

Expression of SOX3 and SOX9 Genes in Gonads of Blue Gourami

Gad Degani^{1,2}

¹Faculty of Science and Technology, Tel-Hai Academic College, Kiryat Shmona, Israel

²MIGAL-Galilee Technology Center, Kiryat Shmona, Israel

Email: gad@migal.org.il

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Abstract

In vertebrates, SOX (SRY-related HMG box) genes are thought to be due to major gene duplication events, initially occurring during early stages of metazoan evolution and later during the transition between non-vertebrate chordates and vertebrates. The aim of this study is to examine SOX3 and SOX9 transcription in oogenesis and spermatogenesis in a fish model, the blue gourami (*Trichogaster trichopterus*). In females during oogenesis, SOX9 mRNA levels were lower compared to SOX3 mRNA levels. In males, SOX9 mRNA levels were higher in testes compared to SOX3 mRNA levels, however no significant differences between SOX3 and SOX9 mRNA levels were observed in the gonads of males that were kept under non-reproductive conditions compared to males kept with females under reproductive conditions and that were nest-builders.

Keywords

SOX3, SOX9, mRNA and Blue Gourami

1. Introduction

Many transcription factors of the SOX (SRY-related HMG box) family are critical for a number of developmental processes, most notably sex determination, neural crest development and neurogenesis [1] [2]). SOX genes are characterized by the presence of a single High Mobility Group box (HMG box), a 79 amino acid DNA-binding domain having affinity to the WWCAA motif [3]. These factors are generally expressed in a dynamic, tissue-specific manner, and often interact with other transcription factors [4].

Expansion in the number of vertebrate SOX genes is thought to be due to major gene duplication events, initially occurring during early stages of metazoan evolution and later during the transition between non-vertebrate chordates and vertebrates [5]. Gnathostomes (jawed vertebrates) have undergone two rounds of whole genome

duplications [6] whereas estimates of the number of rounds of duplication in agnathans like lamprey range from one to two.

SOX genes were first classified by [7] using partial sequences from mouse SOX genes. This study defined the six paralogous groups (A - F) that were the basis of the current classification [7]. Four more groups were subsequently added to include recently identified paralogs. Nevertheless, members of the same groups did not always have similar roles or expression patterns, indicating that recent paralogs could adopt new functions with relative ease [8] [9]. A comparative amino acid analysis between lamprey and other vertebrate SOX genes provides insight into the evolutionary history of early vertebrates and duplication events occurring early in the vertebrate lineage.

When SRY was identified, it was initially assumed to be unique to the Y chromosome. A search for SRY in kangaroos, however, identified a homolog on the X chromosome, termed SOX3. The sequence of the HMG-box in SOX3 most closely resembled that of SRY, so it was suggested that SOX3 was the ancestor of SRY [10]. Most other genes on the Y (for example, RBMY, TSPY), were subsequently found to have homologs on the X from which they had obviously evolved [11].

SOX3 was expressed strongly in gonads and the central nervous system, at least in mice and humans, but its deletion or duplication in human males affected fertility and intelligence rather than sex determination [12]. Its sequence similarity to SOX9 initially prompted the suggestion that SOX3 in its ancestor was originally a dosage-regulated inhibitor of SOX9 in females, and so a null mutant of SOX3 could have permitted activation of SOX9 and male development. Truncation of the null SOX3 allele was suggested to have subsequently turned it into an inhibitor of normal SOX3 and thus an activator of SOX9 [13]. However, it now seems more likely that SRY interacts with steroidogenic factor 1 (SF1) to activate SOX9 directly [13], suggesting that acquisition of a testis-determining function is due to a changed SOX3 expression pattern or to its association with different binding partners. SOX3 is also found on the sex chromosomes of two distantly related non-mammalian vertebrates, the frog *Rana rugosa* [14] and the fish *Oryzias dancena* [15]. However, it has not yet been shown to be sex-determining in either species, and is not known to be sex-linked in any other vertebrate species. Mouse knockouts indicate that SOX3 is involved in spermatogenesis and is a developmental regulator like SOX1 and SOX2, specifying neuronal fate in fish and mammals.

