J. Ocean Univ. China (Oceanic and Coastal Sea Research)
https://doi.org/10.1007/s11802-019-4237-3
ISSN 1672-5182, 2020 19 (1): 171-182
http://www.ouc.edu.cn/xbywb/
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Half Smooth Tongue Sole (*Cynoglossus semilaevis*) Under Low Salinity Stress Can Change Hepatic *igf2* Expression Through DNA Methylation

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(Received May 16, 2019; revised July 16, 2019; accepted October 9, 2019)

Abstract Salinity is a crucial environmental stress that severely affects fish growth and survival. Under environmental stress, DNA methylation plays an important role in gene expression and genome function. To better understand the epigenetic regulation mechanism of *igf2* under low salinity stress, we analyzed the DNA methylation at 5'UTR, exon1, intron1, and exon2, and analyzed the relationship of DNA methylation with mRNA abundance as well as the special single CpG sites methylation patterns of *igf2* in the liver of half smooth tongue sole under low salinity (15) for 0, 7, and 60 d. When exposed to low salinity, DNA methylation at 5'UTR and exon2 remained stable, while it was up-regulated firstly and then down-regulated at exon1 and intron1. Some single CpG sites of *igf2* associated with low salinity, and most of these sites with significantly changed methylation levels ($P<0.05$) are located in intron1 area. The discrepant variation of single CpG sites methylation levels and *igf2* expression further revealed that females and males showed different response to low salinity. Remarkably, the 38-CpG site of intron1 servers as a sexual marker. Additionally, our integrative analysis demonstrated that regional DNA of *igf2* methylation had highly complex interplay on gene expression. The single CpG sites in intron1 were indispensable epigenetic markers under external environmental stress. Above all, to resist the low salinity stress, half smooth tongue sole liver can regulate the expression of *igf2* through methylation of CpG sites in intron1.

Key words salinity; hepatic *igf2*; DNA methylation; gene expression; half smooth tongue sole

1 Introduction

Salinity is one of the most basic physicochemical characteristics of aquaculture water. It rapidly changes and pulsatory fluctuates due to the influence of thermodynamic properties, such as heat capacity, vapor pressure, and density (Kültz, 2015). In aquaculture industry, habitat salinity could change drastically because of the flooding associated with coastal rainstorms, changes of tide, or terrigenous drainage (Drake *et al*., 2013; Duggan *et al*., 2014). This abiotic stress causes indirect drought in physiology, and alter osmotic pressure regulation and metabolism, as well as biochemical processes *in vivo* and *in vitro* of the cells (Choi and An, 2008; Hasenbein *et al*., 2013; Nie *et al*., 2017). It stresses on fish growth, survival, development, and reproduction. Sometimes it even results in huge economic losses. For its economic importance, salinity has been widely studied by aquaculture science (Boeuf and Payan, 2001; Jeremiah and Joseph, 2008; Eddie and Norman, 2009; Martins *et al*., 2014; Viviana *et al*., 2015). Most fish species adapted to tolerate some degree of salinity stress. However, stenohaline species have narrow salinity tolerance range (Wurts and Stickney, 1989; Kültz, 2015). For example, *P. major* is relatively sensitive to low salinity exposure. To cope with salinity changes, some euryhaline teleost developed their complex and unique osmotic regulation mechanism to adjust themselves to a wide range of external salinities (Jung *et al*., 2012; Kültz, 2015). Like *L. maculatus*, it survives at salinity from 0 to 38, while its optimal salinity is 16–17 (Du *et al*., 2013; Zhang *et al*., 2017). In response to salinity variation, these fish species mediate digestive enzyme activities, physiological functions of hormones (GH, TH, and IGFs) and transmembrane proteins (calcium-sensing receptor and ion channels) to regain homeostasis of osmoregulation (Barton and Iwama, 1991; Mustafayev and Mekhtiev, 2008; Martins *et al*., 2014; Kültz, 2015). In addition, euryhaline fish species increase cell proliferation and remodel extensive epithelial of gills in response to salinity changes (Laurent and Dunel, 1980; Chretien and Pisam, 1986).

As a kind of typical euryhaline fish species, half smooth tongue sole (*Cynoglossus semilaevis*) mainly distribute in Bohai Sea and Yellow Sea, China. It gains rapidly appeal

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as aquaculture candidate in China for its high commercial value and good taste. It lives in the aquatic environment with a salinity from 14 to 37, while the optimum salinity is 26 (Wang *et al*., 2003). Half smooth tongue sole is an optional model for studying salinity adaption. It has been proved that when subjected to different salinity conditions, half smooth tongue sole would obtain adaptability by changing its gene expression, non-specific immunity enzymes activities, key carbohydrate metabolism enzymes, body composition, and physical and chemical indexes of blood (Fang, 2013; He, 2016; Li *et al*., 2017a, 2017b; Si *et al*., 2018). Moreover, we have found that when subjected to low salinity of 15, the number of white blood cells, red blood cells, albumin, and alkaline phosphatase levels became significantly higher to enhance the immune function to adapt the low salinity environment (He, 2016). Increasing evidence have demonstrated the involvement of the GH/liver-IGF1 axis in osmoregulation when fish are under salinity stress. For example, the expression of *GH* mRNA level was significantly deceased while *PACAP* and *GHR1* mRNA levels increased, causing weight gain rate significantly decreased when half smooth tongue sole was under low salinity (Si, 2019). Meanwhile, our previous study also demonstrated that hepatic *IGF1* mRNA expression significantly decreased under long-term low salinity treatment (Li *et al*., 2017a). Karl *et al.* (2010) found that both IGF1 and IGF2 in the liver crucially participated in fish osmoregulation with organ-specific manners in tilapia. Meanwhile, IGF2 seemed to mimic the osmoregulation function of IGF1 with different manners when fish was under salinity stress (Reinecke and Collet, 1998; Reinecke *et al.*, 2005; Codina *et al.*, 2008; Karl *et al.*, 2010). Moreover, Norman *et al*. (2011) found that IGF2 may also affect salinity tolerance capacity as suggested by a genome-wide QTL on linkage group 19 in *Salvelinus alpinus*. However, the potential physiology role of IGF2 in fish osmoregulation still need further research.

