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Low salinity affects cellularity, DNA methylation, and mRNA expression of *igf1* in the liver of half smooth tongue sole (*Cynoglossus semilaevis*)

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Abstract Animal growth depends on feedback regulation of hormone levels and environmental conditions. Insulin-like growth factor-1 (Igf1) promotes cell growth and differentiation and represses apoptosis and is highly regulated by the environment. Moreover, animals modify physiological homeostasis under stressful conditions through epigenetics and genetic regulatory mechanisms. Therefore, a comprehensive understanding of the effects of salt on fish growth is needed. In this study, half smooth tongue sole (*Cynoglossus semilaevis*) were subjected to 15‰ salinity for 0, 7, and 60 days (D) to assess the effects of low salinity on liver cellularity and growth. The results show that low salinity changed liver morphology, suggesting an increase in energy expenditure to recover from the osmotic disruption. *igf1* was upregulated in female fish under 15‰ salinity after 7D and may participate in molecular repair. *igf1* was downregulated after 60D of salt stress, resulting in retarded growth. Methylation levels were opposite to those of gene expression, suggesting inhibited regulation. Furthermore, three exons in the *igf1* gene had significantly different methylation levels in fish under salt stress. Notably, more putative transcription factor binding sites were located in CpG sites at higher methylation levels. *igf1* is not a sex-related gene, as no difference in methylation level was detected between males and females in the control group. These results clarify liver damage and changes in DNA methylation and mRNA expression of *igf1*, providing insight into the adverse effects of low salt on growth of *C. semilaevis* and the epigenetics and regulatory mechanisms involved in stressful conditions.

Keywords Salinity stress · *igf1* · Morphology · DNA methylation · mRNA transcription

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Introduction

Salinity represents the content of dissolved salt, an inherent physicochemical composition in water. Most fishes have the ability to tolerate salinity variation through their dynamic osmoregulatory mechanisms, including salt absorption and excretion, water secretion, and retention (narrow for stenohaline and wide for euryhaline) (Kültz 2015). The majority of euryhaline fish species have a superior tolerance as salinity rapidly changes and fluctuates, to 30–40 ppt in marine or <0.5 ppt in freshwater (IAL and IUBS 1958; Kültz 2015). Meanwhile, salinity is a crucial environmental factor that greatly alters osmotic pressure regulation and metabolism, as well as biochemical processes inside and outside cells to threaten fish. In response to variable salinity, fish mediates physiological functions and digestive enzyme activities associated with stress response by releasing the hormones that act in the somatotropic axis, such as GH, TH, IGFs, and transmembrane proteins such as the calcium-sensing receptor and ion channels phospholipase A2 (Barton and Iwama 1991; Chen et al. 1998; Mustafayev and Mekhtiev 2008; Martins

et al. 2014; Kültz 2015). In addition, direct ionic and osmotic formed from salinity stress have great effect on stability of DNA and protein (Kültz 2012). Moreover, cell proliferation and turnover, an osmoregulatory strategy, allow fish to adjust themselves to the external salinity environment (Conte and Lin 1967; Laurent and Dunel 1980; Chretien and Pisam 1986). Many studies have investigated the effects of varying salinity on fish. For example, the liver of *Oncorhynchus keta* is severely injured by low salt, and fat particles degenerate and accumulate (Liu and Zhi et al. 2010). Wang et al. (2006) showed that both low and high salt conditions disrupt cell density and particle cell structure of the head kidney and spleen in rock fish. Expression levels of genes, such as *TRPV4*, *PRLI*, *NKAα1*, *PtAQP*, and *PtCRT*, also change in response to salinity stress in *Oreochromis mossambicus*, *O. hornorum*, and their hybrids, as well as in *Portunus trituberculatus* to re-establish proper osmotic pressure (Liu 2014; Wang et al. 2014). Salinity stress has been implicated in changes in hematological and biochemical indices and enzymatic activities in fish (Zhang 1991; Altinok et al. 1998; Vander et al. 1999; Handeland et al. 2003; Liu et al. 2010). Many studies have suggested that salinity is one of the most extensive and fundamental factors affecting fish growth. Now the subject is over-studied for its economic importance (Martins et al. 2014).

One important group acting in the somototropic axis is the insulin growth factors (IGFs), the regulatory growth peptides (Martins et al. 2014). They act on target tissues controlled via the endocrine and autocrine/paracrine pathways (Bower et al. 2008) and involved in insulin-like metabolism and promotion of mitosis during fetal and postnatal growth (Jones and Clemmons 1995). Igf1 is a 70-amino acid long, 7.5-kD polypeptide synthesized and secreted by the liver. The bioactivities of Igf1 are mediated by the Igf1 receptor which is expressed in various cell types (Czech 1989), activating the phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling pathways to promote cell growth and differentiation and repress apoptosis (Jones and Clemmons 1995). Functional studies indicate that Igf1 functions are conserved in many teleost species (Gray and Kelley 1991; Moriyama et al. 1993; Cheng and Chen 1995; Takagi and Bjornsson 1996; Upton et al. 1996). Increasing evidences suggest that fish Igf1 expression is highly sensitive to salinity variation of external milieu. Li and Miao et al. (2015) explored the effects of salt on *igf1* expression in *Pseudosciaena crocea* and reported that *igf1* expression increases significantly in response to low salinity stress, indicating that *igf1* may be involved in changing serum osmolality, ion concentrations, and Na^+/K^+-ATP enzyme activities so fish can adapt to various salinities (Kelly et al. 1999; Liu and Tong 2004).

Increasing evidence suggests that environmental stimuli may contribute to heritable phenotypic variations through changes in DNA methylation that are directly involved in animal growth and development. Surveys of epigenetic markers have demonstrated a critical role for DNA methylation in the regulation of gene transcription (Ansel et al. 2003; Holliday 2006), beyond regulation of X-inactivation (Allen et al. 1992), genomic imprinting (McGrath and Solter 1984; Tycko 1997; Henckel and Arnaud 2010), DNA replication, and memory and aging (Tserel et al. 2014). CpG methylation has an inhibitory role in gene transcription by inhibiting transcription factor binding or allowing methyl-binding proteins to incorporate into methylated DNA sites leading to "closing" of the chromatin structure (Ziller et al. 2013). CpG methylation levels in the *dmrt1* and *cyp19a* gene promoters of Japanese flounder (*Paralichthys olivaceus*) gonads inhibited the expression in male and female fish (Wen et al. 2014). Furthermore, methylation of the *cyp19a* promoter in European sea bass causes lower expression (Laia et al. 2011).

