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Expression of the genes *cyp19a1a* and *sox9a* in the early hours of development of *Catla catla* and its role in sex determination and differentiation

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Abstract
Sex determination and early gonadal development depends upon specific embryonic gene program that activate differentiation of the bipotential primodium. Many studies reveal that *cyp19a1a* and *sox9a* are significant for the sex determination and differentiation of ovary and testes development in vertebrates. As the research specimen *Catla catla*, Indian major carp is an important edible fish and has great market demand, it is imperative that the basic developmental mechanisms of sex determination are further studied in this species. It is subjected to gene expression studies in order to find out the influence of the genes *cyp19a1a* and *sox9a* in controlling the sex determination and differentiation during embryonic stages. In the present investigation *cyp19a1a* and *sox9a* are on track to express from 12th and 30th hpf respectively. It is seen that the expression of these genes initiated in two different times and both gene expressions were in progress throughout the study phase. This kind of expression pattern disclose the role of these genes in *C. catla* for sex determination and differentiation which would help to unlock the innovative fraction of research as gene manipulation programmed for increasing the mass production.

Keywords: *cyp19a1a*, *sox9a*, hpf (hour post fertilization), sex determination and differentiation

1. Introduction
Fish have a more complicated mechanism of sex determination and differentiation than mammals, and it is currently a popular animal model in this area of research. It is an ancient question among biologists that how sex is determined. In *Caenorhabditis elegans* the genes dependable for sex determination are well characterized [1]. Many mechanisms subsist by which sex is confirmed in vertebrates. It can be controlled by sex chromosomes, sometimes due to a single sex determination gene, as is the case in mammals [2]. Many cases of polygenic sex determination also exist in vertebrates and are well documented in fish [3, 4]. In addition, in some animals the environment controls the sexual fate [5].
In vertebrates a wide variety of sex determination mechanisms exist, including genetic and environmental mechanisms [6]. Many genes known to play a role in mammalian sex determination are also dimorphically expressed in the gonads in nonmammalian vertebrates [6], [7]. Dimorphic *sox9* expression is frequently seen in the male gonad during sex determination of vertebrates suggesting a general role of this gene in testis development [7]. In mammals, *Sox9* is indispensable for testis differentiation [8] and *sox9* is the first gene to show male sex-specific expression in many species [9]. In fish, both *sox9* gene copies were found to be very much expressed in developing testis [10]. The *cyp19a1* is measured as one of the essential genes in vertebrate sex determination [11, 12]. It is the sensitive gene that reacts to temperature changes and it has been implicated with sex change in fish [13, 14] and it is one of the initial genes to show sex differences in developing tilapia [15]. *Cyp19a1* is a basic component in the estrogen pathway and it has been recommended that hormonal actions are so important in sex determination [16]. All individuals first initiate oogenesis forming an immature nonfunctional ovary before developing a fully differentiated ovary or testis [17]. Sex differentiation in fish can be experimentally controlled by *in vivo* treatments with sex steroids [18] like in reptiles and amphibians and to some extent in birds [19, 20, 21].
Sex determination and premature gonadal development depends upon unambiguous embryonic gene programs that trigger differentiation of the bipotential primodium. These processes crux upon the expression of both sex linked and autosomal genes that endorse or antagonize male or

female cell fate pathways. The products of some genes are essential for female and male sex-determination in mammals [22, 23]. In mammals, as well as teleosts, DAX-1, FOXL2, CYP19a1, MIS, SF-1, SOX-9, WT-1 and many other gene products contribute in directing sex-determined fates, as well as to subsequent gonad development and function [24, 25, 15]. Out of these genes *cyp19a1a* and *sox9a* are leading for the sex determination and sex differentiation of ovary and testes development and the present study is accomplished to know the possible role of these genes in *C.catla*.