Recently, the dynamic process of DNA methylation under environmental stresses has evoked the interest in epigenetic adaptive regulation in the abnormal conditions. When situating in the adversity environment, aquatic organisms depend on the gene expression and reprogramming metabolism to regain the physiology equilibrium for development, growth, and survival (Takei *et al.*, 2014; Yang *et al.*, 2016). DNA methylation plays important roles in gene expression and cellular differentiation, it may adjust genome function to adapt to changed environment (Szyf, 2012; Alvarado *et al.*, 2014). Alterations in DNA methylation patterns induced by salt could enable hatchery-reared trout to acclimate to seawater conditions and increase their survival rate (Morán *et al*., 2013). Associated with gene silence or super-activity, DNA methylation can respond to stress quickly with a diversity without changing the DNA sequences (Habu *et al.*, 2001; Bird, 2002). DNA methylation regulates gene expression by repressing the binding of transcriptional factors or incorporating some specific proteins into methylated CpG sites to modify chromatin structure (Ziller *et al.*, 2013). Anastasiadi *et al.* (2017) found that temperature increase resulted in stage-dependent alterations in global DNA methylation and gene expression levels in European sea bass. In addition, we also had explored that DNA methylation of *igf1* in the liver of half smooth tongue sole have significantly increased and down-regulated the gene expression level when fish was subjected to low salinity. Furthermore, the polymorphism of DNA methylation adjust itself to obtain equilibrium (Li *et al.*, 2017a, 2017b).

From the aspect of how epigenetic regulation was involved in fish growth and adaptability under adverse environment, we investigated the methylation levels of regional *igf2* (including 5'UTR, exon1, intron1, and exon2) in the liver and analyzed its function on mRNA expression, as well as some special single CpG sites variation when half smooth tongue sole were subjected to low salinity exposure. These results explain the function of *igf2* in regard to DNA methylation and mRNA expression when fish is under low salinity stress. Additionally, the analysis of differential methylation levels of four regional DNA and its correlation with mRNA expression provided additional evidence for the transcriptional regulation of genomic DNA. The methylation of specific single CpG sites in hepatic *igf2* further improve our understanding of the function of *igf2* in regard of the epigenetic regulation under low salinity stress.

2 Materials and Methods

2.1 Animal Maintenance and Stressing

The 10-month old half smooth tongue sole (*Cynoglossus semilaevis*) (body weight 101.58±32.07 g; body length 25.39±3.52 cm) were maintained in a local commercial fish farm. Three groups of fish were selected. In each group forty healthy fish were randomly raised in a 5 m×5 m×1 m tank filled with salinity 30 seawater. By mixing seawater with fresh well-water at the speed of decreasing 5 every day, the low salinity 15 environment was achieved (Wang *et al.*, 2003). The fish obtained from natural seawater (salinity 30), namely treated with salinity 15 for 0 d (D), were defined as the control group. The fish further exposed to salinity 15 for 7 d (D) and 60 d (D) respectively were considered as treatment groups. The fish were cultured with appropriate temperature (22–24℃) and continuous aeration (DO>6 mg L^{-1}). Additionally, compound feed with 5% of body weight was used to feed fish twice a day before water was replaced to ensure the optimal aquaculture conditions. For each treatment, six individuals from each tank were anesthetized with 0.15% MS-222 (Sigma, St. Louis, MO) and the livers were collected. Three females and three males were used for further analysis in this study. The study was approved by the respective Animal Research and Ethics Committees of Ocean University of China. The field studies did not involve endangered or protected species.

2.2 Analysis of *igf2* mRNA Abundance Under Stress Treatments by qRT-PCR

Total RNA from the liver of fish under different salin-

ity stresses was extracted with RNAiso Reagent Kit. The PrimeScript^{TM}RT Reagent Kit with gDNA Eraser (Takara, Japan) was applied to synthesize the first-strand cDNA at 37℃ for 15 min. Then *igf2* (Accession: NC_024312.1) expression was determined by qRT-PCR using specific primers, *igf2*-F and *igf2*-R (Table1). Meanwhile, the *β-actin* (Accession: KP033459.1) (Table 1) was used as an endogenous reference (Liu *et al*., 2014). The specified amplification efficiency was ensured by conducting five 10-fold serial dilutions of cDNA to produce the standard curves of cDNA samples. Then 1 μL of 100× diluted cDNA template with three triplicates, as well as endogenous reference gene was simultaneously subjected to the Roche LightCycler 480 (Germany) to run the qRT-PCR process. The $2^{-\Delta\Delta CT}$ method was applied to analyze the relative quantification (Livak and Schmittgen, 2001).

Table 1 Nucleotide sequences of primers used in the experiment

Primer name	Sequence (5' to 3')	Application
igf2-P1	F: AGTAATTGGGAAATTAATTTATTTGT R: ATTACAATATCAAAACATCCCTCC	BS-PCR
igf2-P2	F: GGAGGGATGTTTTGATATTGTAAT R: ACAATCTCACAAAAACAAAACACC	BS-PCR
igf2-P3	F: GGTGTTTTGTTTTTGTGAGATTGT R: AACACAAATCTCCAACTATCACTA	BS-PCR
igf2-P4	F: TAGTGATAGTTGGAGATTTGTGTT R: CCAAAACTAAACAAATACAACCAC	BS-PCR
igf2-P5	F: GTGGTTGTATTTGTTTAGTTTTGG R: CTATCTTCACAAACAAACTACAAC	BS-PCR
igf2	F: AGTCCTTCGCTGCTGTT R: ACGCCTGTTGCTACCC	qRT-PCR
β-actin	F: GCTGTGCTGTCCCTGTA R: GAGTAGCCACGCTCTGTC	qRT-PCR

2.3 Analysis of *igf2* DNA Methylation in Control and Experimental Groups by BSP Sequencing

Genomic DNA in the liver tissue was isolated by using Marine Animal Genomic DNA Kit (TransGen, China). The quality and integrity were guaranteed by the nucleic acid analyzer (Biodropsis BD-1000, OSTC, China) and 1.5% agarose gel. The BisulFlash DNA Modification Kit (EpiGentek, USA) could deaminate the unmethylated Cytosine (C) to become Uracil (U) while the methylated Cytosine (mC) remained unchanged. Therefore, we can distinguish the U from C to analyze the methylated C sites in *igf2* by sequencing. One microgram of DNA was modified to serve as methylation-specific amplification templates. Five pairs of primers were specifically designed by online MethPrimer design software (http://www.urogene.org/methprimer/) to amplify the 5'UTR, exon1, intron1, and exon2 regions of *igf2*, which are predicted CpG-rich regions. The PCR products were separated from agarose gel and purified by EasyPure Quick Gel Extraction Kit (TransGen, China). Then the amplification fragments were cloned into a vector by pClone007 Simple Vector Kit (TsingKe, Beijing) and sequenced. In each sample, 7–10 individual clones were tested to present the CpG sites and the methylation level of *igf2*. All the primers used in the experiment are listed in Table 1.

