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Starvation-induced changes in sex ratio involve alterations in sex-related gene expression and methylation in Pacific oyster *Crassostrea gigas*

Dongfang Sun [a], Hong Yu [a], Qi Li [a,b,*]

[a] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

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ABSTRACT

Aquatic animals are subject to varying degrees of starvation stress in their natural habitats due to food limitations. Consequently, starvation is a crucial environmental factor for sex determination in many species; however, limited research has been conducted on the effects of starvation on sex determination in shellfish. Here, four full sibling families of Pacific oysters were established and subjected to starvation stress. The results demonstrated that starvation caused the sex ratio (female to male) to change from 1:0.78 to 1:1.44 and resulted in a delay in gonadal development. Further studies revealed that the expression levels of DNA methylation-related genes *Dnmt1* (DNA methyltransferase 1), *Dnmt3a*/b (DNA methyltransferase 3a/b) and *Tet3* (tet methylcytosine dioxygenase 3) decreased under starvation stress. Conversely, the upregulation of *Dmrt1* (doublesex and mab-3 related transcription factor 1), a gene typically associated with males, in females suggests that the increased proportion of males may be linked to starvation-induced high expression of this particular gene. In addition, the gene *Dgkd* (diacylglycerol kinase delta), which is involved in the regulation of second messenger protein kinase C, was differentially methylated between males and females, with the methylation level of this gene gradually increasing with male development, while the methylation level of this gene decreased under starvation stress. Correlation analysis of *Dgkd* methylation levels with expression levels showed a negative correlation between DNA methylation and gene expression. Finally dual fluorescence reporter experiments confirmed that DNA methylation suppressed *Dgkd* expression in vitro. In summary, the results suggest that starvation may regulate *Dgkd* gene expression through DNA methylation and thus affect *Dmrt1* expression, thereby causing sex reversal in the Pacific oyster. The outcomes resolved how environmental factors are involved in sex reversal from an epigenetic perspective and provided a theoretical basis for sex control breeding in the Pacific oyster.

1. Introduction

Sex control of aquatic animals provides a significant application value for improving the efficiency of aquaculture. Male and female individuals of many aquatic animals differ significantly in economic traits (e.g., growth rate, size). In fish culture, the growth rate of most fish slows down after sexual maturity, and natural reproductive activities also bring growth stagnation and reduction of the edible part of body tissues (Cnaani and Levavi-Sivan, 2009; Martinez et al., 2014; Li et al., 2022). In addition, the study of sex control in aquatic animals is biologically significant for the elucidation of theoretical issues such as sex differentiation and sex determination mechanism.

The Pacific oyster (*Crassostrea gigas*) is a world-wide cultured species and economically important in the oyster farming industry. As a species with sequential hermaphroditism, the sex determination mechanism of Pacific oyster has been extensively studied. So far, the sex chromosomes have not yet been identified in oysters. *Foxl2* (forkhead box l2) and *Dmrt1* (doublesex and mab-3 related transcription factor 1), key homologous genes that are conserved in the animal kingdom, exhibit sexual dimorphic expression and serve an essential role in gonadal differentiation and gender maintenance (Naimi et al., 2009; Yue et al., 2021; Zhang et al., 2014). In addition to the genetic influence on sex, environmental factors also contribute definitively to the sex of oysters. Under warm and well-fed conditions, the sex ratio is biased toward females, and vice versa toward males (Quayle, 1988). As a sedentary intertidal species, oysters are subject to varying degrees of starvation stress due to tidal influences. Besides, the increase in shellfish aquaculture production leads to a progressive strain on food resources in

* Corresponding author at: Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China.
E-mail address: qili66@ouc.edu.cn (Q. Li).

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cultured marine areas, which may eventually alter the sex ratio of natural populations. Periodically skewed sex ratios, especially those biased toward males, could reduce the number of effectively reproducing populations (Baeza et al., 2010) and make populations vulnerable to other external factors that could lead to population declines. Therefore, a balanced sex ratio is important for population dynamics, and consequently, oyster ecology. As food stress impacts sex ratio, it is therefore an important factor to consider in assessments of sex determination in oysters.

