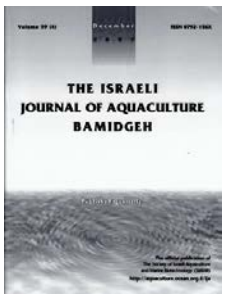

Expression of Sox5, Sox8 and Sox9 Genes in Golden Pompano *Trachinotus ovatus* (Linnaeus 1758) Larvae During Ontogeny and in Response to Water Temperature

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Keywords: ontogeny; temperature; sox genes; gene expression; golden pompano larvae

Abstract

Sox5, Sox8, and Sox9 genes in golden pompano larvae were cloned and analyzed in this study. In the first trial, the expression of Sox genes during ontogeny of fish larvae in the first 18 days was examined, after which the expression of Sox genes in tissues was evaluated on 18 days post hatching (DPH). Subsequently, the response of Sox genes to water temperature (23, 26, and 29°C) on day 12 DPH and 18 DPH were compared. The expression levels of Sox8, Sox9a, and Sox9b peaked around 2 DPH before first feeding during fish ontogeny, and remained at low levels from 3-18 DPH. The expressions of Sox5 and Sox9a were affected by water temperature on 12 DPH and 18 DPH, while the expression of Sox8 was not affected by water temperature on 12 DPH and 18 DPH. The expression of Sox9b was affected by water temperature on 12 DPH. This study detected gene expression of Sox5, Sox8, and Sox9 at the early stage of development of golden pompano larvae. The time dependent expression of Sox genes in fish larvae is important for the understanding of bone ontogeny of fish larvae in early life.

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Introduction

The Sox gene family was initially recognized in 1990 as a set of genes correlated to the mammalian testis determining factor Sry based on conservation of the single HMG box, which encodes a 79-amino acid DNA-binding HMG domain (Gubbay et al., 1990). Research has revealed that the Sox gene family is a group of transcription factors involved in numerous developmental processes and sex determination in vertebrates (Hett and Ludwig, 2005; Wegner, 2010). Sox genes demonstrate a wide spectrum of temporal and spatial expression patterns during early development of vertebrates. They appear to be important in cellular development in numerous developmental processes (Fukada et al., 1995; Russell et al., 1996; Wright et al., 1993).

Based on results of sequence comparisons, Sox proteins have been classified into 6 subgroups (SoxA, B, C, D, E, and F). Sox proteins in the same group have similar biochemical properties and tend to function redundantly when co-expressed. In contrast, Sox proteins from different groups usually perform different functions even when co-expressed (Wegner 2010). SoxD proteins, including Sox5, Sox6, and Sox13, possess a leucine zipper and a coiled-coil domain causing them to form constitutive dimers in solution, interacting with a spliceosomal complex (Wegner, 2010). Sox5 is involved in the regulation of embryonic development and in the determination of the cell fate. The encoded protein may act as a transcriptional regulator after forming a protein complex with other proteins. Sox5 gene expressed during spermatogenesis and chondrogenesis, and the involvement of Sox5 in chondrogenesis, was supported by interaction with Sox9 (Denny et al., 1992; Lefebvre et al., 1998).

Sox8 and Sox9 genes belonging to the SoxE group have been examined over the past 20 years to assess their possible involvement in physiological development. Sox8 was initially proposed as a regulator of male sex determination, testicular differentiation, or germ cell development (Schepers et al., 2002; Schepers et al., 2003). However evidence has demonstrated that the interference of Sox8 can cause poor tarsal development and low bone density (Schmidt et al., 2005; Sock et al., 2001). Although, Sox8 is expressed in fetal CNS, brain, branchial arches, limb, heart, dorsal root ganglia, and testes, there is no direct functional evidence (Pfeifer et al., 2000; Schepers et al., 2000). Sox9 functions in the development of cartilage, bone, placodes, and crest (Wegner, 2010; Yan et al., 2004). Sox9 promotes crest-like behaviors in neural plate cells and biases cells towards glial and melanocyte fates, helps determine crest-derived chondrogenic lineage, and functions in morphogenesis and differentiation of cartilage and bone (Cheung and Briscoe, 2003; Mori-Akiyama et al., 2003; Zelzer and Olsen, 2003).