There is not as much of study on sex determination in fish and the genetic mechanisms behind this remain largely unknown. While the developmental mechanisms by which the mammalian gonads are formed have been thoroughly studied and several genes involved in this have been identified, only a few of these genes have been recognized in fish. As *C.catla* is an important edible fish and has great market demand it is imperative that the basic developmental mechanisms of sex determination are further studied in this species. The present study discusses the role of genes involved in sex determination with a focus on the potential role of the genes *cyp19a1a* and *sox9a* in *C.catla* sex determination and differentiation.

2. Materials and methods

2.1 Sample collection

Induced fish breeding is done every year in the Tamil Nadu Fisheries Department Corporation (TNFDC), Sathanoor Dam, situated about 200 km south of Chennai. Usually injection for induced breeding was given during the time of evening and the fishes were kept in different happas. A female fish with two male fish was kept in each happas in order to facilitate proper fertilization because there will be millions of eggs in a single fish. Eggs were collected from the happas in the early morning of the next day of injection. And these eggs were collected in buckets along with little water and transferred to circular tanks where water was always circulating in order to give an artificial climate for hatching. And the third day this fish seed was transported to different places. Samples were collected from this induced breeding site after fertilization in between every 6 hours. 10 samples were collected from this site and it was included egg stage to different stages of hatchlings. These samples were pooled in eppendorf tubes with RNA later and stored in $-20°C$ until RNA extraction.

2.2. Total RNA extraction

Total RNA was isolated from the 100 mg of tissue using TRIzol method. The quantity and quality of RNA were determined by UV absorbance at 260 and 280 nm wave length. Agarose gel electrophoresis has done and the clear 18S and 28S RNA band has visualised by UV illuminator and gel documentation has done with the computer.

2.3. cDNA synthesis

One microgram of total RNA from the sample tissue was reverse transcribed using M-MuLV RT- PCR Kit according to manufacturer's instruction. The RT-PCR products were quantified and qualified by using spectrophotometer and gel electrophoresis.

2.4. Polymerase Chain Reaction

The cDNA strand synthesized was taken directly for PCR by using suitable primers for the genes *cyp19a1a*, sox9a and the housekeeping gene *beta actin.*

2.5. Details of primers used for PCR

F: 5′-TGGTGAGGARACTCYCATC-3′
cyp19a1a R: 5′-ACTBTCTTCTGNCAGGTGT-3′
sox 9a F: 5′-TGAAGRGCTACGAYTGGACG-3′
sox 9a R: 5′-CCCTCTCGYYTCAGATCAACTT-3′
Beta Actin F: 5′- CGGTTATCGTTGTAGGCACG-3′
Beta Actin R: 5′-CACTGCCTGCACAAAGAACT-3′

2.6. Preparation of PCR reaction

PCR Master Mix kit was used from the Sigma Aldrich Chemicals Pvt. Ltd., Bangalore. Successful PCR amplification result was confirmed for all samples by performing PCR amplification of *Beta actin* as endogenous control and verified with AGE.

3. Results

In this investigation we possibly exposed the expression of both sex specific genes *cyp19a1a* and *sox9a*. The emergence of *cyp19a1a* is not perceptible in the 6th hour post fertilization (hpf). But in the 12th hpf we could notice a negligible expression of *cyp19a1a* (Fig.1). In the 18th hpf and 24th hpf, a gradual increase in the expression of this gene is distinguished. From 30th hpf to 60th hpf there was a high-flying expression of this gene. This gives an idea about the significance of this gene in sex determination and differentiation. The expression of *β actin* was obvious in all the samples and this gene was vastly apposite in using as endogenous control. And the expression of *β actin* in the 6th hpf divulge the absence of *cyp19a1a* expression for the same period.

The expressions of *sox9a* in the same samples were to some extent different from the outward show of *cyp19a1a*. There was not a bit expression of *sox9a* from 6th hpf to 24th hpf (Fig.2). In actual fact, expression of the gene *sox9a* is detectable as a very light band in the 30th hpf. And from the 36th hpf onwards apparent expression can be seen up to 60th hpf. On account of the expression of *β actin* in all the sample results, it is unambiguous that *sox9a* is not expressed from 6th hpf to 24th hpf and the expression found only in the 30th hpf. This domino effect shows that there is a momentous role for *sox9a* in the early developmental stages.