Environmental induced changes in DNA methylation pattern appear to be driving various phenotypes, including sex-ratio (Lim et al., 2021; Riviere, 2014). For example, ocean acidification can affect gene expression by influencing the addition or removal of methyl groups through epigenetic modification processes, ultimately leading to altered phenotypes in oysters (Dang et al., 2023; Lim et al., 2021). Epigenetic modifications and gender-specific gene expression alterations have been linked to the impact of starvation (Sakae et al., 2020), leading to prolonged reproductive cycles and suppression of gametogenesis in invertebrates (Teaniniuraitemoana et al., 2016), and gonadal degeneration in non-mammals (Fan et al., 2019) In addition, in zebrafish, medaka and bivalves, sex ratio is also influenced by starvation (Fan et al., 2019; Sakae et al., 2020; Stenyakina et al., 2010). The process of sex determination/gonad development in invertebrates is accompanied by dynamic regulation of DNA methylation, with significant contributions from both DNA methyltransferases and demethylases (Li et al., 2019; Yang et al., 2014). The function of DNMT1 is to maintain DNA methylation levels in vivo, while DNMT3a/b is a “de novo” DNA methylation enzyme in mammals and arthropods (Bestor, 2000; Du Preez et al., 2020). The demethylase TET3 is a dioxygenase that promote DNA demethylation by oxidizing the methyl group of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) in mammals and arthropods (Du Preez et al., 2020; Wu and Zhang, 2017). The activity of DNA methylation related enzymes is associated with nutrient metabolites in mammals (Kadayifci et al., 2018), indicating that organisms attain the capability to respond to the external environment factors through epigenetic regulation. In oysters, differentially methylated genes between the sexes are mainly related to metabolism (Sun et al., 2022a), such as diacylglycerol kinase gene. DGK-delta (diacylglycerol, DGKD) is a member of the diacylglycerol kinase family that catalyzes the conversion of DAG phosphorylation to phosphatidic acid, and DAG, and as such has an essential function in the AMPK signaling pathway and lipid metabolism in mice and nematodes (Jiang et al., 2016). However, whether DNA methylation differences between sexes are influenced by starvation stress and thus alter sex ratio bias is uncertain.

Here, we performed starvation stress experiments by constructing four whole sibling families. We calculated the sex ratio and the expression levels and methylation levels of DNA methylation-related enzyme genes and sex-related genes after stress. In addition, we explored the methylation and expression patterns of the *Dgkd* promoter under starvation stress. The findings have significant ecological and commercial implications for comprehending the phenomenon of sex change induced by starvation in oysters.

2. Materials and methods

2.1. Oysters rearing and starvation experimental design

Both the germline construction and starvation stress experiments were conducted in the Laizhou breeding nursery farm in Yantai City, Shandong Province, China. Four female oysters and four male oysters were dissected at gonadal maturity and then artificially fertilized one-on-one to form four groups of fully sibling lineages. Fertilized eggs were incubated and larvae were raised in 80 L buckets. The attachment substrate was placed when 50% of the larvae reached the eye-spot stage, and then the attached larvae were placed in the sea area to culture in Rongcheng, Shandong Province, China (37.11°N , 122.35°E) (Fig. 1).

After 9 months, four families of oysters (shell height: 74.1 ± 8.9 mm, weight: 37.3 ± 7.7 g) were taken back to the farm from the sea area for a week of temporary rearing. The experiments were set up as feeding (control) and starvation groups, each consisting of 100 oysters per family. The oysters were placed in 1000 L plastic buckets under the controlled temperature of 20 °C and the whole amount of seawater (30 psu) was replaced every two days. During the starvation stress period, the oysters were managed normally, with the control group fed *Platymonas subcordiformis* (25,000 cells mL^{-1}) every 6 h by using an automatic feeding machine, and the starvation group not fed. On days 10 and 20 of starvation stress, gonadal tissue was taken from ten oysters per family in the starvation and control groups, respectively. Three portions of gonadal tissue were sampled from each oyster and preserved in Bouin’s fluid, RNAstore and 95% alcohol, respectively (Fig. 1). After 20 days of starvation stress, the starvation group was fed to develop gonads to the point where males and females could be identified in order to count sex ratios. On day 40, all oysters (at least 40 oysters per family line) were dissected and sex identification was performed by microscopic examination facilitating assessment of the female to male sex ratio.