During the larval phase, marine teleosts undergo rapid development along with dramatic changes in morphology, metabolism, and metamorphosis into juvenile stage. Regulation of gene expression is widely thought to be a key mechanism underlying the management of biological processes required for healthy development throughout this phase of life (Darias et al., 2008). Golden pompano belongs to the family of Carangidae and is a good candidate species for aquaculture due to its rapid growth and suitability for culture. Information on Sox genes in golden pompano is scarce. As the primary functional gene in fish development, understanding the expression of Sox genes during fish ontogeny will improve knowledge of fish development. This study was designed to explore the expression of Sox genes during the skeleton ontogeny of golden pompano larvae in the first 18 days post-hatch (DPH), and the response of Sox genes to water temperature on 12 and 18 DPH. The expression pattern of Sox genes may provide essential information on osteogenesis of golden pompano larvae. Such knowledge should improve our understanding of bone formation in golden pompano, and provide potential indicators for prevention of skeleton malformation in hatchery rearing of this species.

Materials and Methods

Experiment 1: Expression of Sox genes during the first 18 days of golden pompano larvae development. Fertilized eggs of golden pompano were obtained from Guanghui Aquaculture Hatchery, Hainan Provence, P.R. China, transported to Lingshui Town and hatched in 500 L fiberglass incubators at 26.5^{o}C with a hatching rate of 97.5 ± 1.5% (mean ± SD). On 2 DPH, larvae were stocked into three 1000 L larval rearing tanks.

Larval rearing tanks were supplied with filtered seawater (5 µm pores) from the bottom of each tank through upwelling with a daily exchange rate of 200% tank volume. Water was discharged through an outlet screen (300 µm) on the upper side of each tank, and the screen was cleaned daily to reduce clogging. Two air stones were used in each tank to maintain dissolved oxygen close to saturation level. Light intensity was maintained at 2,400 lux, and the light regime was controlled at 14 h light:10 h dark. Salinity was maintained at 33±0.8 ‰ and rearing temperature was 26.5 ± 1.0^{o}C throughout the experiment.

Rotifers *Brachionus rotundiformis* at a density of 10-20 ind/ml were used to feed the larvae from 2 DPH to 10 DPH. Rotifers fed with baker yeast were enriched with DHA protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) for 12 h before they were added into the larval rearing tanks. Instant microalgal paste (*Nannochloropsis* sp.) was also added into larval fish tanks to create a green-water background. *Artemia* nauplii were first introduced at 0.1 nauplii/mL on 10 DPH, and then added with daily increments of 90%. After five days co-feeding, *Artemia* nauplii were gradually phased out at a daily reduction of 20% until the co-feeding period ended. *Artemia* nauplii were enriched with DHA Protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) following the manufacturer's instruction.

Experiment 2: Response of Sox genes to rearing temperature.

Fertilized eggs of the same batch were obtained from Lingshui, Hainan Province, and transported to the Tropical Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Xincun Town. Upon arrival, all eggs were transferred to 500 L incubators and hatched at 26^{o}C. Three constant temperatures 23, 26, and 29^{o}C were tested with three replicates each. On 2 DPH, yolk sac larvae were acclimatized at each desired temperature for 5 h, and then stocked in 500 L fiberglass tanks at a density of 60 fish/L. Apart from the rearing temperature, all feeding protocols and rearing conditions were the same as experiment I.

Total RNA extraction and reverse transcription. On 0, 1, 2, 3, 4, 5, 12, and 18 DPH, approximately 300 mg (wet weight) fish larvae were sampled from rearing tanks in triplicate. 50 individuals were collected in triplicate on 18 DPH. In the tissue expression assay, a total of 100 individuals were collected in triplicate, and dissected under a dissecting microscope for tissue expression analysis. Total RNA was extracted using TRIzol (Invitrogen, USA). RNA integrity was verified by electrophoresis on a formaldehyde-agarose gel (1.2%). RNA concentration was measured at absorbance of 260 nm and purity was determined at OD 260/280 ratio and agarose gel electrophoresis. RNA was reverse-transcribed to cDNA with oligo (dT) primers using a PrimeScript 1st strand cDNA synthesis kit (TaKaRa Biotechnology, Dalian Co., Ltd). The cDNA was used as a template in subsequent PCR.