Notably zebrafish has duplicated SOX19 genes [16] whereas in most other teleosts for which SOX19 sequences are available, only a single copy appears to be present. Among vertebrates, orthologs in different species are highly similar to each other. Most of these groups are represented by a single gene in the invertebrate model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*, suggesting the expansion of this single gene into multiple related genes during vertebrate evolution [8]. The blue gourami (*T. trichopterus*) is a tropical fish. It serves as a useful model for studying the role of endocrine regulation on reproduction since it is a multi-spawner with a synchronic ovary development [17]-[19] [20].

Understanding the physiological perspectives of SOX3 and SOX9 gene systems on reproductive endocrinology will contribute to the general comprehension of basal mechanisms regulating reproductive activity and their relationships with other endocrine systems in non-mammalian vertebrate species.

2. Materials and Methods

2.1. Fish and Sampling

Trichogaster trichopterus (blue gourami) males and females purchased from local pet stores were separated into different containers and grown for about 2 - 3 months in aquaria at 27°C under a light regime of 12 h light: 12 h darkness, fed twice a day with commercial tropical fish food (TetraBits, 47.5% protein, 6.5% fat) and once a day supplemented with frozen live food (*Artemia salina*). Males were divided into two groups: (1) grouped mature males (isolated from females); and (2) individual mature males under reproductive conditions, including plants adapted for nest-building. Females were divided into three groups: (1) non-mature females (BW = 1.12 ± 0.37) (n = 10), the ovaries of which contained mostly oocytes at their previtellogenic stage; (2) mature females that were kept in groups without males (6.80 ± 1.14) (n = 10); and (3) mature females that were kept with nest-building males for 24 hours (7.15 ± 1.10) (n = 6). The latter showed oocytes at their final maturation stage.

Fish were sampled and the gonads (whole testis or small pieces of the ovary) were dissected. The follicles were then isolated accurately using forceps under a microscope to form an enriched sample of the following follicular stages: previtellogenic follicles (from non-mature females), vitellogenic oocytes (from mature grouped

females), and follicles at the final oocyte maturation stage (from mature paired females) [21]. The samples were kept in RNA save (Biological Industries, Israel) solution until RNA purification.

2.2. RNA Purification and cDNA Synthesis

Total RNA was extracted from female follicles ($n = 26$) using Tri-reagent (Invitrogen, CA), according to the manufacturer's recommendations. First-strand cDNA was synthesized using the Verso-Reverse-IT 1st Strand Synthesis Kit (ABgene, UK) from 0.5 - 2 μg total RNA, with an incubation of 1 h at 57°C, followed by 2 min at 94°C.

2.3. SOX3 and SOX9 Partial Cloning

Gene-specific primers of SOX3 gene were designed for cloning of the partial cDNA sequence based on the full-length sequence of *Lates calcarifer* (accession number DQ915951), *Epinephelus coioides* (accession number DQ219298), *Acanthopagrus schlegelii* (accession number EF605272) and *Amphiprion melanopus* (accession number EU908060) (Table 1).

Gene-specific primers of SOX9 gene were designed for cloning of the partial cDNA sequence based on the full-length sequence of *Epinephelus coioides* (accession number GQ232762), *Epinephelus akaara* (accession number AY676309) and *Oreochromis aureus* (accession number EU373500) (Table 1). The amplified PCR products were cloned into the pGEM-T vector (Promega, USA) that propagated in *Escherichia coli* cells. The recombinant plasmid was then extracted using the SV miniprep (Promega, USA), and the sequence of the amplified product was determined (HyLabs, Israel). Only one transcript of SOX3 and SOX9 was identified (Figure 1 and Figure 2, respectively). The polymerase chain reaction (PCR) was carried out in a total volume of 15 μL consisting of 0.166 μM of each primer and 17.5 μL of Absolute Blue SYBR Green ROX Mix (ABgene). The PCR products were visualized on a UV-transilluminator following electrophoresis on a 1.5% agarose gel containing ethidium bromide. To confirm the specificity of the PCR reaction, the identity of each PCR product was verified by sequencing (Figure 2).