2.4 The Prediction of Genetic Structure and CpG-Rich Regions

The *igf2* sequence was submitted to Splign software (http://www.ncbi.nlm.nih.gov/sutils/splign) and the four function areas, including 5'UTR, exon1, intron1 and exon2 were analyzed. The MethPrimer design software (http://www.urogene.org/methprimer/) was employed to forecast the CpG-rich regions. Moreover, the open reading frame (ORF) was found by online ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The transcription factor and the binding site of *igf2* were analyzed by PATCH™ public 1.0 online software (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi).

2.5 Statistical Analysis

The data of *igf2* mRNA expression and methylation levels in the liver of half smooth tongue sole under salinity stress were analyzed by SPSS 19.0 software, followed by one-way ANOVA within Duncan's multiple range test ($P \leq 0.05$). Independent *t*-test was performed to compare the difference between female and male fish. The Pearson correlation coefficient (R) was conducted to compute the correlation of the DNA methylation and the mRNA expression level.

3 Results

3.1 Prediction of *igf2* Structure and CpG-Rich Regions

The structure schematic of *igf2* was shown in Fig.1. With the length of 5727 bp, *igf2* contains four exons and three introns, and the initiation codon and termination codon are located at positions 141 bp and 4957 bp, respectively. The three predicated CpG islands in *igf2* are 484 bp, 210 bp, and 124 bp in length and are embraced in 5'UTR, exon1,

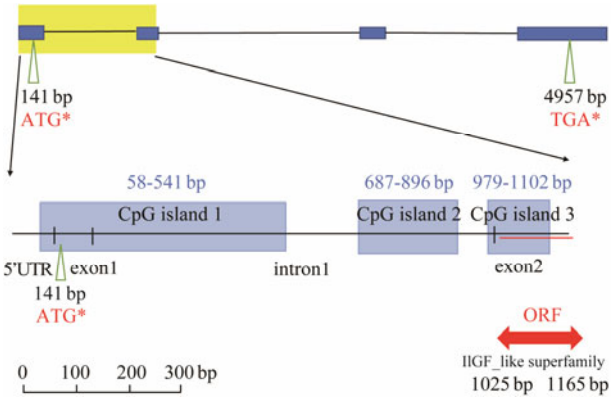

Fig.1 Structure distribution of *igf2* within BSP-amplification. The *igf2* gene contains four exons (blue frame) and three introns (black line); the initiation and termination codons were located at 141 bp and 4957 bp, respectively, labeled in red font. The yellow frame exhibits the amplified *igf2* with 5'UTR, exon1, intron1 and exon2, with three predicated CpG islands depicted in blue boxes. The red box indicates the open reading frame (ORF), locating 1025–1165 bp.

Fig.2 The CpG sites and forecasted transcription factors binding sites on the four functional areas of *igf2*. The underlined red letters show the CpG dinucleotide sites on 5'UTR, eoxn1, intron1 and exon2. The stained frames indicate the binding sequences of forecasted transcription factors with transcription factors marked upon it.

intron1 and exon2 areas. Analyzing the sequences of these four functional areas, there are 3, 3, 47, and 10 CpG sites, respectively. The open reading frame (ORF, locating at 1025–1165 bp) encodes a 46-amino acid IGF-like superfamily domain. Additionally, as shown in Fig.2, a large number of forecasted transcription factors are gathered around the CpG sites, including GR, Sp1, AP-1, and NF. They play important roles on the cellular proliferation and differentiation, metabolism and cell apoptosis, as well as regulating organism development and immunization.

3.2 The Changes of *igf2* DNA Methylation in the Liver Under Salinity Stress

As a particular indicator, DNA methylation of CpG sites is susceptible and dynamic to the external environment (Szyf, 2012). Since the predicted CpG islands mainly covered the 5'UTR, exon1, intron1, and exon2 regions, we carried out BSP sequencing to analyze the methylation levels of these four functional areas to investigate how the methylation level of *igf2* DNA specifically responded to low salinity stress. Considering the adult females are two to three times larger than male ones (Chen *et al.*, 2007), we analyzed the females and males respectively to eliminate the sexual difference. Interestingly, the DNA methylation levels among 5'UTR, exon1, intron1, and exon2 of *igf2* had no significant difference. Meanwhile, the methylation levels of four functional areas showed the same change tendency. They all increased firstly and then decreased when fish were exposed to short- and long-term low salinity stresses.

In female fish, when under normal salinity environment, the CpG methylation status of the four functional areas was quite low, averaging between 4.95% and 8.89% with no significant difference (Fig.3A, $P>0.05$). Subjected to low salinity stress (Fig.4A), 5'UTR CpG methylation levels increased initially and then decreased at a certain level ($P>0.05$), which were 8.89%, 23.33%, and 6.67%, respectively. Exon1 methylation ratio was 5.55% under low salinity at the beginning (0D), and then significantly up-regulated to 23.33% ($P<0.05$) under 7D stress, followed by a dramatical decline under 60D stress ($P<0.05$). The CpG methylation in intron1 significantly raised ($P<0.05$) and then reduced along with stress time prolonged (4.95%, 20.75%, and 10.00%, respectively). Subjected to salinity 15, the methylation level of exon2 was 8.33% at 0D, 8.81% at 7D and 6.33% at 60D, showing a slightly upper and then lower changes with no significance ($P>0.05$). In general, the methylation level of *igf2* was 5.70% under at 0D, significantly increased to 18.99% (7D, $P<0.05$) and then gently decreased to 8.99% (60D, $P>0.05$).