As a euryhaline fish, half smooth tongue sole (*Cynoglossus semilaevis*) can inhabit salinity range of 14–37 ppt and the optimal is 26 ppt (Wang et al. 2003). Since a breakthrough in artificial breeding technology in 2003 (Liu et al. 2006a, b), it has gained rapid appeal as an aquaculture candidate in China due to their taste, commercial value, ease of domestication, and lack of the natural resource. The females are two to three times larger than males. Epigenetics is the missing link between genetics, endocrine function, and the environment (Zhang and Ho 2011). However, how epigenetic regulation is involved in fish growth and adaptability is not well understood. As both epigenetics and genetics work together to determine genomic diversity, Hellman and Chess (2010) recommended investigating the mechanism of gene transcription from these two aspects. The present study was carried out to characterize salt-induced tissue damage and changes in DNA methylation and *igf1* messenger RNA

(mRNA) expression in *C. semilaevis* maintained under low-salt conditions. Our results depict the effects of low salt on liver tissues and the molecular mechanisms involved in the stress response through epigenetic and genetic regulation.

Materials and methods

Animal drawing and salinity treatments

Ten-month-old female and male half smooth tongue sole (*C. semilaevis*) (body weight 101.58 ± 32.07 g) were obtained from a commercial fish farm (Qingdao, China). Before salinity challenging test, the seawater was diluted with fresh well water at a rate of decreasing 5‰ salinity daily, until reaching the experimental ranges of 15‰ (Wang et al. 2003). Fish were randomly distributed into six tanks with 40 each, which were maintained in natural seawater (salinity 30‰) and were considered as the control group (salinity 15‰ 0D), and those exposed further to salinity 15‰ for 7D and 60D were regarded as treatment groups. The experimental fish were carried out at 22–24 °C, with continuous aeration (DO >6 mg/L), fed twice a day with compound feed (3–5% of body weight) before half seawater was replaced daily. The animals (three females and three males) from each treatment were harvested and anesthetized with 0.15% MS-222 (Sigma, St. Louis, MO). Liver tissues from six individuals (three females and three males) of each treatment were removed under sterile conditions. The liver tissues were preserved at −80 °C till further analysis to determine the methylation status and gene expression. Additionally, samples were fixed in Bouin's liquid for histology analysis.

Histology observation

Liver tissues were dissected and fixed in freshly prepared Bouin's liquid for 8–12 h at room temperature, then subsequent clearing in 70% alcohol solution. After a series of dehydration in graded alcohols, the tissues were rinsed in xylene using standard techniques, and then embedded in paraffin. The paraffin was cut at 6 μm thickness, and stained with hematoxylin-eosin (HE). Sections were coverslipped with neutral resin, and an optical slice was examined under an Olympus DP73 microscope.

DNA extraction, bisulfite modification, and sequencing

Genomic DNA was extracted from the fish liver using Marine Animal Genomic DNA Kit (TransGen, China).The DNA concentration was quantified by the nucleic acid analyzer, Biodropsis BD-1000 (OSTC, China), and the integrity was analyzed with 1.5% agarose gel.

One microgram of genomic DNA was modified using the BisulFlash DNA Modification Kit (EpiGentek, USA) following the manufacturer's protocol. The genomic sequence of *igf1* (GenBank accession no. NM_001294198.1) was submitted to the online MethPrimer design software (http://www.urogene.org/methprimer/) to achieve CpG-rich regions and candidate CpG loci. Primers of exons 1, 2, and 3 were separately designed according to the known sequences using Oligo 6.0 (Table 1). After amplification with methylation-specific PCR, the products were separated by agarose gel and bands were purified by EasyPure Quick Gel Extraction Kit (TransGen, China). Purified products were, subsequently, cloned into a pEASY-T1 vector (TransGen, China), and transformed into *Trans1*-T1 Phage Resistant Chemically Competent Cell (TransGen, China). For each animal, 7–10 individual clones were sequenced to present the CpG dinucleotide positions in the three exons and the methylation status were computed.

To evaluate the efficiency of bisulfite conversion, the percentage of converted cytosine on the total number of cytosines (not in the context of a CpG dinucleotides), was determined in different clones and all tested individuals. For *igf1*, the mean percentage was 97.87 ± 0.45%.

RNA isolation, reverse transcript, and expression analysis

The relative expressions of *igf1* in the liver under salinity stress were detected by quantitative real-time PCR (qRT-PCR). Total RNA was isolated using an RNAiso Reagent Kit (TaKaRa, Japan) according to the manufacturer's instruction. The synthesis of the first-strand complementary DNA (cDNA) was conducted by using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) for 15 min at 37 °C. Gene-specific primers, *igf1*-F and *igf1*-R, were designed to amplify *igf1* (Accession: NM_001294198.1), and the β-actin (Accession: KP033459.1) served as an endogenous

Table 1 Nucleotide sequences of primers used in the experiment

Primer name	Sequence (5′ to 3′)	Length (bp)	Tm (°C)
igf1 exon1(BS-PCR)	F: AGTTGTTTTTTGTTGAAAATGTTTG R: AAACATCCCAAAAATACCACTAAA	298	61.4
igf1 exon2(BS-PCR)	F: TGTGTTGTATTTTTTGTAGTTATAT R: AATAAAAACCTCTCTCTCCA	150	56
igf1 exon3(BS-PCR)	F: ATTAATAGGTTATGGTTTTAATTTA R: TATTTTTTATCTTTTCTAACTACTA	248	49.4
igf1 (qRT-PCR)	F: CATCGCATCTCATCCTCTT R: CAGCACATCGCACTCTTG	171	55
β- actin (qRT-PCR)	F: GCTGTGCTGTCCCTGTA R: GAGTAGCCACGCTCTGTC	184	55

reference gene (Table 1) (Liu et al. 2014), to normalize the mRNA expression. The specificity and integrity were verified by PCR products which were submitted to gel electrophoresis. An SYBR Green RT-PCR assess with triplicate was carried out to determine the *igf1* mRNA expression. The PCR conditions and temperature profile were specified by the SYBR® Premix Ex Taq™ (Tli RNase H Plus) Kit (TaKaRa, Japan) on a Roche LightCycler 480 (Germany) of the qRT-PCR system. Each sample with triplicate was preformed simultaneously with internal control gene under the same conditions in the qRT-PCR system. The amplification efficiency was calculated as 1.02 by the standard curves according to serial dilutions of the original cDNA, to ensure that all the efficiency of the value ranges from 0.9 to 1.05. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was calculated to analyze the relative expression levels of *igf1*, while the threshold cycle (C_T) value was achieved using StepOne Software v2.3. Statistically significant difference was considered as $p < 0.05$.