This research draws attention to the significance of these both genes *cyp19a1a* and *sox9a* in the gonadal development and also opens the path of new investigate areas for manipulating these genes in a more appropriate way. Since these two genes are having significant role in the ovary and testes fate determination, and this kind of study is very rarely conducted in the Indian major carps, the present study leads to new inventions to the fish reproductive biology.

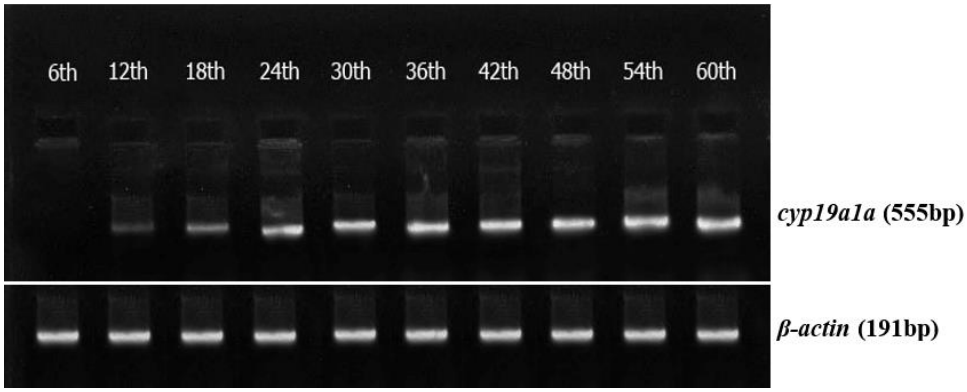

Fig 1: Bands showing the expression of *cyp19a1a* and *β actin* during 0 to 60th hpf (hour post fertilization) in *Catla catla*

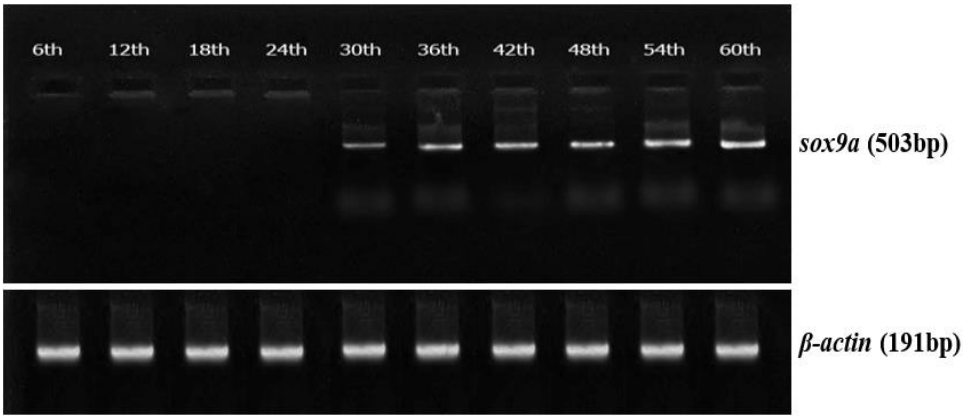

Fig 2: Bands showing the expression of *sox9a* and *β actin* during 0 to 60th hpf (hour post fertilization) in *Catla catla.*