In male fish, the methylation levels of all these four regional *igf2* had no difference, which were 3.33%, 2.22%, 4.74% and 6.22%, respectively (Fig.3B, $P>0.05$). In Fig.4B, 5'UTR methylation occupied 3.33% under 15 salinity at 0D. Then it increased to 5.33% and then recovered to 3.33% ($P>0.05$). When the time of stress prolonged, the methylation status of exon1 showed a significant increase followed by a significant decrease ($P<0.05$), presenting 2.22%, 12.22%, and 3.33% respectively. Intron1 had the methylation level of 4.74% on day 0, 9.50% on day 7 ($P<0.05$) and 4.16% ($P>0.05$) on day 60 with the consistent tendency of firstly rising and then dropping. There was litter discrepancy of exon2 methylation under stress ($P>0.05$), which were 6.22%, 8.67%, and 7.33% respectively. Above all, with 0D salinity stress, the methylation level of *igf2* was 4.78%; when the stress prolonged to 7D and 60D, it apparently was up-regulated to 9.21% and down-regulate to 4.60% ($P<0.05$) with a recovery.

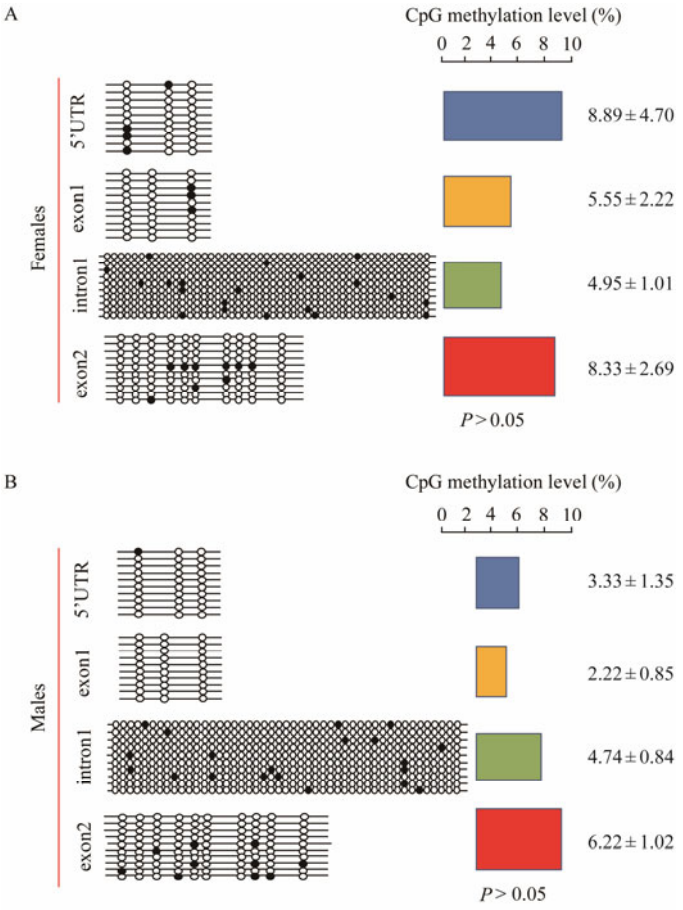

Fig.3 The presentation of the CpG methylation levels in four function areas of *igf2* in the liver of untreated females (A) and males (B). One fish representative of the methylation level is demonstrated. A filled or open circle indicate CpG positions methylated or unmethylated in the CpG site, respectively. Ten clones per fish were used to determine the average methylation levels, which was specifically calculated outside the bar. Data represents mean±SD, $P>0.05$ shows no significant differences between function areas with Duncan's t test.

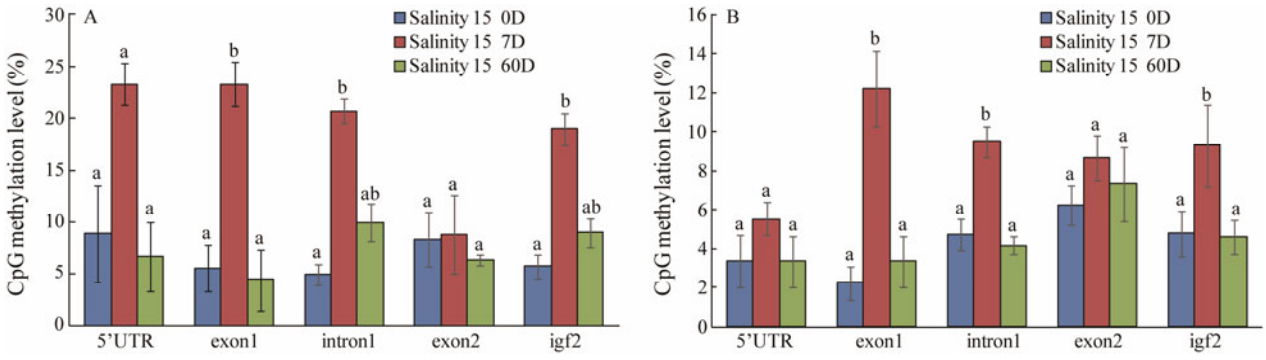

Fig.4 The changes of *igf2* methylation in female (A) and male (B) half smooth tongue sole under short-(7D) and long-(60D) term of low salinity stress. Different lowercase letters indicate the significances when subjected to stress ($P<0.05$, Duncan's t test).

3.3 The Influence of Low Salinity Stress on Single CpG Site of *igf2*

Furthermore, the single CpG sites of *igf2* were found to be sensitive to the low salinity stress. When we analyzed the methylation of single CpG sites (Fig.5), we found they were quite constant in 5'UTR area, while exhibited dramatic changes in exon1, intron1, and exon2 areas under treatments. Overall, the methylation levels of these significantly changed CpG sites mainly displayed firstly increased under stress for 7D and then decreased to recover under stress for 60D.

In female fish (Fig.5A), low salinity stress had a great effect on some particular single CpG sites in exon1 (two CpG sites), intron1 (12 CpG sites) and exon2 (one CpG site) of *igf2*. The methylation level of the 37-CpG site in intron1 exhibited a continuous rising when subjected to salinity stress and showed a significant change with 60D stress ($P<0.05$). Moreover, the other CpG sites all exhibited the same methylation tendency that firstly upgraded

and then descended with significant difference ($P<0.05$). Particularly in intron region, the methylation levels of 2-, 21-, 27-, 39-, 43- and 45-CpG sites were all 0.00% under 15 salinity at 0D. And the methylation levels of 1-, 21-, 31-, 35-, 39- and 40-CpG sites declined to 0.00% after 60D under low salinity stress.

