantitative real time PCR as this is the first study in *L. thermalis* of Southern Region.

The expression of *cyp19a1* was observed in the late gastrulation period of cat fish. During sex differentiation in the larval stage, expression of *cyp19a1a* and *cyp19a1b* increased. The ovarian expression of *cyp19a1a* and *amh* (Antimullariean Hormone) increased with increase in plasma estradiol levels during vitellogenesis. The expression of testis-specific *cyp19a1b* supports the importance of estrogen in the spermatogenesis, while abundant expression in the male and female brain is probably related to the continuous neurogenesis. The dimorphic expression patterns of *cyp19a1* paralogs show the complexity of the genetic network regulating sexual development in fish (Hanne Johnsen *et al.*, 2013). *cyp19a1* gene encodes the estrogen synthesizing aromatase enzyme cytochrome P450 (family 19, subfamily A, polypeptide 1) which has an important role in sexual differentiation of the vertebrate gonads and brain (Rouiler-Fabre *et al.*, 1998; Carreau *et al.*, 2006; Stocco, 2008; Le Page *et al.*, 2010). Gonadal development in both sexes is negatively regulated by the action of AMH inhibiting the ovarian expression of *cyp19* in the granulosa cells, by blocking the differentiation of Leydig cell precursors and decreasing the expression of steroidogenic enzymes (Josso *et al.*, 1998; Racine *et al.*, 1998; Rey *et al.*, 2003).

11β- hydroxylase (11*βH*), steroidogenic enzyme involved in biosynthesis of 11-Ketotestosterone (11-KT) is not expressed at early stages of testis development or during male sex determination. The role of 11-KT the expression and dehydrogenase activity of 11*β-HSD* might be important for testicular differentiation (Rasheeda *et al.*, 2010). In catfish 11*βhsd* enzyme has indicated its expression in testicular differentiation and seasonal testicular cycle (Rasheeda *et al.*, 2010).

3*β-hsd* gene belongs to short-chain dehydrogenase or reductase (SDR) family. Type I 3*βhsd* activity is found in placenta and peripheral tissues like brain, skin and kidney, whereas the type II 3*β*hsd is an isoenzyme and is predominantly expressed in the adrenal gland, ovary and testis. 3*βhsd* controls the critical steroidogenic reaction in the adrenal cortex, gonads, placenta and a variety of peripheral target tissues. In cat fish, 3*βhsd* plays an important role in both spermatogenesis and oogenesis and the transcript were detected much earlier in undifferentiated gonads. Later the expression was observed in both male and female gonads (Raghuveer and Senthilkumaran, 2012).

Materials and Methods

Animal sampling

Indian spiny loach at different age groups were collected from river and reared in fresh

water tanks, maintained at ambient photo thermal conditions with circulating aeration system. The fishes were fed with live blood worms, rice bran and commercially available fish feed gonadal tissues were collected from fishes at various developmental stages for this study. The Indian spiny loach (*Lepidocephalus thermalis*) of different age groups (small size fish: 50-90 days; medium size fish: (90-150 days) and large size fish: (150- 250 days) were collected from river and fresh water farm. Mesonephric gonadal complex (MGC, 10 to 50 days) (5 hatchlings per sample; n=3) and adult gonad tissues (7 fish per sample; n=3) from the same age group of fish were pooled out and used for total RNA isolation using TRI-reagent method from Sigma.

Estimation of RNA purity

The concentration and purity of total RNA was estimated by measuring its absorbance at 260nm using NanoDrop spectrophotometer. The purity of RNA was assessed by checking the absorbance (A 260/280) ratio which was found to be in the range of 1.8 to 2. The range indicates that the total RNA was pure. Concentration of total RNA was calculated using the formula= A260 × 40 × Dilution factor.

First strand complementary DNA (cDNA) synthesis- RTPCR

First strand cDNA synthesis was performed using Applied Biological Material Easy Script ™ cDNA synthesis kit. The RTPCR was performed in Gene AmpR PCR 9700 of Applied Biosystem machine. First strand cDNA fragment was associated with Total RNA range from 1microgram to 2 microgram, Oligo nucleotide was 1 micro liter, dNTPs are 10 micro liter along with nuclease free water than followed by adding EasyscriptRtase enzyme for cDNA synthesis. The cDNA mixture was stored at -20ºC for real time PCR.

Real-Time Quantitative PCR

The target genes of 3*β-hsd, 11-βhsd and cyp19a1* were analyzed by using endogenous control of (18s rRNA). The primer sequence is mentioned in Table 1. The expression of different gonad-related and steroidogenic enzyme genes were analysed by real time PCR using SYBR Green detection method. Total RNA was extracted by using Sigma TRI-reagent method from testis, ovary and muscle tissue samples that were collected at different age groups (small (MSG complex): 70 days; medium: (90-150 days) and large: (150- 250 days) and quantified by using a NanoDrop spectrometer. Successful reverse transcription was confirmed for all three sample performed by PCR amplification of 18s rRNA. Quantitative real-time PCR analysis was carried out with 20µl reactions in triplicates using power SYBR Green PCR master mix (Applied Biosystem) at an initial hold of 95ºC(15s) and 60ºC(1 min) for 40 cycle according to the manufacture's protocol.