Genetic structure analysis of *igf1*

The gene structure was analyzed by Splign software (http://www.ncbi.nlm.nih.gov/sutils/splign). The online MethPrimer design software (http://www.urogene.org/methprimer/) was used to achieve CpG-rich regions and candidate CpG loci. In addition, online ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was conducted to find the open reading frame (ORF). Transcription factor and the binding site were predicted using PATCH™ public 1.0 online software (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi).

Statistical analysis

Methylation levels and *igf1* expression data in liver under salinity stress were submitted to one-way ANOVA within Duncan's multiple range tests ($p \leq 0.05$), using the SPSS 19.0 software. Independent samples *t* test was conducted to compare the difference between females and males in CpG dinucleotide methylation and expression of *igf1*. The main outcome measure was the Pearson correlation coefficient (r) between the methylation level and the mRNA expression level. In all cases, statistical significance was accepted as $p \leq 0.05$.

Results

Salinity stress and liver morphology

We characterized liver structure by hematoxylin and eosin staining to detect damage due to salinity stress. Our data show that the liver tissues were severely damaged when fish were cultured in low salinity (Fig. 1). The liver was a snuff color at the beginning of the experiment on 0D in 15‰ salinity and was covered with a layer of compact connective tissue membrane. The polygonal-shaped hepatocytes (Fig. 1b) were large, cytoplasm-rich, and possessed a large round nucleus inclined toward the hepatic sinusoid. The hepatocytes formed a significant boundary and located radially around the central vein. After 7D, the liver manifested

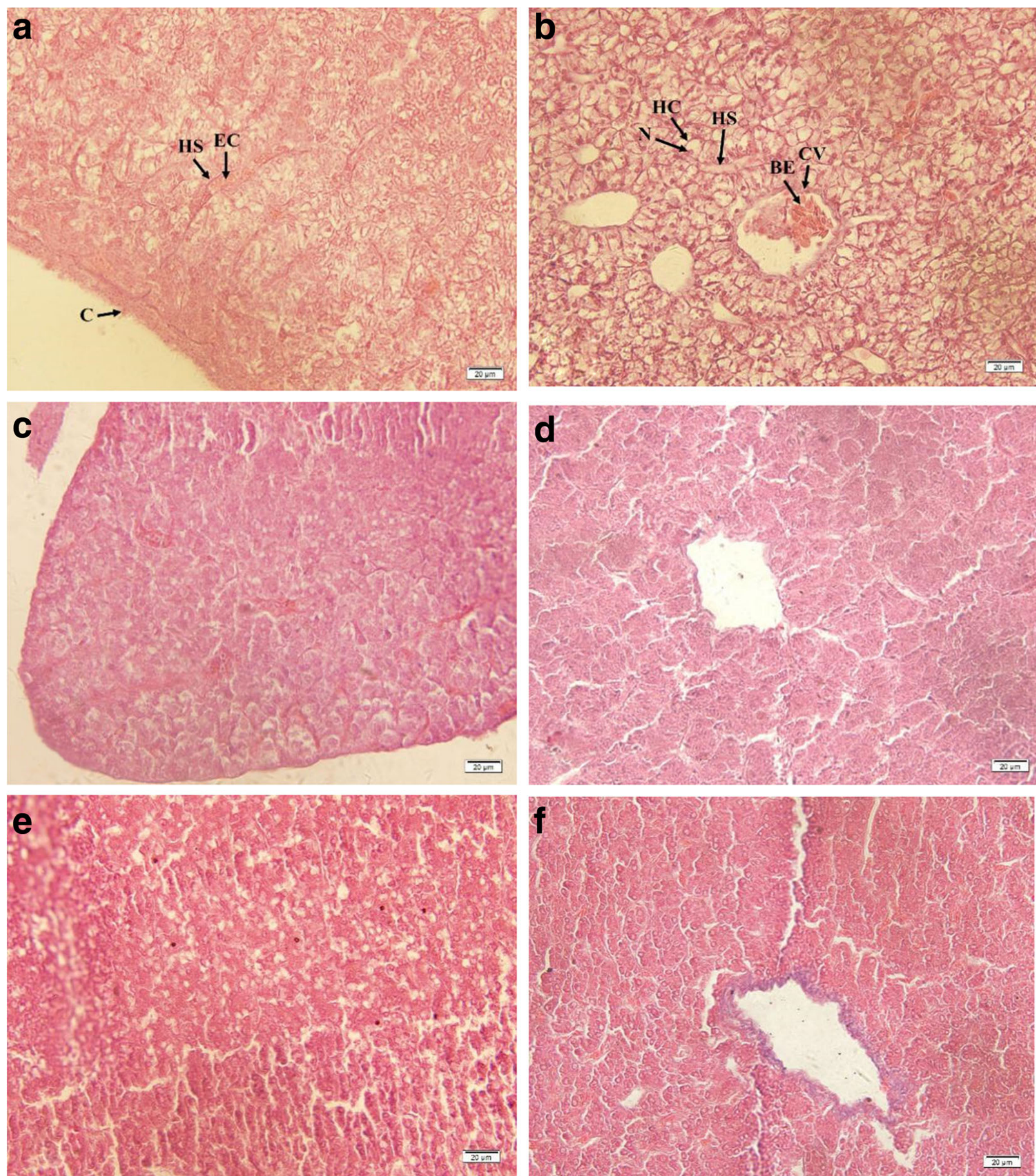


Fig. 1 Detail of the liver micrographs in half smooth tongue sole. **a**, **b** The liver under salinity 15‰ 0D. **c**, **d** The liver under salinity 15‰ 7D. **e**, **f** The liver under salinity 15‰ 60D. *C* capsule, *HS* hepatic sinusoid, *EC* erythrocyte, *HC* hepatocyte, *N* nucleus, *CV* central vein, *BE* blood erythrocyte. *Bars* 20 μm

a flea bite and some of the hepatocytes had swollen and were irregularly shaped (Fig. 1c, d). Moreover, the cytoplasm was less enchymatous, leading to neither a dense arrangement nor a well-aligned structure. Hepatocellular (Fig. 1e, f) injury was clearly visible after 60D exposure to 15‰ salinity. Almost all lipid vacuoles within hepatocytes had lost their normal structure and were swollen with degenerated fat.