4. Discussion and conclusion

In general, the primordial germ cells in growing embryos are measured to be sexually bipotent. They differentiate into male germ cells or female germ cells in the developing gonads. In non-mammalian vertebrates, the sex reversal of gonads has been confirmed in both sexes [26]. In teleost fishes a complete sex reversal of gonads in both sexes can be stimulated by the administration of sex steroid hormones, and in some cases, by modulating environmental factors [27]. A key enzyme regulating the ratio of steroid hormones is Cyp19 (aromatase) which is the significant enzyme in the steroidogenic pathway. It converts androgens into estrogens. Therefore, suitable expression of this enzyme is vital for sex differentiation and reproduction in vertebrates. Most vertebrates have a single *CYP19* gene, however, the zebrafish has two genes (*cyp19a1a* and *cyp19a1b*) encoding different proteins. In universal, the gonadal form *cyp19a1a* is more abundant than the brain form *cyp19a1b*. The expression of the two *cyp19* genes has previously been detected from 0–41 days post fertilization which is the predictable time of sex determination and differentiation in zebrafish. Expression of *cyp19a1a* was uppermost shortly after hatch from 4–8 days post fertilization [28].

In the present study the expression analysis of *cyp19a1a* on the samples from induced breeding site shows that this gene is expressed from the 12th hour of post fertilization onwards and this expression is gradually increasing in different stages. This result points out that this gene has a significant role in the sex determination and sex differentiation. Expression of *cyp19a* was observed in unfertilised common carp eggs; however, the level of expression was lower than that observed in the whole ovary, consistent with the findings in zebrafish [29]. Expression of *cyp19a1a* was found in undifferentiated gonads of zebrafish at 17 dpf which is expected based on the juvenile ovarian gonads. However, at 31 dpf the differentiated ovary revealed the expression of *cyp19a1a* in cells surrounding the oocytes. These studies show the importance of this gene in early gonadal developmental stages of female.

The *Sox* gene family encodes an important group of developmental regulators occupied in sex determination. It has been shown that the *SRY*-related gene *Sox9* is necessary and sufficient to cause testicular differentiation in mammals [30]. The expression of *Sox9* during gonadal differentiation is up-regulated in testis and down-regulated in ovaries in mammals, birds and turtles [31]. However, the organisation and purpose of the Sox gene family is less understood in other types of vertebrates and in spite of the wide distribution of *sox9* genes in fish, only few have been investigated [31, 32]. In zebrafish, two *sox9* genes are present (*sox9a* and *sox9b*) and in adult zebrafish the *sox9a* transcript was observed in testis but not in ovary. Conversely, *sox9b* transcripts were detected in ovary, but not in testis [31]. In Nile tilapiais sex reversal is easily inducible using hormones, and sex-reversed males (XX) and supermales (YY) have been produced [33]. Consequently, all-male or all-female populations can be easily produced using these males. In tilapia the *sox9a* is expressed in XY gonads specifically after the appearance of sex differences in histological architecture, such as the formation of intra testicular efferent duct or ovarian cavity and also in mature gonads, *sox9a* is expressed in males but not in females [34].

In the present investigation *sox9a* is expressed from 30th hpf and the expression is increasing gradually. In previous studies Stickleback embryos at 32 hours post fertilization shows *sox9a* expression and zebrafish embryo approximately at the same stage of development shows strong *sox9a* expression. Findings shows that *sox9a2* expression in several stages during gonad development in medaka (*Oryzias latipes*) and at

30 days after hatching (dah), *sox9a2* expression could only be detected in developing testes but not in developing ovaries [35]. *Sox9* also plays a role in the female-to-male sex reversal [36]. When we compare the present study with the previous studies it reveals the importance of *sox9a* in the sex determination and sex differentiation of male.

In summary, this study is of major value for understanding the role of *cyp19a1a* and *sox9a* during sexual differentiation process of *C. catla*. In this experiment it is seen that the expression of these genes initiated in two different times. These results indicate that *cyp19a1a* and *sox9a* may have an important role in the early gonadal development. Out of these two genes *cyp19a1a* is expressed first. After 18 hours the other gene *sox9a* was expressed. This reveals that if the ovary specific gene *cyp19a1a* fails to be expressed, the testis gene *sox9a* remains will be expressed. This kind of expression pattern indicates the significant role of these genes in sex determination and sex differentiation during the time of early developmental stages of *C. Catla,* which would help to unlock the innovative fraction of research as gene manipulation programmed for increasing the mass production.

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