In male fish (Fig.5B), five CpG sites in intron1 altered under stress, while the low salinity stress showed no influence on the methylation of CpG sites in 5'UTR, exon1, and exon2. The methylation of 1-, 5-, 8- and 11-CpG sites in intron1 all increased firstly and then declined with significant differences ($P<0.05$). The methylation level of 38-CpG site kept continually declined to 0.00% under stress for 7D and 60D ($P<0.05$). Additionally, the methylation levels of 5- and 8-CpG sites presented 0.00% at 0D of stress.

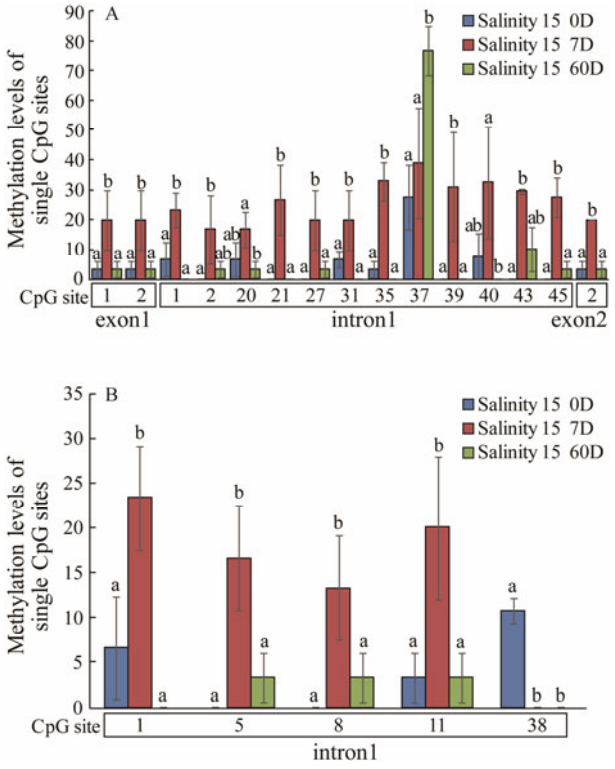

Fig.5 The effect of low salinity on the methylation of *igf2* single CpG sites in female (A) and male (B) half smooth tongue sole. Different lowercase letters indicate the significances when subjected to stress ($P<0.05$, Duncan's t test).

3.4 The *igf2* mRNA Abundance Under Stress and Its Correlation with DNA Methylation

Surveys of epigenetic markers had proved that DNA methylation participates in transcriptional regulation (Ansel *et al*., 2006). However, the correlation of regional DNA methylation levels and mRNA expression were rarely discussed. To test whether this epigenetic mechanism evokes plastic transcriptional programs of *igf2*, especially the relationship of methylation level with mRNA expression, when half smooth tongue sole was under low salinity stress, we quantified the *igf2* mRNA expression by RT-qPCR technology. Its correlation with the methylation level was figured out by correlation coefficient (R) analysis. The *igf2* mRNA expression (Fig.6) significantly declined as low salinity stress prolonged. Compared with salinity 15 at 0D, the *igf2* mRNA expression levels in female fish liver significantly decreased to 3.6 times ($P<0.05$) at 7D and 5.1 times ($P<0.05$) at 60D, respectively. In male fish liver, *igf2* mRNA expression significantly dropped under 7D stress, which was 2.5 times ($P<0.05$) less than at 0D. When subjected to low salinity for 60D, the relative quantity slightly raised, which was 2.5 times ($P<0.05$) less than for 0D, given the significance of DNA methylation in regulating gene expression we tested.

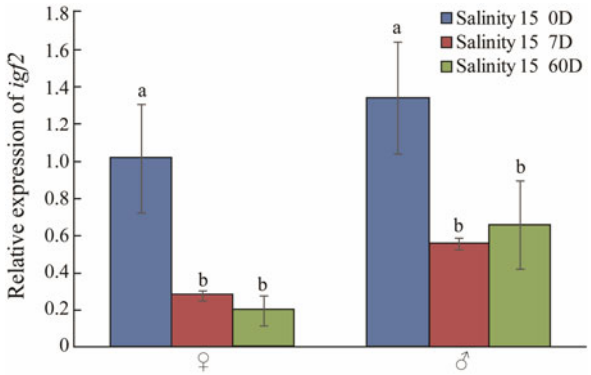

Fig.6 The relative expression of *igf2* in half smooth tongue sole under salinity stress (salinity 15). Different lowercase letters indicate the significances when subjected to stress ($P<0.05$, s ($P<0.05$, Ducan's t test).

In female fish liver (Figs.7A and B), the CpG methylation levels of 5'UTR, exon1, intron1, and *igf2* negatively correlated with mRNA expression, were −0.299, −0.319, −0.425, and −0.239, respectively. Among them, intron1 occupied the greatest correlation coefficient. However, the R between exon2 methylation and mRNA expression presented a positive relationship with 0.239. In male fish liver (Figs.7C and D), 5'UTR, exon1, intron1, exon2, and *igf2* showed a negative correlation with mRNA transcription with R −0.126, −0.588, −0.313, −0.188, and −0.341, respectively. And the R of exon1 exceeded other functional DNA areas.

3.5 Analysis of Gender Difference of *igf2* and Single CpG Site in the Liver of Stressed Fish

Half smooth tongue sole exhibits significant sexual dimorphism as females are two to three times larger than males (Chen *et al.*, 2007). Thus, it is necessary to analyze the sexual difference. In this study, the analysis manifested that the DNA methylation levels of *igf2* at its four functional areas (5'UTR, exon1, intron1, and exon2) in the liver under salinity stress had no gender difference (Table 2). Although the *igf2* expression under low salinity stress showed no significant sex difference under salinity 15 for 0D ($P>0.05$), dramatic differences were observed under stress for 7D and 60D, as the *igf2* expression in the liver of male fish was significantly higher than female fish to 2 times ($P<0.05$) and 3.3 times ($P<0.05$), respectively.