At the beginning of the experiment (0D), the central vein in the liver was thin, and many erythrocytes were seen (Fig. 1b). The central vein became deformed after 7D (Fig. 1c, d) and was severely distorted with a broken wall after 60D exposure to 15‰ salinity (Fig. 1e, f).

The hepatic sinusoids were irregularly shaped and stacked in the interstitial space between the hepatic cord at the beginning of the experiment on 0D (Fig. 1a), and

many nucleated erythrocytes were observed. The hepatic sinusoids extended slightly after 7D, resulting in a larger space compared to that in the control group (Fig. 1c, d). The hepatic sinusoids were severely distorted, and hemorrhaging was evident after 60D (Fig. 1e, f). Taken together, these data indicate that low salinity culture evoked a stress response that significantly altered liver structure.

Predicted *igf1* structure

A schematic representation of the *igf1* gene structure is shown in Fig. 2. The *igf1* gene contains five exons (GenBank Accession no. NM_001294198.1), which encode an mRNA of 907 bp in length with a termination codon at position 803 bp. The predicted CpG-rich regions in *igf1* were 102 and 281 bp in length and included exons 1–3. The predicted open reading frame was located from 119 to 719 bp and encoded a 200-amino acid IGF-like protein. The predicted transcription factor binding sites are shown in Fig. 3. A sequence analysis of coding region of *igf1* exons 1–3 identified 13, eight, and 17 CpG sites, respectively. These sites were located at or near binding sites for a number of transcription factors that have a variety of transcriptional regulatory functions controlling cell proliferation and apoptosis, differentiation, and cancer, such as POU1F1, AP-2alpha, Ap-1, Sp1, ZAC-1a, AhR, HES-1, NF-1/L, CTCF, WT1-KTS, and others.

CpG methylation levels and *igf1* expression status are correlated with low salinity rearing

To determine whether low salinity altered the *igf1* CpG methylation levels, we carried out CpG DNA methylation and mRNA expression analysis of the igf1 gene. The *igf1* CpG methylation levels increased significantly in response to salinity stress. As expected, the analysis showed that CpG methylation had a negative correlation with *igf1* mRNA expression levels.

The methylation levels differed significantly among the three exons. Figure 4 shows the CpG methylation levels of exons in females and males at the beginning of the experiment. Exon 1 had the lowest methylation level, occupying 8.37%, whereas the methylation level of exon 2 was moderate at 46.53%. The vast majority of CpG sites was methylated in exon 3 and were as high as 89.33% in females. A similar CpG methylation pattern was observed in males with methylation levels in exons 1, 2, and 3 of 7.49, 50.21, and 88.23%, respectively. Methylation levels of the *igf1* exons in the livers of male and female half smooth tongue sole were in the order of exon 1 < exon 2 < exon 3 ($p < 0.001$).

The association between low salinity and methylation status was investigated by bisulfite sequencing, and the methylation levels of each exon were determined. As shown in Fig. 5a, CpG methylation levels in exon 1 of females decreased significantly after 7D in 15‰ salinity ($p < 0.05$), followed by a continuous decline after 60D ($p < 0.05$). Interestingly, CpG methylation in exon 2 decreased initially and then increased significantly ($p < 0.05$). In contrast, the methylation ratio of exon 3 was slightly upregulated and prolonged in the 15‰ salinity treatment. As shown in Fig. 5b, males subjected to low salinity treatment had similar methylation levels in exon 1 after 7D compared to those at the beginning of the experiment (0D). However, CpG methylation decreased significantly after 60D ($p < 0.05$). Exon 2 in male fish showed a similar dynamic CpG methylation pattern as that of females. CpG methylation in exon 2

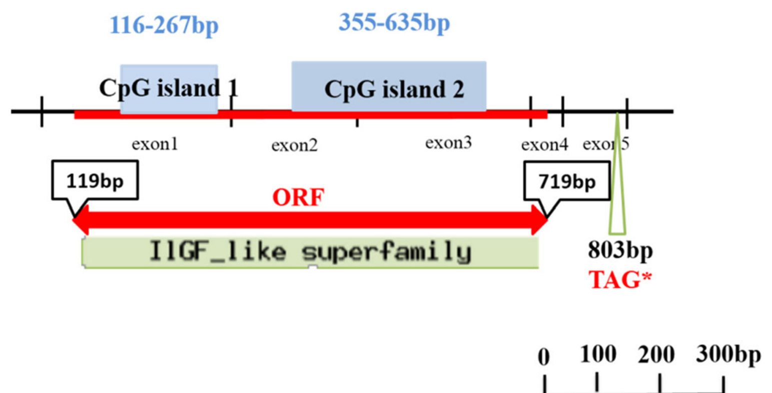


Fig. 2 Schematic representation of *igf1* mRNA structure exhibiting the distribution of five exons. In this study, the *red box* indicates the open reading frame (ORF), locating 119–719 bp. The two predicated CpG islands are depicted by *blue boxes*; a termination codon of TAG was located in 803 bp, labeled in *red font* (color figure online)

Fig. 3 The gene structure analysis of *igf1* (exon 1, exon 2, and exon3). The *underlined red letters* show the CpG dinucleotide sites on exon 1, exon 2, and exon 3. The stained frames indicate the binding sequences of forecasted transcription factors with transcription factors marked upon it

exon1

POU1F1
GTTGAAAATGTCTGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATCCG
1CpG