Cloning of the gene cDNA and real-time PCR. Based on unpublished golden pompano transcriptome sequences (Illumina HiSeq2000, annotated by NR, KOG, kegg, and Swissprot), the primers for gene cloning were designed with Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) based on golden pompano sequence data previously measured in our laboratory (Table 1). The PCR reaction systems were: 1 µL of golden pompano larval cDNA, 1 µL of gene-specific forward primer (F), 1 µL of gene-specific reverse primer(R), 0.5 µL of ExTaq, 5 µL of PCR buffer, 4 µL of dNTP mixture (2.5 µM), 37.5 µL of ddH_2O, in a total volume of 50 µL. The PCR conditions were: denaturation at 94^{o}C for 1 min, 35 cycles of 94^{o}C for 30 s, annealing temperature of each gene for 30 s, 72^{o}C for 4 min, followed by a 10 min extension at 72^{o}C. PCR products were cloned into PMD-19T vector (TAKARA, Japan), and sequenced.

Quantitative real-time PCR (qPCR) was used to analyze the expression levels of Sox genes in golden pompano larvae. Gene specific primer pairs for Sox genes (Table 1) were amplified in LightCycler480 II (Roche, Switzerland). EF-1α was used as the internal reference and amplified. Cycling conditions for Sox genes and EF1α were: 1 min at 95^{o}C, followed by 40 cycles at 95^{o}C for 15 s, and at 60^{o}C for 1min. Dissociation curves were employed to ensure that only one single PCR product was amplified in each gene reaction. Three replicates were performed for each test. Relative quantification (RQ) was

calculated using $^{\Delta\Delta}$CT (comparative threshold cycle) method ($^{\Delta}$CT = CT of target gene - CT of EF-1α, $^{\Delta\Delta}$CT = $^{\Delta}$CT of any sample - $^{\Delta}$CT of calibrator sample). Efficiencies of the primers (E) were E_{Sox5} = 0.999, E_{Sox8} = 1.003, E_{Sox9a} = 1.002, and E_{Sox9b} = 0.998.

Table 1 Oligomeric primers used in PCR and real-time PCR

Name	*Gene*		*Primer sequence (5'-3')*	*Products (bp)*
SOX5-F	Sox5	Sense	CCCTCGGAGCATTCAAGC	129
SOX5-R		Anti	ACTGTCGTCCAGCCGTAGC	
SOX8-F	Sox8	Sense	AGTCCATCAGGCACAAACAGC	203
SOX8-R		Anti	GGCACCAAGGACCAGTCGTAT	
Sox9a-F	Sox9a	Sense	GGACACCGAGAACACCCG	143
Sox9a-R		Anti	GCACCAGCGTCCAGTCGT	
Sox9b-F	Sox9b	Sense	TCTCCCACATTGAGACCTTTGA	189
Sox9b-R		Anti	CCTGCTGGTTCTGGCTTTTAG	
EF1a-F	EF1a	Sense	CCCCTTGGTCGTTTTGCC	101
EF1a-R		Anti	GCCTTGGTTGTCTTTCCGCTA	

Statistical analysis. Data were all expressed as mean ± SD, and compared with one way ANOVA (PASW Statistics 18.0, Chicago, SPSS Inc.). Tukey's test was used for multiple range comparisons with the level of significant difference set at $P<0.05$. All data were tested for normality, homogeneity, and independence to satisfy the assumptions of ANOVA.

Results

Expression of Sox5, 8, 9a, and 9b during ontogeny. At hatching, expression of Sox 5 was nearly 2 times higher than the expression level on 5 DPH (Fig. 1). On 1 DPH, the expression of Sox5 increased significantly ($P<0.05$), and rose on 2 DPH. On 3DPH, the expression of Sox 5 was similar to 2 DPH. On 4 DPH Sox5 reached the highest level after which expression of Sox 5 remained at low levels until 18 DPH. The expression of Sox8 increased sharply from 0 DPH to 1 DPH, and the highest expression ($P<0.05$) of Sox8 was observed on 1 DPH. After that expression of Sox8 remained low until completion of experiment.

Expression of Sox9a reached the highest level on 1 DPH, and remained there on 2 DPH after which expression of Sox9a dropped significantly ($P<0.05$) from 2 DPH to 18 DPH. The lowest expression of Sox9a was observed on 12 and 18 DPH ($P<0.05$). Expression level of Sox9b was low at hatching, and sharply increased from 0 DPH to 2 DPH. The highest expression level of Sox9b was observed on 2 DPH ($P<0.05$). Afterward, expression level of Sox9b dropped sharply, and was not significantly different from 3 DPH to 5 DPH ($P>0.05$). On 12 DPH, the expression level of Sox 9b reached the lowest level ($P>0.05$) and remained so until the end of the experiment (see Fig. 1).