2.4. Real-Time PCR

To compare mRNA levels of GnRH1, GnRH3, SOX3, SOX9, βLH and βFSH in the gonads of individual gourami, the relative abundance of their mRNA was normalized with the endogenous reference gene, the 18S subunit of rRNA (18S rRNA), using the comparative threshold cycle (C_T) method according to Pfaffl *et al.* [22]. The relative amount of each gene was calculated by the formula $2^{-\Delta C_T}$, where ΔC_T corresponds to the difference between the C_T measured for each target hormone and that determined for 18S rRNA. To validate this method, serial dilutions were prepared from a gonad (testis and ovary) cDNA sample and the efficiencies of each target gene and 18S rRNA amplifications were compared by plotting ΔC_T versus $\log(\text{template})$ according to the method of Muller *et al.* [23]. Linear regressions of the plots showed the following R^2 values and efficiency, respectively: 0.99 and 1 for GnRH1; 0.99 and 0.9 for GnRH3; 0.99 and 0.94 for 18S rRNA; 0.99 and 1 for βLH ; 0.99 and 0.87 for βFSH ; 0.99 and 1 for SOX3; and 0.99 and 0.94 for SOX9. Gene-specific primers for real-time PCR were designed using Primer3 Software. Table 2 summarizes the primers used in the real-time PCR. To each of the above PCR mixtures were added 7.5 μL of the Syber Green Master Mix (ABgene) in a final volume

Table 1. Nucleotide sequences of primers for partial cloning for SOX3 and SOX9 genes.

Gene	Primer Name	Sequence 5'-3'	PCR Product Length
SOX3	SOX3(fw)300	CCGGATTATAAATACCGTCC	552 bp
	SOX3(rev)854	GTGCGGATGGACGCTGTGAACCG	
SOX3	SOX3(fw)145	CAGCGGAGGAAGATGGCTCA	618 bp
	SOX3(rev)775	CTTATCATATCCCTTCAGGTC	
SOX9	SOX9(fw)10	CTCGACCCTTACCTGAAGATGAC	339 bp
	SOX9(rev)359	TGAGCCCACACCATGAATGCGTTCAT	

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1 AGCGGGAGGAAAGATG GCTCAAGAGAATCCC AAAATGCACAACCTCT GAGATCAGCAAAGCGG CTCGGCGCCGACTGG
1 S G R K M A Q E N P K M H N S E I S K R L G A D W
76 AAATTCTGACCGAC GCCGAAAAGAGGCGC TTCATCGACGAGGCC AAGCGTCTACGCGCC ATGCACATGAAGGAG
26 K L L T D A E K R P F I D E A K R L R A M H M K E
151 CATCCGGATTATAAA TACCGTCCCGGCAGG AAGACCAAGACCCTG CTCAAGAAAAGACAAG TACTCCCTGCCCGGA
51 H P D Y K Y R P R R K T K T L L K K D K Y S L P G
226 GGACTGCTGGCGCCA GGAGCCAAACGCCGTC AACAACTCGGTGTCG GTGGGGCAGCGCATG GACGGTTACGCGCAC
76 G L L A P G A N A V N N S V S V G Q R M D G Y A H
301 ATGAACGGCTGGACG AACAGTGCCTACTCC CTCATCGACGAGCCAG CTGGCGTACCCTCAG CATCACAGCATGAAC
101 M N G W T N S A Y S L M Q D Q L A Y P Q H H S M N
376 AGCCCCCAGATCCAG CAGATGCACCCGGTAC GAGATGGCGGGGCTC CAGTACCCGATGATG TCCTCGGCGCAGACG
126 S P Q I Q Q M H R Y E M A G L Q Y P M M S S A Q T
451 TACATGAACGGCGCT TCCAGTACAGCATG TCCCGGCGGTACAG CAGCAGAGCACCAAC GCCATGGCTGTAGC
151 Y M N A A S T Y S M S P A Y T Q Q S T N A M G L S
526 TCCATGGCGTCCGTG TGCAAGACCGAGCCC AGCTCGCCACCCGCG GCCATTACGTCCCAC TCTCAGCGGGCGTGT
176 S M A S V C K T E P S S P P P A I T S H S Q R A C
601 TTGGGGCACCTGAGG GATATGATAAGCATG TACCTGCTCCCGGC GGGGACAGCGCGGAG CATTCTCCTCGCAG
201 L G D L R D M I S M Y L P P G G D S A E H S S L Q
676 AGCAGCCGGTTCACA GCGTCCATCCGCACA
226 S S R F T A S I R T