TCTCCTGTTCGCTAAATCTCACTTCTCCAAAACGAGCCTGCGCAATGGAACAAA
2CpG 3CpG 4CpG

LUN-1 NF-ATp
GTGGGAATATTGAGATGTGACATTGCATCGCATCTCATCCTCTTTCTTTCCTCCTC
5CpG 6CpG

Gbx2 RAR-alpha1
GGCCCCGTTTTTTAATGACTTCAAACAAGTTCATTCTCGCCGGGCTTTTGACTT
7CpG 8CpG 9CpG

AP-2 AP-2alpha HrpF
GCGGAGACCCGTGGCCGTGGGGATGTCCATCTCTGCTCCGTCCTTCCAGTGGC
10CpG 11CpG 12CpG 13CpG

ATTTCTGGGATGTTCTCAAG

exon2

Sp1
TGTGCTGTATCTCCTGTAGCCACACCCTCTCACTACTGCTGTGCGTCCTCAC
1CpG

AP-1 Sp1 Sp1 ZAC-1a
CCTGACTCAGGCGGCAGCAGGGGCGGGCCCGGAGACCCTGTGCGGGGC
2CpG 3CpG 4CpG 5CpG 6CpG

LBP-1 AhR
GGAGCTGGTCGACACGCTGCAGTTTGTGTGTGGAGAGAGAGGCTTTTAT
7CpG 8CpG

TTCA

exon3

HES-1 NF-1/L NF-Y CTCF
ACGGCGGTCTCGTGGCATCGTGGACGAGTGCTGCTTCCAAAGCTGTGAG
1CpG 2CpG 3CpG 4CpG 5CpG

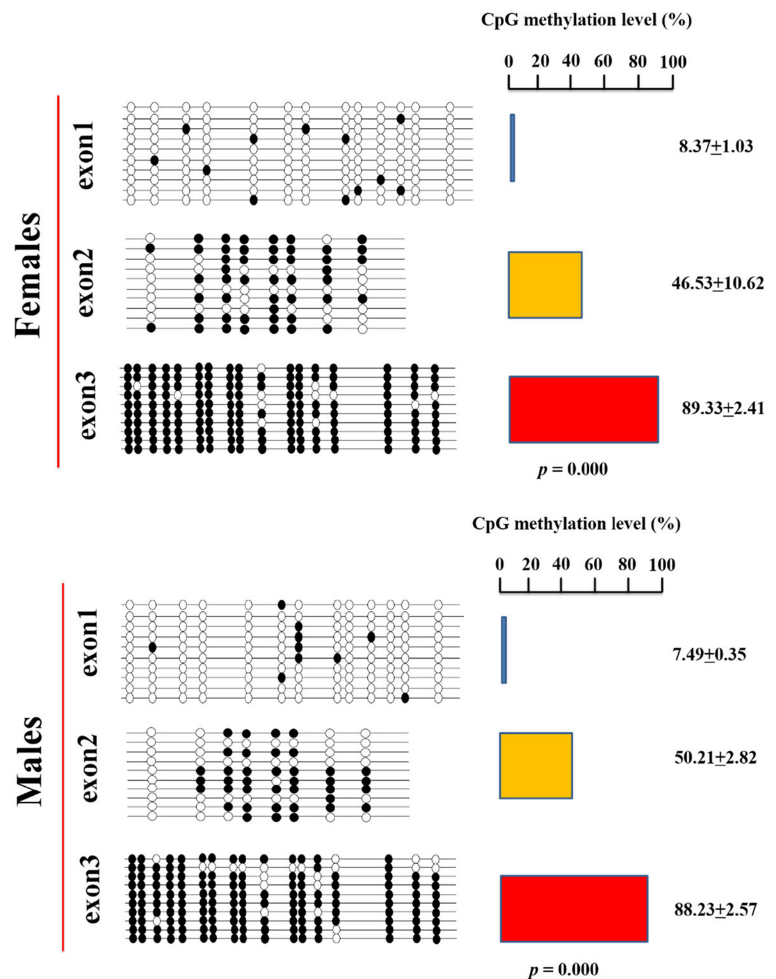
WT1-KTS LBP-1
CTGCGGCGCCTGGAGATGTACTGCGCGCCAGCCAAGACTGGCAAAGCAG
6CpG 7CpG 8CpG 9CpG

Sp1 TR2-11
CTCGTTCTGTGCGCGCACAGCGCCACACGGACCTCCCCAGAGCACCTAA
10CpG 11CpG 12CpG 13CpG 14CpG

Sp1 MAPF2
GGTCAGCACCCCAGGGCACAAGGCGGACAAAGGTTAGGAGCGTAGGAT
15CpG 16CpG

NF-E
AGCGTAGTAGTTAGAAAAGATAAAAAATA
17CpG

Fig. 4 The presentation of the CpG methylation levels in three exons of *igf1* in the liver of untreated females and males. One fish representative of the methylation level is demonstrated. A *filled* or *open circle* indicates CpG positions methylated or unmethylated in the exon site, respectively. Ten clones per fish were used to determine the average methylation levels, which were specifically calculated outside the *bar*. Data represents mean ± SD; $p = 0.000$ shows the significant differences between exons with Duncan's test



decreased slightly after 7D and then increased moderately after 60D. CpG methylation in exon 3 increased significantly compared with that of exon 1. This increase was positively correlated with the low salinity stress, showing all too frequent aggrandizement after 60D ($p < 0.05$). Collectively, these data suggest that mean *igf1* CpG methylation levels in females declined slightly from the beginning of the experiment (52.62%) to 7D (50.33%, $p > 0.05$) and then spiked significantly after 60D (57.61%, $p < 0.05$).

To test whether alterations in CpG methylation due to salinity stress affect igf1 gene expression, we analyzed *igf1* expression by quantitative polymerase chain reaction (qPCR) analysis. The correlation between *igf1* methylation level and gene expression is shown in Fig. 6a. A highly negative correlation was detected between CpG methylation and *igf1* expression ($r = -0.795$, $p < 0.05$). Relative *igf1* expression in the liver increased and then decreased significantly in all treatments ($p < 0.05$), with values of 1.00, 1.22, and 0.02, respectively. The mean *igf1* methylation level at the beginning of the experiment (0D) in males (Fig. 6b) was 52.60%, which increased slightly to 52.90% after 60D and then to 55.91% ($p < 0.05$). igf1 gene expression was as high as 1.17 at the beginning of the experiment (0D), decreased significantly to 0.16 after 7D ($p < 0.005$), and continued to decrease to 0.01 after 60D. Taken together, these data suggest that the increase in the *igf1* methylation level may have repressed its gene expression after 60D in low salinity ($r = -0.413$).