We also detected some single CpG sites which showed significantly different methylation levels between stressed female and male fish (Fig.8). Under salinity 15 for 0D, the

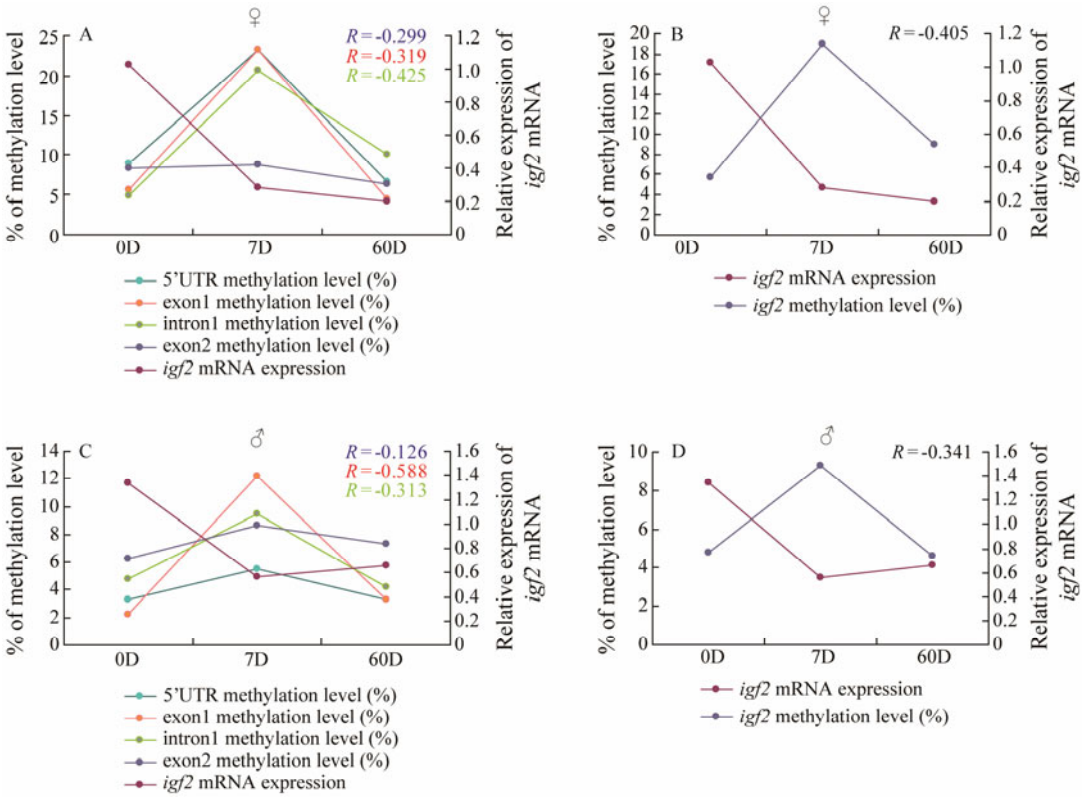

Fig.7 The correlation between CpG methylation and mRNA expression of *igf2* in female and male fish when exposed to low salinity treatments (salinity 15). A and C: The correlation between the four functional areas (5'UTR, exon1, intron, and exon2) methylation levels with *igf2* mRNA expression in female (A) and male (C) fish. B and D: The correlation between *igf2* DNA methylation level with *igf2* mRNA expression in female (B) and male (D) fish.

Table 2 Comparisons of methylation and expression levels of *igf2* between female and male fish under low salinity treatments

Treatments	Gender	Methylation level (%)					Expression level (Fold change)
		5'UTR	exon1	intron1	exon2	Total	*igf2*
Salinity 15 0D	♀	0.09±0.08	0.06±0.04	0.05±0.01	0.08±0.06	0.06±0.01	1.02±0.29
	♂	0.03±0.03	0.02±0.04	0.05±0.01	0.06±0.04	0.05±0.01	1.34±0.30
Salinity 15 7D	♀	0.23±0.12	0.23±0.09	0.21±0.11	0.09±0.05	0.19±0.09	0.28±0.03a
	♂	0.06±0.04	0.12±0.02	0.10±0.04	0.09±0.01	0.09±0.03	0.56±0.03b
Salinity 15 60D	♀	0.07±0.03	0.04±0.05	0.10±0.07	0.06±0.01	0.09±0.05	0.20±0.08A
	♂	0.03±0.03	0.03±0.03	0.04±0.00	0.07±0.03	0.05±0.01	0.66±0.24B

Notes: The difference between females and males in regards of methylation levels of 5'UTR, exon1, intron1, exon2 and total *igf2*, as well as expression level of *igf2* mRNA under different salinity treatments are presented in the table. Data are expressed as mean±SD; different lowercase (a and b) or uppercase (A and B) letters represent significance difference between females and males when subjected to different salinity stress ($P<0.05$, t test).

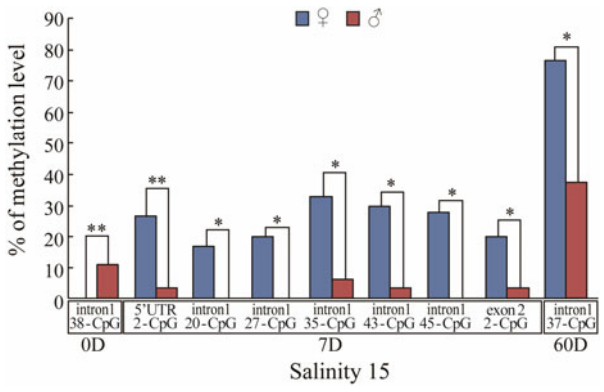

Fig.8 The significant difference between female and male fish in single CpG site under salinity stress. * indicates the significances when subjected to stress (* <0.05, ** <0.01, t test).

methylation level in males significantly exceeded females at the 38-CpG site of intron1 ($P<0.01$). For 7D stress, the 2-CpG of 5'UTR, 20-, 27-, 35-, 43- and 45- CpG of intron1 and the 2-CpG of exon2 all had a significantly higher methylation status in female fish than in male fish ($P<0.05$). As time prolonged to 60D, only the 37-CpG of intron1 had an evident difference in methylation and females were significantly higher than in males ($P<0.05$). Thus, the results implied that *igf2* might play different osmotic-regulation roles in females and males.

4 Discussion

Salinity is an important environmental stimulating factor. It irreversibly affects physiological and biochemical

functions, endocrine systems, molecular receptor of osmotic pressure, as well as the stability of DNA and proteins in organism to affect fish growth, reproduction and ecological distribution (Barton and Iwama, 1991; Kültz, 2012, 2015; Martins *et al.*, 2014). By regulating different gene expressions for adjusting genomic functions, the potential role of DNA methylation in adaptation mechanism under environment contexts has been proved to be important (Szyf, 2012). As a commercial euryhaline marine fish in China, half smooth tongue sole significantly changed DNA methylation levels and gene expression of GH-IGF axis genes to adjust itself to low salinity stress (Li *et al.*, 2017a, 2017b; Si, 2019). However, how DNA methylation of heptic IGF2 changes and how it regulates mRNA expression under low salinity stress has not studied thoroughly. Based on the background, we carried out the analysis to figure out how regional DNA methylation of *igf2* regulates gene expression to serve as genome adaptation mechanism when half smooth tongue sole is exposed to low salinity.