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(a)

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1 CTCGACCCTTACCTG AAGATGACAGAAGAA CAGGAGAAGTGTAC TCCGACGCTCCAGC CCAAGCATGTCTGAG
1 L D P Y L K M T E E Q E K C H S D A P S P S M S E
76 GACTCCGCAGGCTCG CCGTGCCCGTCCGGG TCCGGCTCGGACACT GAGAACACTCGGCCG TCCGACAACCACCTC
26 D S A G S P C P S G S G S D T E N T R P S D N H L
151 CTCGGGGTCCAGAC TACAAGAAAAGAGGC GAGGAAGAGAAGTTC CCGTGTGEATACGG GACGCGTCTCCAG
51 L G G P D Y K K E G E E E K F P V C I R D A V S Q
226 GTGTTGAAGGGTTAC GACTGGACGCTGGTG CCAATGCCGGTGC GC GTCAACGGCTCCAGT AAAAGCAAAGCCTCAC
76 V L K G Y D W T L V P M P V R V N G S S K S K P H
301 GTCAAAAAGACCCATG AACGCATTCATGGTG TGGGCTCAA
101 V K R P M N A F M V W A Q

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(b)

Figure 1. SOX3 (a) and SOX9 (b) partial nucleotide and deduced amino acid sequences. —localization of primers for cDNA cloning. —localization of primers used for real-time PCR.

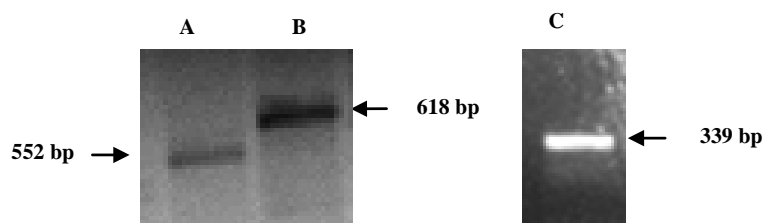


Figure 2. Detection of SOX3 and SOX9 transcription in blue gourami RT-PCR. Total RNA from gourami testis or ovary was extracted, reverse transcribed and amplified by PCR. PCR products were separated on 1.5% agarose gel containing ethidium bromide. A: SOX3 partial fragment (primer sets: SOX3(fw)300 and SOX3(rev)854); B: SOX3 partial fragment (primer sets: SOX3(fw)145 and SOX3(rev)775); C: SOX9 partial fragment (primer sets: SOX9(fw)10 and SOX9(rev)359).

of 15 μ l. Amplification was carried out in a RotorGene 3000 Sequence Detection System (Corbett Research, Sydney, Australia) under the following conditions: for GnRH1—initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 52°C for 20 s, 72°C for 20 s and 82°C for 115 s; for GnRH3—initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 53°C for 20 s, 72°C for 20 s and 82°C for 10 s; for β LH—initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 25 s and 84°C for 15 s; for β FSH—initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for

Table 2. Nucleotide sequences of primers for real-time PCR.