2.2. Histological observation of the gonads

Oyster gonad tissues sampled on day 10 and 20 were fixed using Bouin’s fluid for 12 h and then replaced with 70% alcohol. The tissues were dehydrated in xylene, embedded in paraffin and cut to 5 μm, and finally stained with hematoxylin-eosin. Gonadal development was staged according to previous studies (Lango-Reynoso et al., 2000).

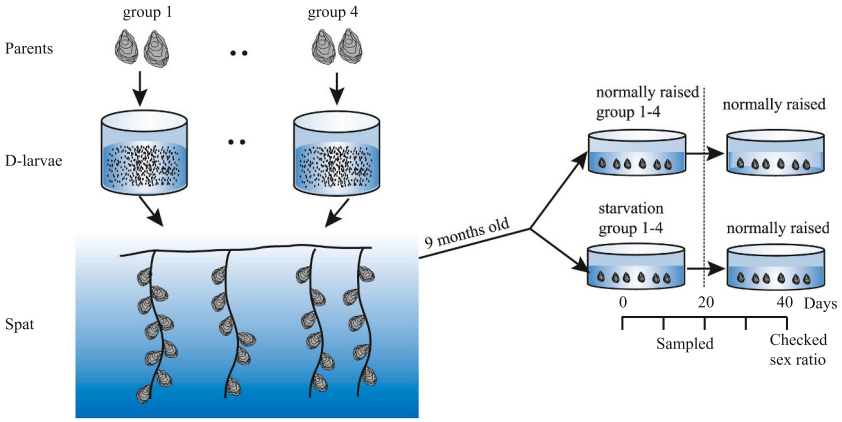

Fig. 1. Experimental design of the Pacific oyster starvation treatment. Four full sibling families were constructed and raised to adult, and then treated with starvation stress experiment.

2.3. Relative gene expression

cDNA templates of different developmental periods of the gonads were used from previous experiments (Sun et al., 2022b). Gonadal tissues (50 mg) from 10 and 20 days of starvation and controls were homogenized in 1 mL TRIzol using a high-speed low temperature grinding machine (Servicebio, China). Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration and integrity of RNA were detected utilizing nanodrop 2000 micro-UV spectrophotometer and 1% gel electrophoresis. The synthesis of cDNA was performed using HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, China) in the 20 μL reaction system containing 1 μg of total RNA. PCR efficiency and proper dilution of cDNA was determined for each primer pair by constructing a five points standard curve from 5-fold serial dilutions of a male or female gonad template. The melting curves of PCR products (60 to 95 °C) were performed to ensure the detection of a single specific product. The cDNA was diluted 10-fold and analyzed by qPCR for *Foxl2* (LOC105319597), *Dmrt1* (LOC105337844), *Tet3* (LOC105322168), *Dnmt1* (LOC105330054), *Dnmt3a/b* (LOC105334030) and *Dgkd* (LOC105324494), respectively. The 20 μL reaction system containing 2 μL of diluted cDNA was configured with ChamQ SYBR Color qPCR Master Mix (High ROX Premixed) as described in the manual. qRT-PCR was performed on a LightCycler 480 II detection system (Roche, Switzerland) with the following program: initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s, and a final dissociation curve analysis of 1 cycle at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Three biological replicates, with each replicate being pooled by five oysters and two technical replicates per gene were utilized for qPCR analysis. The elongation factor I (*Ef1*) (Yue et al., 2018 and 2021) was adopted as an internal reference gene and the relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.4. Relative DNA methylation levels detection