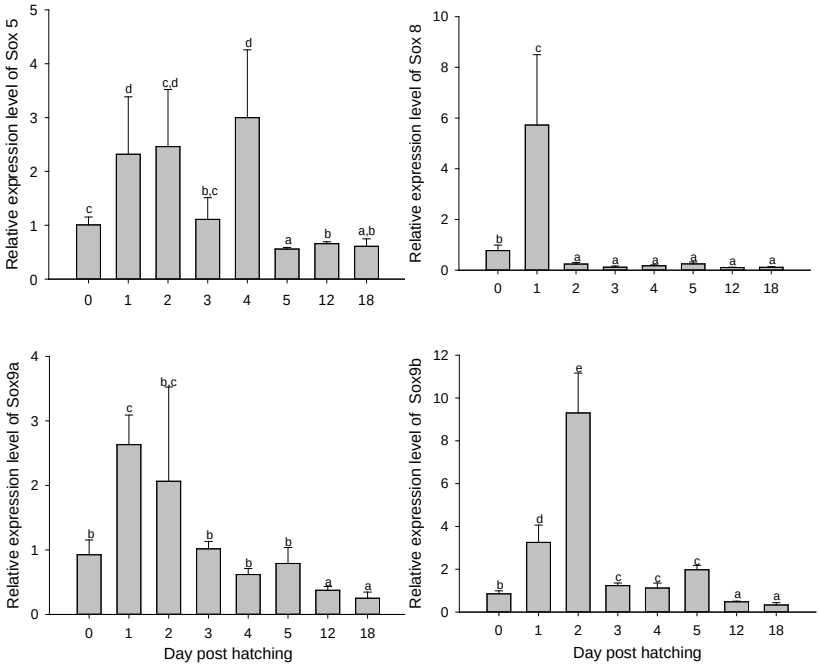

Fig. 1 Relative expression levels of Sox 5, 8, 9a, and 9b during the early development of golden pompano *T. ovatus*. Data with different letters were significantly different (P<0.05).

Expression of Sox5, 8, 9a, and 9b in fish tissues. The highest expression of Sox5 was observed in fish heart and brain on 18 DPH. An intermediate expression level of Sox5 was recorded in fish gills, liver, and intestine. The expression level of Sox5 in the fish eye, head-kidney, flesh, spleen, stomach, and kidney was low. The highest expression of Sox8 was observed in fish kidney (P < 0.05), followed by the liver. The lowest expression level of Sox8 was observed in eyes, gills, spleen, and stomach (Fig. 2).

The highest expression of Sox9a was observed in fish spleen on 18 DPH (P < 0.05). The expression level of Sox9a in head-kidney, liver, and stomach was not significantly different (P > 0.05). The lowest expression level of Sox9a was observed in the fish eye, flesh, and kidney. On 18 DPH, the highest expression level of Sox9b was observed in the fish spleen, and followed by head-kidney, liver, and stomach (Fig. 2). The lowest expression level of Sox9b was observed in the fish eye and flesh.

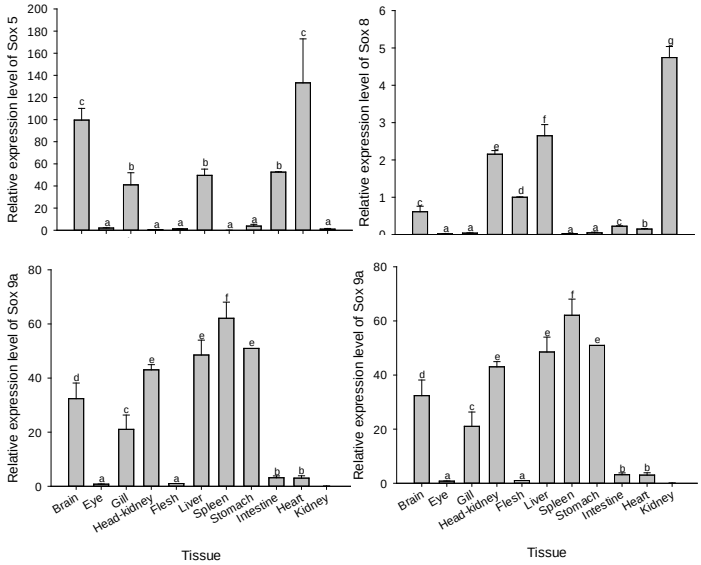

Fig. 2 Relative expression levels of Sox 5, 8, 9a, and 9b in the tissues of 18 DPH golden pompano *Trachinotus ovatus*. Data with different letters were significantly different (P < 0.05).