Gene	Primer Name	Sequence 5'-3'	Reference
βFSH	GFSHexfor1	GTTGTCATGGCAGCAGTGT	Levy <i>et al.</i> , 2009
	GFSHexrev1	CCTCGTGGTAGCAATGTCCT	
βLH	GLHexfor1	CCTGACTGTCTCTCTGGTGT	Levy <i>et al.</i> , 2009
	GLHexrev1	TTTGCTTTTGGTTTGGCTGTG	
GnRH1	Expsbfw	TCCAGGAGGAAAGAGGGGTCTGGA	Levy <i>et al.</i> , 2009
	Expsbrev	TGCGTCCATTTCCTCTGTCAAGTGT	
GnRH3	sal48fw	TGGAGGCGCGAAGCAGAG	Levy <i>et al.</i> , 2009
	sal244rev	CTCTTGGGTTTGGGCACTT	
18S	expG18Sf	CCGTCGTAGTTCGACCATA	Levy <i>et al.</i> , 2009
	expG18Sr	CCCTTCGGTCAATTCCTTTA	
SOX3	SOX3(fw)400	GTCAACAACCTCGGTGTCGGT	
	SOX3 (rev)563	CATCATCGGGTACTGGAGCC	
SOX9	SOX9(198)fw	CGAGGAAGAGAAGTCCCGTGT	
	SOX9(359)rev	TGAGCCCACACCATGAATGCGTT	

10 s, 57°C for 20 s and 72°C 20 s; for SOX3—initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 63°C for 20 s, 72°C for 20 s and 85°C for 15 s; for SOX9—initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 63°C for 20 s, 72°C for 25 s and 85°C for 10 s; and for 18S rRNA—initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 20 s, 64°C for 20 s and 71°C for 20 s. Amplifications of each target gene and the reference gene 18S rRNA cDNAs were performed simultaneously in separate tubes in duplicate, and the results were analyzed using Q-Gene software (BioTechniques Software Library at: www.BioTechniques.com). Dissociation-curve analysis was run after each real-time experiment to ensure that there was only one product. To control for false positives, a non template negative control was run for each primer pair.

2.5. Statistical Analysis

Data are presented as mean ± SEM. The significance of the differences between group means of hormone mRNA levels was determined either by a one-way analysis of variance (ANOVA) followed by an a posteriori Bonferroni post-hoc test or a student T-test in males using SPSS 17.0 software. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. SOX3 and SOX9 Gene Expression in the Gonads of Blue Gourami

In order to determine if SOX3 and SOX9 are expressed in the gonads of blue gourami, total RNA from the organs of gourami fish was reverse-transcribed. Reactions using cDNA derived from ovary RNA samples using two sets of primers specific to partial segments of the gourami SOX3 revealed two bands: 552 bp and 618 bp. One primer set of primers specific to a partial SOX9 segment revealed a 338 bp band (**Figure 2**). Similar results were obtained from samples derived from testis (data not shown).

3.2. Variations in SOX3 and SOX9 mRNA Levels in Oocytes during Different Stages of Oogenesis

SOX9 mRNA levels were lower in oocytes compared to SOX3 mRNA levels (33.24 fold) ($P < 0.05$, by student

t-test) (**Figure 3(a)**). Lower levels of SOX3 were measured in vitellogenic and FOM oocytes compared to previtellogenic oocytes (11- and 52-fold, respectively) (**Figure 3(b)**), whereas lower levels of SOX9 were measured in vitellogenic oocytes compared to previtellogenic oocytes (3.3-fold) (**Figure 3(c)**) ($P < 0.05$, by ANOVA, Bonferroni post-hoc test). However, the level of SOX9 transcription in ovary found in FOM was significantly higher than during vitellogenesis.