To explore the dynamics of DNA methylation in gonadal development, we examined the relative methylation levels of *Dnmt1*, *Dnmt3a/b*, and *Tet3* during resting, proliferative and mature phases by means of the methylation sensitive McrBc-qPCR (Huang et al., 2019). Meanwhile, the DNA methylation patterns of *Dnmt3a/b*, *Tet3* and *Dgkd* were investigated on day 10 and 20 in the starvation and control groups. The period of gonadal development, starvation and control groups corresponded to the samples analyzed by qPCR. Three biological replicates, with each replicate representing tissues pooled from five oysters were used for McrBC analysis for each gonadal developmental stage, starvation group and control group, respectively. DNA templates of different developmental periods of the gonads were used from previous experiments (Sun et al., 2022b). DNA was extracted from starvation-treated gonadal tissues and control group with DNA extraction kits (Tiangen, China). DNA concentration and integrity were detected utilizing a nanodrop 2000 micro-UV spectrophotometer and 1% gel electrophoresis. Primers were designed by selecting the region containing more CpG sites within the upstream of the translation start site (TSS) -2 kb (Table 1). Treatment of 250 ng of DNA with the McrBC enzyme (TaKaRa, Japan) system for 6 h at 37 °C and complemented with GTP, while the negative controls were treated with the same reaction system without the addition of GTP as standard (Huang et al., 2019). The kits and analytical procedures for McrBC-qPCR analysis were consistent with the qPCR analysis described above. For the convenience of analysis, we used $2^{\Delta\Delta Ct}$ instead of $2^{-\Delta\Delta Ct}$ to express the relative methylation levels, so that the relative fluorescence signal intensity is proportional to the methylation levels.

2.5. Detection of sex-related gene methylation levels in different periods of gonad development

Genome-wide methylation data revealed that *Foxl2* and *Dmrt1* are

Table 1
Primers used in this study.

Name	Primers (5′-3′)	Purpose	Amplification efficiencies
qDmrt1 RT F	ACCTGTGGGTCCTTGCCTT	qPCR	1.956
qDmrt1 RT R	GCTCTTGATTGGTGCTCTATGG		
qFoxl2 RT F	CACAGTGTGGTTACAACGCAATGC		1.996
qFoxl2 RT R	CCTGTCAGTCCAGTACGAGTAATGC		
qTet3 F	ACGAGTTTGAGGATGATGACGAT		2.083
qTet3 R	TCTGAATCATGGTTATCCGATTCG		
qDnmt1 F	GTACGACAAGAATACCCACCTGTGT		1.965
qDnmt1 R	GCTGTCCACCATTCATTGATA		
qDnmt3a/b F	TAGACTCCGTTACCCAGTAGACC		2.056
qDnmt3a/b R	CGACTTTCTTAGCGAGTGTTGTG		
qDgkd F	ATACCAGCGGATGAAGGAGTC		1.983
qDgkd R	CCAGGGTGAAAATGGAGAATG		
McrBc-qDnmt1 F	GACAAAGTATACGATAGTGAACG	McrBc qPCR	1.973
McrBc-qDnmt1 R	CTGTCGGCCTCCTTCACTGACTCG		
McrBc-qDnmt3a/b F	ACTCTGACGAACGTGGCACAGAGTC		2.055
McrBc-qDnmt3a/b R	TTGCTACATCATCCGTCATGGAAC		
McrBc-qTet3 F	CTTGTTTGATTGTCCTAGCCCAGC		2.054
McrBc-qTet3 R	CGTGCTTCTAATGATAACCAATAGC		
McrBc-qDgkd F	GTGAACAGGCAGACTACAAAGCA		1.998
McrBc-qDgkd R	TCAAGTCACTCAGTTTGTTTCCTA		
BS-Dgkd F	GTGATTATGATGGGTGAATTTTTTTG	BS PCR	
BS-Dgkd R	CATTAACATAATCAAAAAAACCTAAATT		
BS-Dmrt1 F	ATATTAGGAGATATATAAAGTTTGGA		
BS-Dmrt1 R	TCAATTTCAAATATATATTAAATTAC		
BS-Foxl2 F	GTGTTAAAATGATATGGAGAGAG		
BS-Foxl2 R	CTAATATTACATTCAATTCCCACAA		
Pro1-Dgkd F	ccgctcgag*CCTGATGCGAGGGGTTTATT	Dual Fluorescence Report	
Pro1-Dgkd R	ttgcggccgc*AGGGAAGTGTGAAATCCTGTGTA		
Pro2-Dgkd F	ccgctcgag*GACCCATCGTATGCCAGTTTT		
Pro2-Dgkd R	ttgcggccgc*CGCACAATATGTGTGTACCATACC		
Pro3-Dgkd F	ccgctcgag*CAAGATACTGATATACACCTCCTACCC		
Pro3-Dgkd R	ttgcggccgc*GCACAATATGTGTGTACCATACCAC		