Response of Sox5, 8, 9a, and 9b to water temperature. The expression of Sox5, 9a and 9b was significantly affected by rearing temperatures (P < 0.05, Fig. 3). On 12 DPH, the highest expression of Sox5 was observed at 26°C, and the lowest at 29°C. The highest expression of Sox9a was observed at 29°C, and the lowest at 23°C (P < 0.05).

On 12 DPH, the highest expression of Sox9b was observed at 23°C, but was not significantly different between 26 and 29°C ($P > 0.05$).

On 18 DPH, the expression of Sox5 and Sox9b followed a similar pattern of highest expression at 23°C ($P < 0.05$, Fig. 3). This expression was not significantly different between 26 and 29°C ($P > 0.05$). The expression of Sox9a at 23 and 29°C was not significantly different ($P > 0.05$), but was significantly higher ($P<0.05$)than at 26°C (Fig. 3).

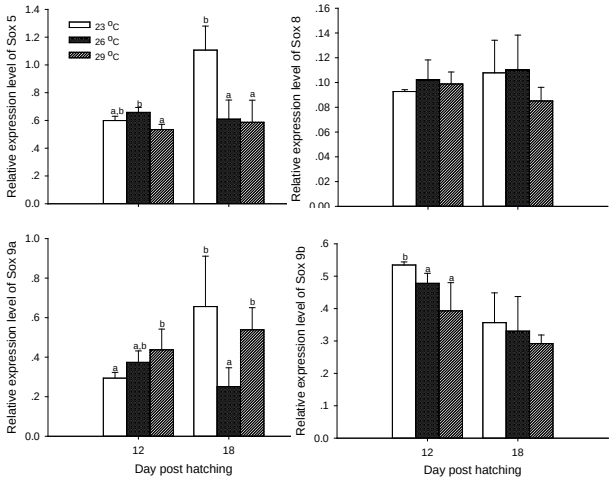

Fig. 3 Response of Sox 5, 8, 9a and 9b to the water temperature of 23, 26, and 29°C on 12 DPH and 18 DPH in golden pompano *Trachinotus ovatus* larvae. Data with different letters were significantly different ($P < 0.05$).

Discussion

Sox5 which belongs to the SoxD subgroup is primarily expressed in cartilage, and its expression has been associated with chondrocytes (Lefebvre et al., 1998). Rescan and Ralliere (2010) suggested a hitherto disregarded role for Sox5 in regulating myogenic cells involved in muscle formation and growth. In the present study, a higher expression of Sox5 was observed in the first 4 days after hatching. During this period, important organs were formed in larval golden pompano (Ma et al., 2014). Higher expression of Sox5 within this period may be consistent with the formation of these organs. The transcript of Sox5 has been found in the brain, kidney, lung, and skeletal muscle in humans (IKEDA et al., 2002). Most existing studies on Sox5 have focused on the embryonic development of fish. There is little information regarding tissue expression of Sox5 in fish larvae. In the present study, the highest expression of Sox5 was observed 18 DPH in the fish heart, as well as in the brain, gills, liver, and intestine. Higher expression of Sox5 in these organs may indicate fast growth of these organs (Rescan and Ralliere, 2010).