Dissociation curve analysis was performed for each sample to check single amplification. During PCR, fluorescence accumulation resulting from DNA amplification was recorded using the ABI Prism 7500 sequence detection system software (Applied Biosystems).

Cycle threshold (Ct) values were obtained from the exponential phase of PCR amplification and 18s rRNA was used as an endogenous control. Comparative Ct method was used to analyze the results. The NPY expression was normalized against 18s rRNA expression, generating a ΔCt value (ΔCt = NPY Ct –18s rRNA Ct). Relative expression was then calculated according to equation 2-ΔCt.

Results and Discussion

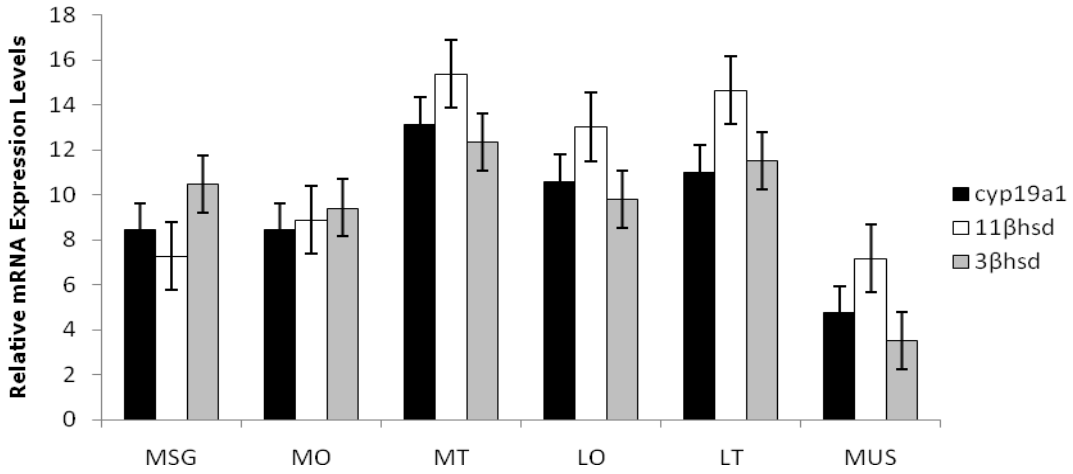
cyp19a1 gene expression shows equal level on maturing stage ovaries and also MSG (Mesonephric gonadal complex) whereas it shows slightly higher expression in large stage (Maure) ovaries. *cyp19a1* gene shows higher expression during spermatogenesis which is evident from the higher expression on medium stage testis and large stage testis, whereas muscle show very low level expression. In *Lepidocephalus thermalis* *cyp19a1* is expressed on spermatogenesis development. In Air breathing cat fish *cyp19a1* is expressed at spermatogenesis. In Atlantic Cod *cyp19a1* sex dimorphic gene expression was during the early stage of both male and females (Hanne Johnsen *et al.*, 2013)

11βhsd gene was hugely expressed in maturing stage testis and adult stage testis for spermination and spermatogenesis development in *Lepidocephalus thermalis*. Similar level of expression was from muscle and undifferentiated gonads (Mesonephric Gonadal Complex). *11βhsd* expression was lower on maturing stage ovaries and adult ovaries than in the testis. Similarly *11βhsd* gene expression plays a major role in testicular differentiation in Air breathing cat fish (Rasheeda *et al.*, 2010).

Table.1 List of primers used for quantitative real-time PCR analysis

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
18s RNA	GCTACCACATCCAAGGAAGGCAGC	CGGCTGCTGGCACCAGACTTG
Cyp19a1	ACAACAACAAGTACGGCAGCA	GTAGAGGAGCTGCTGAGGATGAG
11β-hsd	ATCACAGGGTGCGACTCGGGTTTCGGG	CGGCTGAGTGATGTCCACCTG
3β-hsd	GAGGTAAATGTGAAAGGTACCAA	TAGTACACAGTGTCCTCATGG

Figure.1 Expression level of steroidogenic enzymes



Legend: MSG – Mesonephric Gonadal Complex, MO – Medium sized ovary, MT – Medium sized Testis, LO – Large sized Ovary, LT – Large sized Testis, MUS – Muscles

3βhsd gene expression in *Lepidocephalus thermalis* was higher level in maturing stage testis and almost equal in large testis whereas in ovaries it showed lower level gene expression than in testis and higher expression than in muscles of the animal. In Air breathing cat fish *3βhsd* expressed on both spermatogenesis and as well as oogenesis development in gonads (Raghuveer K *et al.*, 2012)

The comparison between the level of expression of the steroidogenic enzymes *cyp19a1, 11βhsd* and *3βhsd* is shown in Figure 1. The expression of these enzymes is higher during the developing stage in testis.

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