* lowercase letters indicate added restriction sites and protective bases.

demethylated during maturation (Sun et al., 2022a). Therefore, to explore whether sex is influenced by DNA methylation, we examined the methylation levels of sex-related genes *Foxl2* (−852 ~ −437 upstream of the TSS) and *Dmrt1* (−1918 ~ −1517 upstream of the TSS) at different gonadal developmental periods by BS-PCR (bisulfite sequencing PCR). We also examined the methylation levels of sex differentially methylated gene *Dgkd* (−1461 ~ −875 upstream of TSS) at different gonadal developmental periods to explore the dynamics of methylation in gonadal development. Due to DNA damage by sulfite, the amplified sequences were designed to be no longer than 600 bp and to contain as much CpG as possible within −2 kb located upstream of the translation start site (Li and Dahiya, 2002). Specific amplification primers (Table 1) were designed within −2 kb upstream of the TSS of the genes using the online software http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi. DNA templates were treated with EpiArt DNA Methylation Bisulfite Kit (Vazyme, China) as described in the instructions and then amplified with 2 × Taq Master Mix enzyme (Vazyme, China). Three biological replicates were used for the resting, proliferative and mature stages respectively. The target fragments were recovered utilizing the Gel Recycling Kit (Omega, USA) and then cloned into pMD-19 t vectors (Takara, Japan), and finally 10 monoclonal clones were randomly picked for sanger sequencing. Methylation sequencing was processed by applying the BiQ Analyzer software.

2.6. Effects of methylation on Dgkd promoter activity in vitro

The two target sequences upstream of the TSS were amplified separately with the specific primers (Table 1) and then cloned into the pGL3.0-basic plasmid to construct pGL3-promoter1 (−1487 ~ −964) and pGL3-promoter2 (−987 ~ −202). According to the instructions, pGL3-promoter 1 and 2 were methylated by applying SssI methylation enzyme (NEB, US). After methylation, the plasmids were recovered through PCR purification kit. The successfully methylated plasmids were detected following the instructions with McrBC enzyme. Perform transfection experiments utilizing plasmids that were fully methylated.

Methylated and unmethylated plasmids were transfected into 500 μL of HEK 293 T (human embryonic kidney 293 T) cells using Lipofectamine 3000 (Invitrogen, USA) as described in the instructions. Plasmids in the unmethylated groups were transfected as follows 1) 500 ng pGL3.0-basic (control); 2) 500 ng pGL3.0-promoter1; 3) 500 ng pGL3.0-promoter2. The methylated group was transfected with equal amounts of methylated plasmids in the same manner. All groups were transfected with a total of 40 ng pRL-TK as an internal reference. The experiment was performed twice, with three replicates each time. After 10 h, the medium was renewed and the fluorescence values were detected under the Synergy NEO2 instrument using the luciferase assay kit (Promega, US) after 48 h.

2.7. Data analysis

All data were expressed as mean ± standard deviation. Before analysis of variance, data were checked for normality and homogeneity of variance, and all data passed parametric tests. The effects of starvation on the sex ratio were examined for significance using a one-way ANOVA (Analysis of Variance) and Tukey's test. The relative expression data of the starvation and control groups were compared by a one-way ANOVA for significance testing and Tukey's test. DNA methylation levels were calculated by dividing the number of CG sites that underwent methylation by the total number of CG sites. Differences in methylation levels between males and females were tested by utilizing a one-way ANOVA and Tukey's test. DNA methylation levels of genes in the starvation and control groups were tested for significance using a one-way ANOVA and Tukey's test. In the dual luciferase reporter assay, the relative fluorescence activity of the methylated group was normalized to that of the unmethylated group and subjected to one-way ANOVA and Tukey's test for statistical significance. $P < 0.05$ was considered the significant difference.