3.3. SOX3 and SOX9 mRNA Levels in the Testis of Blue Gourami

In males, SOX9 mRNA levels were higher in testes compared to SOX3 mRNA levels (9.21-fold) ($P < 0.05$, by student t-test) (**Figure 4(a)**). However, no significant differences between SOX3 and SOX9 mRNA levels were observed in the gonads of males that were kept in a group compared to males kept with females and that were nest-builders ($P < 0.05$, student T-test)

4. Discussion

In the current study, the transcription of both SOX3 and SOX9 was found in both testis and ovary, but at different levels. The SOX gene signature domain HMG was highly conserved across different vertebrates. Recent studies in teleosts such as zebrafish, medaka, rice field eel and carp also reported two forms of SOX9 [24]-[27].

In the current study, the transcription of SOX9 was high in males and that of SOX3 in females. Moreover, the level of both transcription factors SOX3 and SOX9 in testis changed during oogenesis and the sexual behavior of females but not of males. In catfish, semi-quantitative RT-PCR analysis in adult gonads revealed sexual

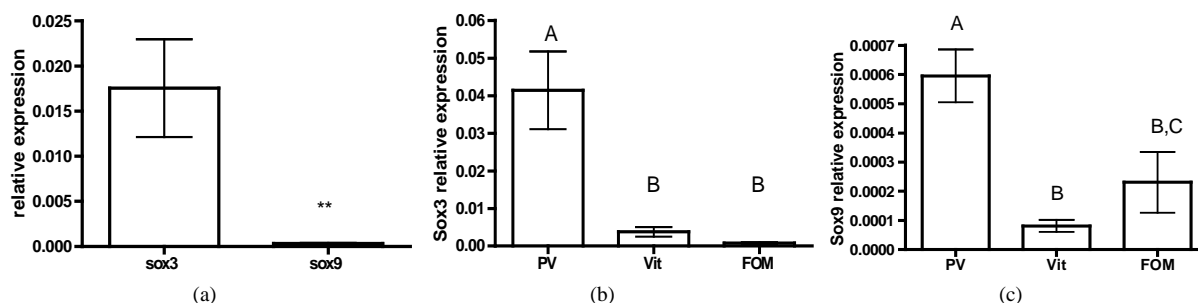


Figure 3. The expression of SOX3 and SOX9 genes in follicles of females (a) and in oocytes at each of the following stages of oogenesis: previtellogenesis (PV), vitellogenesis (Vit) and final oocyte maturation (FOM) (b, c). Females were kept in groups or with males for one day. Total RNA was extracted and reverse-transcribed to cDNA, which was used as a template for real-time PCR. SOX3 and SOX9 mRNA levels were normalized to that of 18S rRNA by the C_T cycle method, where $2^{-\Delta C_T}$ reflects the relative amount of specific gene precursor transcripts. Each histogram represents the average of independent measurements (mean \pm SEM; $n = 6 - 26$). A different letter above the histogram denotes its significant difference from the mRNA levels of the other histograms [$P < 0.05$ student t-test (a) or ANOVA and Bonferroni post-hoc test (b, c)].