Furthermore, the single CpG methylation sites are analyzed with salinity stress. The particular DNA methylation sites in exon 2 significantly change with low salinity (Table 2). The seven-CpG site of exon 2 in females under salinity 15‰ 0D was 41.90%, then decreased to 33.60% for 7D. After 60D low salinity stress,

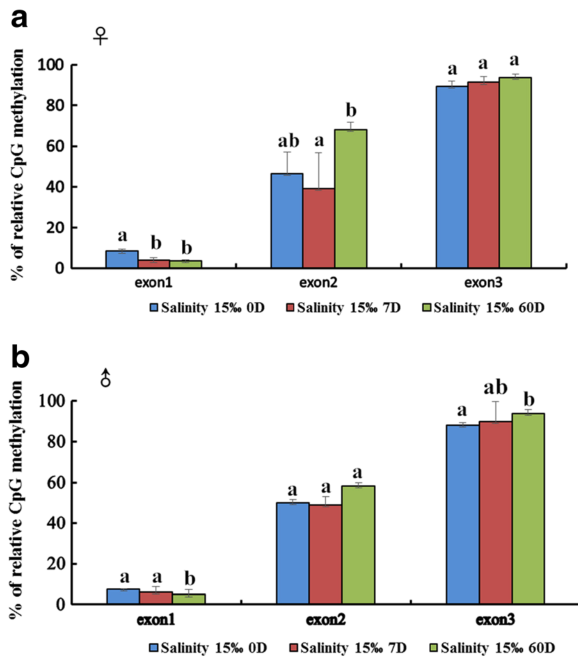


Fig. 5 The differences in methylation levels (%) of three exons in the female (**a**) and male (**b**) half smooth tongue sole liver according to low salinity treatments. *Different lowercase letters* represent significances subjected to salinity stress ($p < 0.05$, Duncan's test)

the methylation level of the seven-CpG site was significantly ($p < 0.05$) increasing to 67.13%. In exon 2 of male fish, the four-CpG site significantly ($p < 0.05$) reduced from 73.33 to 53.33%, then significantly ($p < 0.05$) raised to 65.70% with salinity treatment. The six-CpG site was 73.33%. When subjected to salinity 15‰ for 7D and 60D, it was significantly ($p < 0.05$) lower (60 and 50.97%, respectively). We inferred that these particular DNA methylation sites in exon 2 were more sensitive to low salinity.

Sex bias in methylation and expression levels

To test whether a sex bias exists in methylation and expression levels under various salinities, the methylation levels of *igf1* and its exons, as well as *igf1*

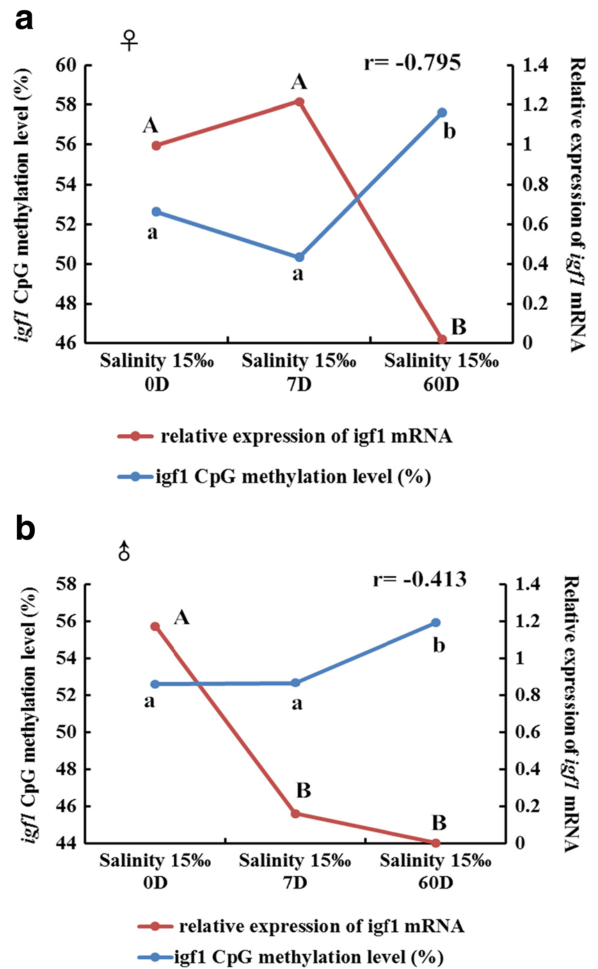


Fig. 6 The correlation between CpG methylation level and mRNA expression of *igf1* in females (**a**) and males (**b**) when subjected to low salinity. *Different lowercase or uppercase letters* represent significances subjected to salinity stress ($p < 0.05$, Duncan's test)

expression were analyzed using the independent sample *t* test. As shown in Table 3, the methylation levels of the *igf1* exons exhibited similar changes in response to the low salinity treatments in both female and male fish ($p > 0.05$). Regardless of sex, methylation levels after 60D were significantly higher than those recorded at the

Table 2 Particular CpG methylation sites in exon 2 significantly vary with salinity stress

Treatments	7-CpG site (♀)	4-CpG site (♂)	6-CpG site (♂)
Salinity 15‰ 0D	41.90 ± 14.35%a	73.33 ± 2.89%a	73.33 ± 2.89%a
Salinity 15‰ 7D	33.60 ± 14.56%a	53.33 ± 5.77%b	60.00 ± 0.00%b
Salinity 15‰ 60D	67.13 ± 6.22%b	65.70 ± 7.45%a	50.97 ± 8.59%b

Data represents mean + SD; different lowercase letters represents significances subjected to salinity stress ($P < 0.05$, Duncan's test)

Table 3 Methylation and expression levels of *igf1* in female and male half smooth tongue sole under salt treatments

Treatments		Methylation level (%)				Expression level
		Exon 1	Exon 2	Exon 3	Total	*igf1*
Salinity 15‰ 0D	♀	8.37 ± 1.03	46.53 ± 10.63	89.33 ± 2.41	52.62 ± 1.41	1.00 ± 0.07
	♂	7.49 ± 0.35	50.21 ± 2.82	88.23 ± 2.57	52.60 ± 1.76	1.17 ± 0.27
Salinity 15‰ 7D	♀	3.76 ± 1.41	39.17 ± 17.55	91.20 ± 2.97	50.33 ± 4.01	1.22 ± 0.27a
	♂	6.01 ± 1.56	49.17 ± 4.02	90.00 ± 1.56	52.67 ± 1.21	0.16 ± 0.06b
Salinity 15‰ 7D	♀	3.59 ± 0.45	68.16 ± 3.61	93.96 ± 1.30	57.61 ± 0.51	0.02 ± 0.00A
	♂	4.87 ± 1.17	58.33 ± 9.55	93.79 ± 2.01	55.91 ± 1.72	0.01 ± 0.00B

Data represents mean ± SD; different lowercase or uppercase letters represent significance difference between females and males when subjected to salinity stress ($p < 0.05$, *t* test)

beginning of the experiment (0D) ($p < 0.05$). Interestingly, a sex bias occurred in *igf1* expression. Although *igf1* gene expression levels were not different under 15‰ salinity at the beginning of the experiment (0D) in female and male half smooth tongue sole, females showed significantly higher *igf1* expression levels than did males after 7D ($p < 0.05$). *igf1* expression levels in females were significantly lower than those of males after 60D, ($p < 0.05$). However, *igf1* expression levels decreased significantly in both females and males after 60D compared with those at the beginning of the experiment (0D).