3. Results

3.1. Alterations in gonad development and sex ratio under starvation stress

Histological analysis showed that gonadal development was significantly inhibited in the absence of feeding. On day 10, oogonium (Fig. 2A) and spermatids (Fig. 2B) were observed in the gonadal tubules. This period was in the late proliferative phase of gonadal development (stage 2). In contrast, a small number of oogonium (Fig. 2C) and spermatocytes (Fig. 2D) were observed in the gonadal tubules in the starved group. The gonads in this condition belonged to the pre-proliferative phase of gonadal development (stage 1). On day 20, the gonads in the control group were developed to the growing phase and oocytes (Fig. 2E) and spermatids (Fig. 2F) were clearly visible in the gonadal tubules (stage 3). Compared to the starvation group on day 10, oogonium (Fig. 2G) and spermatids (Fig. 2H) were clearly observed in the starvation group on day 20, the gonads were in the proliferative phase (stage 2). On day 40, male and female identification was performed by microscopic examination facilitating assessment of the female to male sex ratio. In the four replicate groups, the sex ratio (female to male) changed from 1:0.78 to 1:1.44 (Fig. 2I), which means that the proportion of male individuals increased under starvation stress.

3.2. Variations of sex related and DNA methylation gene expression levels under starvation stress

To confirm epigenetic involvement in the altered sex ratio, we analyzed the expression of DNA methylation-related genes (*Dnmt1*, *Dnmt3a/b* and *Tet3*) and sex-related genes (*Foxl2* and *Dmrt1*) on day 10 and 20 of the starvation treatment. The expression of DNA methylation-related genes after starvation treatment was different between males and females. In female oysters treated with starvation, the expression level of *Dnmt1* was not significantly different from that of the control group at day 10, but its expression level was reduced by 83% ($P < 0.05$) at day 20 relative to the control group. The expression level of *Dnmt3a/b* showed a trend of decreasing and then increasing, decreasing to 0.56 times ($P < 0.05$) that of the control at day 10, while increasing to no significant difference from the control at day 20. The expression level of *Tet3* was downregulated to 0.61- ($P < 0.05$) and 0.49-fold ($P < 0.05$) of the control at days 10 and 20, respectively (Fig. 3). In the male oysters treated with starvation, only the expression level of *Dnmt1* was inhibited at day 10, and its expression level was reduced to 0.74-fold ($P < 0.05$) that of the control (Fig. 3). Surprisingly, starvation significantly suppressed the expression level of *Foxl2* in females and males at day 10, decreasing to 0.63- ($P < 0.05$) and 0.30-fold ($P < 0.05$) that of the control, respectively, while *Foxl2* expression at day 20 was not significantly different from the control. The expression levels of *Dmrt1* increased in females at days 10 and 20 and were 1.5 ($P < 0.05$) and 1.9 times ($P < 0.05$) higher than controls, respectively, whereas the expression levels of *Dmrt1* in males were not significantly different from controls (Fig. 3).

3.3. DNA methylation levels decreased under starvation stress

To investigate whether alterations in gene expression respond to environmental changes through DNA methylation, we first analyzed the methylation patterns of the *Foxl2* *Dmrt1*, *Dnmt1*, *Dnmt3a/b* and *Tet3* during different periods of gonadal development. Since previous studies have shown that *Foxl2* and *Dmrt1* are hypomethylation levels, we used the BSP method to more visually demonstrate their methylation status during gonadal developmental periods. The results showed that the conversion efficiency of bisulfite was >99%. Detection of 17 CG sites within the upstream −2000 bp of TSS demonstrated that *Foxl2* exhibited