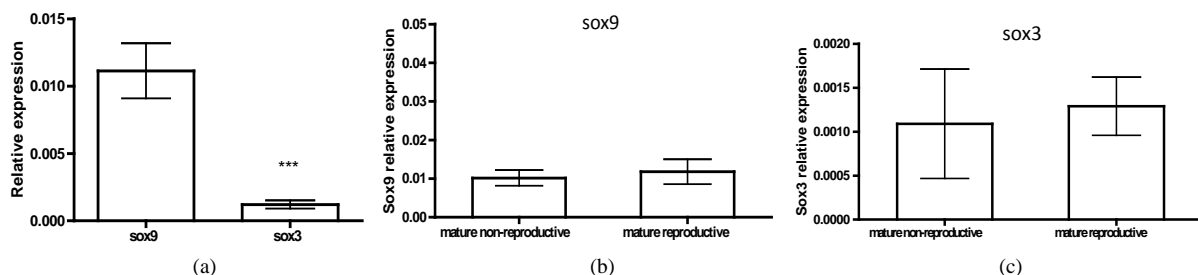


Figure 4. The expression of SOX3 and SOX9 genes in testis (a) and mature males under non-reproductive and reproductive conditions (paired with females) (b, c). Total RNA was extracted and reverse-transcribed to cDNA, which was used as a template for real-time PCR. SOX3 and SOX9 mRNA levels were normalized to that of 18S rRNA by the C_T cycle method, where $2^{-\Delta C_T}$ reflects the relative amount of specific gene precursor transcripts. Each histogram represents the average of independent measurements (mean \pm SEM; $n = 6 - 15$). A different letter above the histogram denotes its significant difference from the mRNA levels of the other histograms [$P < 0.05$ student t-test (a) or ANOVA and Bonferroni post-hoc test (b, c)].

dimorphic expression of SOX9a and SOX9b in adult and developing gonads, whereas SOX9a was expressed preferably in testis, while SOX9b was expressed abundantly in ovary [24]. This type of differential expression pattern of duplicated genes might attribute to more flexible gene regulation and function during catfish development. A similar kind of expression pattern was also noticed in zebrafish using PCR Southern and *in situ* hybridization [28].

Most studies of those genes examined the mRNA levels of SOX3 and SOX9 during sex determination and differentiation, but not during spermatogenesis and oogenesis, as was done in the current study [29]. The results of this study are in agreement with a previous finding that SOX9 is present in both sexes, but in one of the first molecular events in the male pathway is upregulated in Sertoli cell precursors (which are essential for testis differentiation) by the primary sex-determining trigger interacting with an evolutionarily conserved sequence within SOX9 [29]. In mammals, the SOX8 and SOX9 transcription factors are involved, among other things, in sex differentiation, male gonad development and adult maintenance of spermatogenesis [30].

The expression level of the SOX9 gene varied among several organs of adult *Cyprinus carpio*, being highest in the brain and testis [27]. Similar structure and identity of SOX9 and SOX17 genes in mammals, chickens and fish suggest that these genes have evolutionarily conserved roles, potentially including sex determination and differentiation [31]. Based on the current study, we suggested that SOX9 genes were involved in spermatogenesis. In this study, it was found that the level of SOX9 transcription was high in mature males before sexual behavior and reproduction. A high level of sperm was found in the testis in both these stages [32], and spermatogenesis was controlled by gonadotropins (FSH and LH) and androgens hormones [20] [33]. Raghuvveer and Senthikumar [34] observed that in breathing catfish *Clarias gariepinus*, which underwent an annual reproductive cycle, a dimorphic expression pattern of SOX9a and SOX9b was found in both adult and developing gonads using RT-PCR, indicating that SOX9a retained its function in testis while SOX9b might play a new role in ovary, as was revealed in the current study in blue gourami using real-time PCR.

In the current study, it was found that the levels of both SOX9 and SOX3 were high in the ovary where the oocytes were found in PV compared to VT and FOM. In rainbow trout ovary, on the basis of the HMG box amino acid sequence, SOX24 can be categorized into the same subgroup of SOX proteins as SOX4, SOX11 and SOX22. These results suggest that SOX24 plays a role as a transcriptional regulator during oogenesis [35]. Our results regarding the change of mRNA in PV compared to VT and FOM as was found in blue gourami are in agreement with the result for SOX9 transcription in oogenesis in catfish [34].

In mature female catfish, SOX9 expression was restricted to the perinucleolar stage of developing oocytes and pre-vitellogenic oocytes, but not in mature oocytes. Raghuvveer and Senthikumar (2010) suggested that the decrease in SOX9 transcript in mature oocytes might be due to the dilution of transcripts with increasing mass/size of mature oocytes that accumulated large amounts of vitellogenin and maternal RNAs. These results combined indicated that SOX9 might play an important role in early gonadal development and recrudescence.

5. Conclusion

In conclusion, it is suggested that SOX genes that are involved in sex differentiation where cell structure and function change are found during oogenesis and spermatogenesis might be involved similarly as during sex differentiation in fish.

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