Discussion

Salinity is an inherent physicochemical composition in water, representing one of the most important environmental stimulating factors. Studies regarding the effects of salinity on animal growth have been carried out mostly in marine stenohaline and euryhaline fish, particularly species that experience gradual movement from saltwater to freshwater (Boeuf and Payan 2001). Igf1 acts importantly in osmoregulation process, and it is sensible to salinity variation of the external environment (Martins et al. 2014). It is also central to fish development, growth, and reproduction and is expressed in a wide variety of tissues with the highest levels expressed in the liver (Duan 1998). The present study attempts to better understand transcriptional regulation of *igf1* expression and changes in liver morphology in a euryhaline fish held at low salinity. We demonstrated increased DNA methylation and decreased *igf1* mRNA expression in response to low salinity.

Low salinity negatively affects liver histology

As a euryhaline flatfish, half smooth tongue sole shares common liver histology with freshwater and marine teleosts, such as *Cyprinus carpio* (Sáez et al. 1984), *Gadus macrocphalus* (Fujita et al. 1986), *Salmo gairdneri* (Schulz 1986), and *Xiphophorus helleri* (Fang and Lin 2006). The liver of half smooth tongue sole is covered with a layer of connective tissue and has no distinct boundary between the hepatic lobules as observed in higher animals (Ding et al. 2007; Liu and Xing 2011; Que and Lou 2015). Xie et al. (2004b) speculated that these vague boundaries are presumably a common characteristic of the liver of Osteichthyes. Similar results have been reported for *Symphlebia meridionalis* (Liu and Zhang 2001), *Leptobotia elongata* (Chen et al. 2002), *Pelteobagrus vachelli* (Xie et al. 2004a), *Claris fuscus* (Luan et al. 2001), and *Muraenesox cinereus* (Xie et al. 2004b). The liver of Osteichthyes has been described completely; it has no typical portal area and randomly distributed veins, bile duct, and arterioles. The liver arterioles and bile duct of *S. gairdneri* form an arteriole-bile duct system, and arterioles are rare compared to the bile duct and veins (Hampton et al. 1985, 1988; Rocha et al. 1994; Guo and Lu 1994). This structure was supported by our light microscopic observations.

The liver is an important digestive gland in fish, and it maintains reserves of lipids, sugars, and protein. The liver is central in maintaining and stabilizing the equilibrium of energy metabolism. Liver tissue has been studied in detail because of its complex functions, such as detoxification and defense (Hinton and Lauren 1990). The detoxification function of the liver is limited in fish exposed to long-term environmental stress, as stress

impedes normal physiological functioning and can lead to structural lesions, accompanied by weight variations and abnormal hepatocyte morphology. Many internal and external factors, such as temperature, salinity, dietary composition, heavy metals, and external extrusion pressure, contribute to hepatotoxicity (Xu et al. 2005; Liu et al. 2010; Du 2014; Tang et al. 2014). The effects of salinity stress on fish growth and physiology have been widely studied (Likongwe et al. 1996; Tian et al. 2010). Here, we showed that salinity stress severely damaged the liver of half smooth tongue sole. We observed the loss of normal hepatocyte structure, such as degenerated lipid vacuoles, swelling, and degenerated fat. The hepatic sinusoids and central vein were severely distorted accompanied by hemorrhage. Longer-duration exposure to low salinity stress led to more severe liver damage. These findings are consistent with Choi et al. (2008) who reported that salinity changes disrupt osmotic homeostasis in *O. keta* (Liu et al. 2010). Energy must be expended for osmoregulation though breakdown of glycogen to regain homeostasis (Sangiao-Alvarellos et al. 2003). Excessive production of reactive oxygen species and lipid peroxidation can cause apoptosis and necrosis of hepatocytes (Choi et al. 2008; Yin et al. 2011). The vacuoles we observed in hepatocytes may have been caused by disrupted synthesis and release of compounds by the liver (Gingerich 1982). Taken together, low salinity destroyed the alexipharmic and energy metabolic functions of the liver, resulting in aberrant intracellular homeostasis (Lundebye et al. 1999).

Low salinity alters *igf1* DNA methylation and mRNA expression levels

Igf1 is a single-chain 70-amino acid polypeptide (7.5 kD) that includes the B, C, A, and D functional domains. Igf1 is exclusively synthesized in the liver and secreted into the circulating system where it acts as a key regulator of fish growth, through cell metabolism, cell proliferation, and differentiation, and immune-related hormone secretion (Jones and Clemmons 1995; Liu and Tong 2004). As we demonstrated in this study, the predicted *igf1* CpG-rich regions were 102 and 281 bp in length, including exon 1, exon 2, and exon 3, respectively. The methylation levels of these three exons in the livers of male and female fish were significantly different. The methylation level of exon 3 was significantly higher than that of exon 2 ($p < 0.001$), and that of exon 2 which was significantly higher than the methylation level in exon 1 ($p < 0.001$). Interestingly, more putative transcription factor binding sites were located in the CpG sites away from the promoter, such as in exons 2 and 3. Notably, the DNA methylation patterns in these three exons were completely different after the fish were exposed to 15‰ salinity for 7D and 60D. Methylation in exon 1 decreased continuously, whereas that in exon 2 declined initially and then increased, whereas methylation in exon 3 increased gradually. Methylation has three statuses in eukaryotic cells: sustained hypomethylation (methylation level of housekeeping genes), induced demethylation (modification in developmental-stage-specific genes), and hypermethylation (inactivated X chromosome in women) (Kang et al. 2013). Li et al. (2012) reported that genomic hypomethylation in the human germline is associated with selective structural mutability, as hypermethylation affects the DNA strands and transcription, resulting in inactivation of genes (Kang et al. 2013). In addition, Yano et al. (2003) found that DNA methylation regulates tissue-specific gene expression. These results suggest that the DNA methylation pattern may have its own genetic characteristics. Hypomethylation of exon 1, which was in close proximity to the promoter, is indispensable for promoting gene transcription and expression. Moreover, the different hypomethylation patterns in fish maintained in low salinity suggest that different gene structures might be involved in diverse regulatory mechanisms. In addition, CpG methylation at the transcription factor binding sites could block binding of transcription factors and repress gene expression. We speculate that hypermethylated sequences far from the promoter had more binding sites located in CpG sites.