4.1 The Diversity Characteristics of DNA Methylation in Four Functional Areas of *igf2*

DNA methylation has well established its role in tissue differentiation and development as a mechanism for the genome to express diverse phenotypes (Choe, 2008; Szyf, 2012). Additionally, different regional DNA presented its specific methylation pattern, which has been proved to greatly affect gene expression (David *et al.*, 2014). Structure prediction showed *igf2* contains three CpG islands. For 5'UTR, exon1, intron1, and exon2 areas of *igf2*, there were 3, 3, 47, and 10 CpG sites, respectively. The analysis indicated that the methylation levels of *igf2* four functional areas were polymorphic in the liver of half smooth tongue sole. It is relatively higher in exon1 and is lower in exon2, which may correlate with the different abundances of CpG dinucleotide methylated. Based on the nine cell lines and 15 tissues of human methylation data, Ai *et al.* (2016) found that different functional regions had diverse methylation distributions: first exon region kept in lower state, transcription start site region fluctuated within a certain range, while the medium exon, last exon, intron, and transcription termination site had comparative higher methylation levels. Our previous study also had manifested that methylation in three exons of *igf1* was significantly different with exon1 prominently lower than exon2 and exon3 (Li *et al.*, 2017a). DNA methylated occurs in all creatures and expresses the fundamental dynamics of epigenomes. The diversity of methylation states across individuals resulted in the plasticity, tissue-specific nature, as well as the variability of gene expression (Weiss *et al*., 1996; Yano *et al*., 2003; Christensen *et al*., 2009).

4.2 The Dynamic Response of Methylation and mRNA Abundance of *igf2* in Liver to Salinity Treatments

As a crucial environmental factor, salinity greatly alters osmotic pressure regulation to threaten fish biochemical processes (Boeuf and Payan, 2001). To adapt to this specific environmental cue, the organism gains its ability to maintain tissue-specific function and regulate the gene transcriptional patterns (Alvarado *et al*., 2014). Compared with sequences adjustment, DNA methylation can quickly respond to environmental stimulus as it methylates different fragments to change gene expression quantity or activate new functional genes in specific environmental cues (Steward *et al.*, 2002; Mazzucotelli *et al.*, 2008; Ou *et al.*, 2009). The dynamic response of DNA methylation had emphasized its integral role as an important adaptive mechanism under environment changes (Bird, 1986; Hashida *et al.*, 2006; Zhao *et al.*, 2009).

The four functional areas showed dynamic methylation levels in response to low salinity stress. As we have manifested, the methylation levels of exon1 and intron1 elevated significantly under 7D stress with mRNA expression significantly decreased. As low salinity prolonged to 60D, methylation levels gradually decreased to the original level, serving as a genome adaptation method. Meanwhile, the conservation of DNA methylation suggests to be indispensable in maintaining the genomic stability as well (Alvarado *et al.*, 2014). In our study, the methylation levels of 5'UTR and exon2 of *igf2* had no significant difference under low salinity stress. The single CpG sites, especially in 5'UTR, presenting no significant changes under treatments. These GC enrichment regions remained steady under stress which may involve in maintaining genomic stability to ensure the fish survival and growth. Studies had provided that the loss of DNA methylation could result in the genomic instability and chromosomal aberrations which observed in cancer (Gama-Sosa *et al.*, 1983; Eden *et al.*, 2003). We speculated the normal methylation level of 5'UTR may be integral for *igf2* gene expression. As for exon2 methylation, the higher methylated CpG dinucleotide made it difficult to remethylate. Additionally, previous work indicated that the full-methylation in three tissues (gonad, kidney, and gill) under low salinity stress steadily ranged from 12.06% to 18.64% without significant change, which was considered to be necessary for the stability of gene expression (Li *et al.*, 2017b).

Interestingly, by analyzing the methylation levels of total 64 CpG sites in three CpG islands of *igf2*, the results showed that the significant changes of single CpG sites under salinity stress were found in intron1 region. When studied the DNA methylation changes of rice under drought stress, Pan *et al.* (2009) verified that both the coding and non-coding regions had the similar frequency of DNA methylation. Regarding the mutability of the length and sequence of intron fragments, Wang and Liu (2000) pointed out that most of the mutants occur in intron regions randomly, and intron can diminish the impact on organism to enhance the resistant ability of mutations. In our study, the significant changes of methylation in intron1, especially the single CpG sites further implied the unique adjustment ability and regulation of fish under salinity stress. Moreover, the variation tendency of single CpG sites methylation levels contributed to the functional areas methylation levels, causing the methylation level of *igf2* increases firstly and then decreased.

DeChiara *et al.* (1990) had illuminated the physiological role of IGF2 in cellular proliferation and differentiation, mediating metabolism and inhibiting apoptosis. In consideration of its important role, the balanced expression of *igf2* is imperative for fish survival and growth. Karl *et al.* (2010) had revealed the *igf1* and *igf2* mRNA expressions in tilapia were downregulated in parallel after seawater transfer and recovered ultimately, suggesting that *igf1* and *igf2* are involved in fish osmoregulation with organ-specific manner. In our study, as methylation of *igf1* had no changes under 7D stress and significantly increased under 60D low salinity (Li *et al.*, 2017a), DNA methylation of *igf2* could respond to low salinity more quickly and recover its methylation level as stress time prolonged. Thus, the function regarding epigenetic adaptive mechanism of *igf2* has superior timeliness to *igf1*. Although half smooth tongue tolerates a certain degree of salinity stress, the low salinity down-regulates the expression of *igf2* when the time of stress becomes longer. Wang *et al.* (2003) found that the growth rate was affected when half smooth tongue sole subjected to a lower (for example, 15 or 18) or higher salinity (for example, 32 or 35). Our previous studies also found that the expression of *GH* and *igf1* also decreased and resulting in lower weight gain rate when half smooth tongue was subjected to 15 salinity for 60D (Li *et al.*, 2017b; Si, 2019).