Sox8 is a member of the SoxE subgroup. It exhibits a highly dynamic expression pattern that occurs in a wide range of cell types and tissues predominantly derived from ectoderm or mesoderm (Pfeifer et al., 2000; Schepers et al., 2003; Sock et al., 2001). In the present study, the expression of Sox8 sharply increased in the first 2 DPH, and maintained a low level from 3 DPH to 18 DPH. Sox8 expression reportedly occurs during osteoblast differentiation (Schmidt et al., 2005). Higher expression of Sox8 in golden pompano larvae in the first 2 DPH is correlated with large amounts of osteoblast differentiation in fish larvae at post embryonic stage. The expression profiles of Sox8 have been reported in humans, mice, chicken, trout, and mud loach (Schepers et al., 2003; Xia et al., 2011). In chickens, Sox8 gene is expressed in the developing heart, pancreas, enteric neuron system, limbs, and the neural tube (Bell et al., 2000). In rainbow trout, Sox8 is mainly detected in the brain, ovary, and testis, and less in other tissues (Ito et al., 1995). Most of these publications focus on embryos or adults. Information on the expression Sox8 in the tissue of larval fish is rare. In the present study, the highest expression of Sox8 in larval golden pompano was observed in fish kidney, followed by liver, head-kidney, flesh, brain, and intestine on 18 DPH. It is unclear why a significantly higher expression was observed in the fish kidney, and this may warrant further investigation.

Evidence has indicated that the expression of Sox5 and Sox6 is dependent on Sox9 in developing chondrocytes (Akiyama et al., 2002). In the SoxE subgroup, expression patterns of Sox9a and Sox9b have been reported in several fish species including zebrafish, pufferfish, stickleback, and medaka (Chiang et al., 2001; Kluver et al., 2005; Koopman et al., 2004; Yokoi et al., 2002). The ontogenetic expression of Sox9a and Sox9b in larval golden pompano was similar in the first 18 days of development. At hatching, expression of both Sox9a and Sox9b increased sharply, reaching its peak level on 3 DPH, but dropped to low levels from 5 DPH. Such expression patterns are consistent with ossification time in larval golden pompano during osteological development. Major vertebral ossification processes of golden pompano larvae were initiated from 7 DPH (Zheng et al., 2014).

Information on tissue expression of Sox9 in fish is limited to adults and embryos, and expression pattern depends on species and developmental stage. For instance, in zebrafish, Sox9a is expressed in the adult testis while Sox9b is expressed in adult ovary (Chiang et al., 2001). In medaka, Sox9a is expressed predominantly in the ovary (Kluver et al., 2005). In the present study, tissue expression patterns of Sox9a and Sox9b at 18 DPH larval golden pompano were similar. Highest expression was observed in the fish spleen, followed by head-kidney, liver, and stomach, but lower in the fish eye, flesh, intestine, and heart. Low expression of Sox9 in the fish heart is similar to findings observed in adult allotetraploid fish (Liu et al., 2007).

Cartilage formation is a complex process in vertebrates. Cartilages are obligatory templates for formation of endochondral bones during development, and constitute permanent skeletal structures in articular joints and other organs (Lefebvre et al., 1998).The Sox family of transcription factors is essential during in vivo chondrogenesis (Akiyama and Lefebvre, 2011; de Crombrugghe et al., 2001). Under certain conditions, irregular expression of Sox genes may indicate the possibility of skeletal malformation (Bell et al., 1997; Yan et al., 2004). Evidence has indicated that temperature can regulate expression of Sox genes in Atlantic salmon (Ytteborg et al., 2010). Under hyperthermic conditions, transcription of Sox genes in Atlantic salmon followed a trend of down regulation that potentially delays maturation and mineralization of osteoblasts, leading to vertebral deformities (Ytteborg et al., 2010). In the present study, the expression of Sox5, Sox9a, and Sox9b was affected by ambient temperatures. Expression of Sox9b in larval golden pompano decreased when rearing temperature increased from 23^{o}C to 26-29^{o}C on 12 DPH, but Sox9b expression was not affected by rearing temperature after 18 DPH. Expression of Sox5 in fish larvae decreased on 18 DPH. Our previous study found that increased rearing temperature, from 20^{o}C to 29^{o}C, significantly increased malformation (Yang et al., 2016). Increased malformation at high rearing temperature may correlate to reduced Sox9a on 12 DPH and Sox5 on 18 DPH. However, this may need further verification.

In summary, the present study successfully cloned and analyzed expression of Sox5, Sox8, and Sox9 genes in the first 18 days of golden pompano development. The time dependent expression of Sox genes in fish larvae is important in understanding ontogenetic development and growth of fish larvae in early life. The expression of Sox5 and Sox 9b genes in golden pompano larvae may serve as useful indicators for skeleton malformation, which may help identification of environmental factors causing malformation in fish larvae.

Acknowledgements

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