Growing evidence indicates that environmental and genetic stimuli cooperatively act on heritable phenotypic variation through changes in methylation (Angers et al. 2010). It is now recognized that DNA methylation plays an important role in the stress response (Bird 1986; Hashida et al. 2006; Pilsner et al. 2007). Changes in the DNA methylation pattern have been observed in *Jatropha curcas* L. (Mastan et al. 2012) and two rice genotypes (Wang et al. 2011) under salt stress conditions, which probably induce immediate adaptive responses. In addition, Navarro-Martín et al. (2011) reported that DNA methylation is affected by temperature change, which may shift the sex ratio of European sea bass. We showed that *igf1* DNA methylation levels in the liver of female half smooth tongue sole decreased

under 15‰ salinity stress for 7D, but increased significantly after 60D. In contrast, *igf1* mRNA expression increased initially and then decreased significantly with time. Kovalchuk et al. (2003) showed that hypomethylation regulates gene expression in response to stress, which has been considered an indirect defense mechanism. Our results suggest that low salinity stress was involved in decreasing the *igf1* methylation level and increasing the expression level in females for 7D. We speculate that the expression of *igf1 mRNA* was upgraded to participate in molecular or cellular repair in female half smooth tongue sole and ensure fish survival. A similar conclusion was reached in *P. crocea* that appropriately low salinity increases *igf1* expression and facilitates growth (Li et al. 2015). Bamman et al. (2001) and Hambrecht et al. (2005) reported that locally produced Igf1 increases following acute muscle damage. In the present study, *igf1* methylation levels surged and gene expression plummeted in females exposed to 15‰ salinity, whereas *igf1* methylation levels increased gradually and changed significantly by 60D in males, and gene expression decreased continuously, particularly after 7D. A chronic low salinity environment may inhibit somatic growth of half smooth tongue sole, as energy was expended for osmoregulation rather than growth (Martins et al. 2014). Hormones such as IGF are crucial for osmoregulation and respond to physiological stress (Barton and Iwama 1991; Boeuf and Payan 2001). In the research of Zhang et al. (2015), they found that low salinity may have directly down-regulated GH and combined with growth hormone receptor or other regulators to decrease transcription of GH receptors (GHRs), which repressed downstream *igf1* expression. In addition, *igf1* mRNA expression also decreased significantly in *Oncorhynchus kisutch* in response to low temperature (Larsen et al. 2001). Many studies have demonstrated that nutritional status and growth are positively correlated with *igf1* mRNA expression in *Micropterus salmoides*, *Anguilla japonica*, *Lates calcarifer*, *Ictalurus punctatus*, *Epinephelus coioides*, *O. mykiss*, *Oreochromis niloticus*, and other species (Matthews et al. 1997; Dyer et al. 2004; Small and Peterson 2005; Pedroso et al. 2006; Li and Leatherland 2008; Chen et al. 2010). In our study, gene expression was negatively correlated with DNA methylation levels in females ($r = -0.795$) and males ($r = -0.413$). CpG methylation represses gene transcription by inhibiting gene regulatory elements, particularly transcription factor binding sites, or allowing methyl-binding proteins to incorporate into methylated DNA, resulting in “closing” of the chromatin structure (Ziller et al. 2013). This observation is consistent with previous reports that DNA methylation can strongly impede gene expression (Fitzpatrick and Richards 1991; Hsieh 1997; Boerboom et al. 1999; Fürbass et al. 2001; Irvine et al. 2002). Although female half smooth tongue sole grow two to three times faster than males, we found no differences in *igf1* methylation levels between the sexes under 15‰ salinity for 0D, 7D, or 60D, indicating that *igf1* is not a sex-related gene. *igf1* mRNA expression was not different in males and females at the beginning of the experiment. However, after 7D and 60D low salinity stress, its expression levels in females significantly exceeded those in males, suggesting the differential response of *igf1* to low salinity in female and male fish. The finding that *igf1* expression differed between the sexes, but not methylation, confirms that, in addition to DNA methylation, other genomic epigenetic modifications, including histone modifications, short RNAs, chromatin silencing, and other factors, are also involved in regulating gene expression (Li et al. 2012).

Conclusions

The present study was carried out to characterize salt-induced tissue damage and changes in DNA methylation and *igf1* mRNA expression in *C. semilaevis* maintained under low salt conditions. Our results depict the effects of low salt on liver tissues and the molecular mechanisms involved in the stress response through epigenetic and genetic regulation. We showed that low salt evoked a noticeable hepatotoxic response that affected fish liver. And epigenetic modification plays an important role in fish when it is subjected to an adversity environment. Meanwhile, it appeared that *igf1* DNA methylation was crucial for regulating *igf1* transcription. *igf1* was not a sex-related gene in half smooth tongue sole, as no differences in methylation or expression levels were detected between females and males in the control group. We found that exons 1, 2, and 3 had significantly different methylation levels under salt stress. More putative transcription factor binding sites were located in CpG sites as methylation level increased. More work is needed to confirm changes in organelles and biochemical processes. The present assay is the first to show direct salt damage, combined with changes of DNA methylation and mRNA expression of *igf1*.

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Authors' contributions SL carried out the histology observation, DNA bisulfite modification and sequencing, and RNA expression procedures, and drafted and wrote the manuscript; FH, HW, and JL designed and guided the experiment, and participated in the manuscript modification and coordination; YS performed the fish feeding and sampling; ML and LM are involved in DNA and RNA extraction; and YH took part in methylation data interpretation. All authors read and approved the final manuscript.

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