4.3 The Sexual Difference of *igf2 DNA* Methylation and mRNA Expression Levels

Half smooth tongue sole is an important commercial fish in China which exhibits significant sexual dimorphism as females are two to three times larger than males (Chen *et al.*, 2007). The studies focused on the gonadal development and sex determination mechanism had been widely explored. Sex-related genes, including *Cyp1a1*, *Dmrt1*, *Ubc9*, *Foxl2*, *Sox9*, and *Wnt4* had been characterized during gonadal differentiation (Deng *et al.*, 2009; Dong *et al.*, 2011; Hu and Chen, 2013; Hu *et al*., 2014). Interestingly, methylation modification appeared to be indispensable for sex determination by regulating gene expression (Shao *et al.*, 2014). In our study, the methylation levels in four functional areas of *igf2* had no sexual difference under normal salinity environment, indicating *igf2* is not a sex determination gene. Interestingly, the DNA methylation in males significantly exceeded females in the intron1 38-CpG site under normal salinity environment, which could be considered as a candidate marker to character female and male half smooth tongue sole during breeding. When exposed to low salinity, the DNA methylation of *igf2* had no difference between stressed male and female fish.

However, the number and the site of single CpG which had significantly changed methylation levels are different between female and male fish under salinity stress. Remarkably, we observed that the methylation levels of single CpG sites under low salinity treatment have significant difference between sexes. Meanwhile, the *igf2* expression levels in males significantly exceed those in females under low salinity for 7D and 60D. These differences may reveal that female and male fish have different epigenetic responses to low salinity. Additionally, the sensibility of different methylated CpG sites to low salinity may be different in female and male fish. Furthermore, *igf2* expression level showed the opposite change as *igf1* in females was significantly higher than that in males under salinity stress for 7D and 60D (Li *et al.*, 2017a), suggesting the discrepant expression of *igf1* and *igf2* in female and male fish when they are subjected to low salinity stress.

4.4 The Correlation Between DNA Methylation and Gene Expression

The role of DNA methylation has been extensively characterized in altering transcription pattern temporally and spatially. Mechanistically, it was reported to cause steric hindrance that inhibited transcriptional activators binding to the DNA thus repressing gene expression particularly. Moreover, DNA methylation was suggested to combine with transcriptional repressors such as methyl-binding domain family. These complexes could recruit histone modifying complexes to silence the transcription (Razin and Riggs, 1980; Choy *et al.*, 2010; Alvarado *et al.*, 2014). Most work has focused on the importance of promoter region in repressing gene expression. As the studies of regional DNA in regulating gene expression get deeper, the function of DNA methylation at different functional areas has attracted more attention. Given the comprehensive DNA methylation map of the entire genome in *Arabidopsis*, Zhang *et al.* (2006) reported that over one-third of expressed genes had methylated sites within transcribed regions and only approximately 5% of genes contain methylation sites within promoter regions. In addition, the high methylation ratio of exon1 involved in the inactivation of AR gene in Hela cells (Li *et al.*, 2011). Based on the background, we analyzed the correlation between DNA methylation levels in four regions and mRNA expression. The results showed that the methylation level in exon2 was positive with gene expression, while other regions presented a negative correlation with higher r values in exon1 and intron1. When Eckhardt *et al.* (2006) explored the DNA methylation profile of human chromosomes 6, 20 and 22, they discovered that one-third of the differentially methylated sites in 5'UTR were inversely correlated with gene transcription. Brenet *et al.* (2011) found that methylated exon1 region was tightly linked with transcriptional depression, much more than the promoter region. In our study, the methylation level of exon2 in female fish showed the positive correlation with transcription, which was consistent with the previous result that higher methylation in intragenic CpG islands led to active genes expression (Jjingo *et al.*, 2012). Xiao *et al.* (2014) also observed the positive correlation between intron2 methylation and expression of *GPR120* gene in the subcutaneous adipose tissue and greater omentum of *porcine*, while the DNA methylation might alter the chromosome structure to promote the gene transcription (Lorincz *et al.*, 2004; Jjingo *et al.*, 2012; Kulis *et al.*, 2013).

Salinity is a crucial environmental stimulus to stress an irreversible influence on fish growth, reproduction and ecological distribution (Martins *et al.*, 2014). In response to

salinity stimulus, DNA methylation occurs at different fragments to control gene expression as an important adaptive mechanism (Bird, 1986; Hashida *et al.*, 2006; Zhao *et al.*, 2009). We had manifested the genes of GH-IGF axis changed their expressions through DNA methylation when half smooth tongue sole was under low salinity stress. With a high identity of IGF1, IGF2 plays a role in the osmoregulation with a different manner (Reinecke and Collet, 1998; Reinecke *et al.*, 2005; Codina *et al.*, 2008; Karl *et al.*, 2010). To further understand the *igf2* osmoregulation function from the aspect of DNA methylation and mRNA expression, we sought to identify the different functional regulation of regional DNA methylation on mRNA expression and some special single CpG sites of *igf2* in the liver of half smooth tongue sole that was associated with low salinity. The 5'UTR and exon2 hold the stability of methylation statues under stress, and the significant methylation happened on single CpG sites of intron. The discrepant variation of single CpG sites methylation levels and *igf2* expression in female and male fish under salinity stress further reveal that female and male fish respond to low salinity variously. However, *igf2* was not a sex determination gene as no methylation or expression difference was found between female and male half smooth tongue sole. Remarkably, the methylation levels of intron1 38-CpG could be considered as a candidate marker to differentiate gender during breeding. The exon2 in females showed a positive correlation between DNA methylation and mRNA expression, while DNA methylation in other regions showed negative functions on *igf2* transcription.

Overall, our study firstly indicated that by changing regional DNA methylation and mRNA expression, *igf2* plays a complicated function in response to low salinity stress in half smooth tongue sole liver. More work still needs to be conducted to further understand the modification mechanism of cytidine analog inhibitor of DNA methyltransferases (DNAMTs) on DNA methylation under stress.

Acknowledgements

We thank Shandong Dongying Farm for providing the animals. This research was supported by the Key Laboratory of Mariculture of Ministry of Education, Ocean University of China (No. KLM2018009) and the National Natural Science Foundation of China (No. 31672642). Editors and reviewers are highly appreciated for their comments to greatly improve our manuscript.

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(Edited by Qiu Yantao)