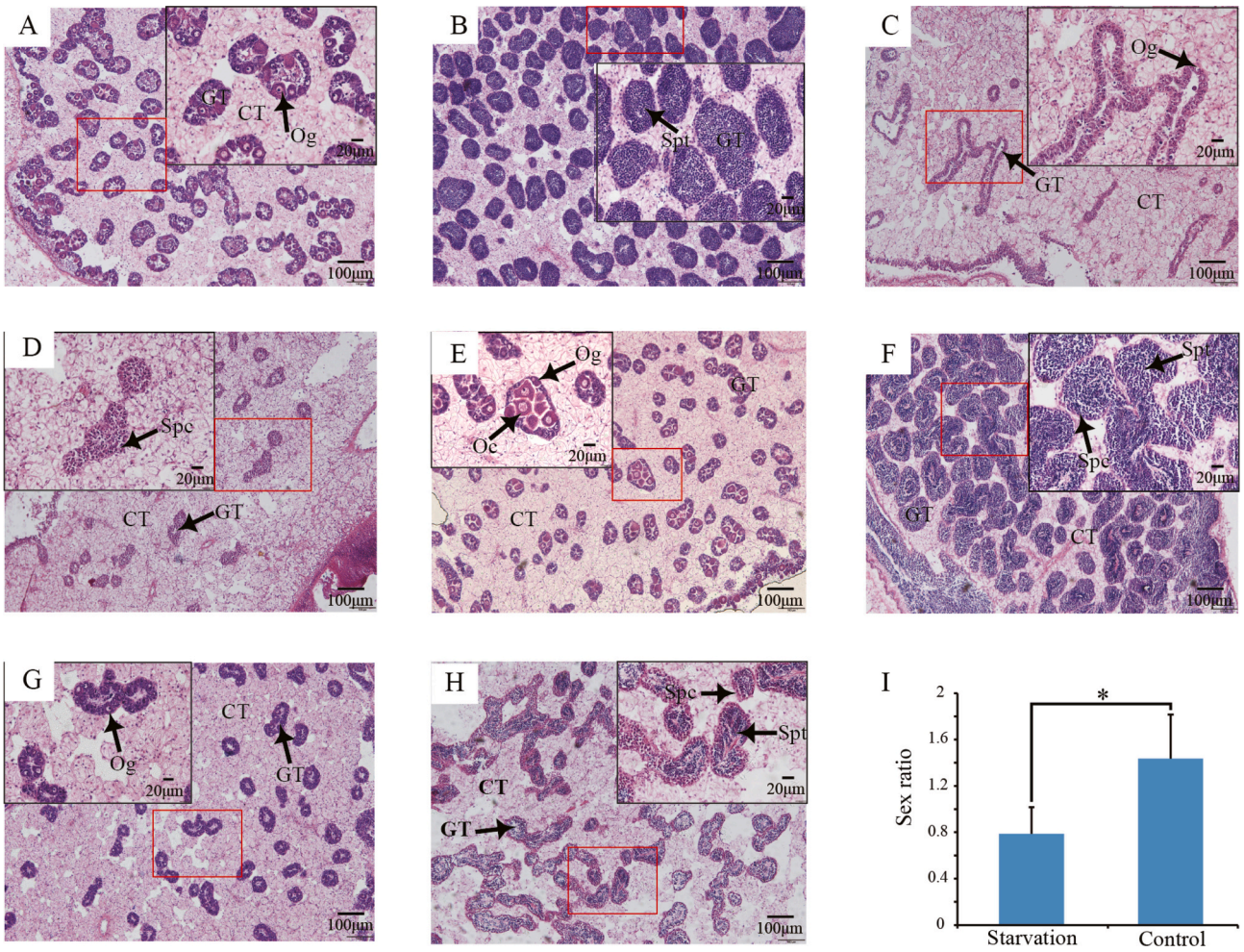

Fig. 2. The histological observation and sex ratio under starvation stress. (A-H) Histological observation of gonad stained by HE. A-D denoted gonadal tissue of female and male oysters starved for 10 days in control (A and B) and starved groups (C and D). *E*-H indicated gonadal tissue sections of female and male oysters starved for 20 days in the control (E and F) and starved groups (G and H). CT: conjunctive tissue, GT: gonadal tubules, Oc: oocyte, Og: oogonia, Spc: spermatocytes, Spt: spermatids. The red box (bar = 100 μm) represents the enlarged position of the black box (bar = 20 μm). (I) Sex ratio (female/male) of the starved and control groups counted at day 40. An asterisk indicates a significant difference ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

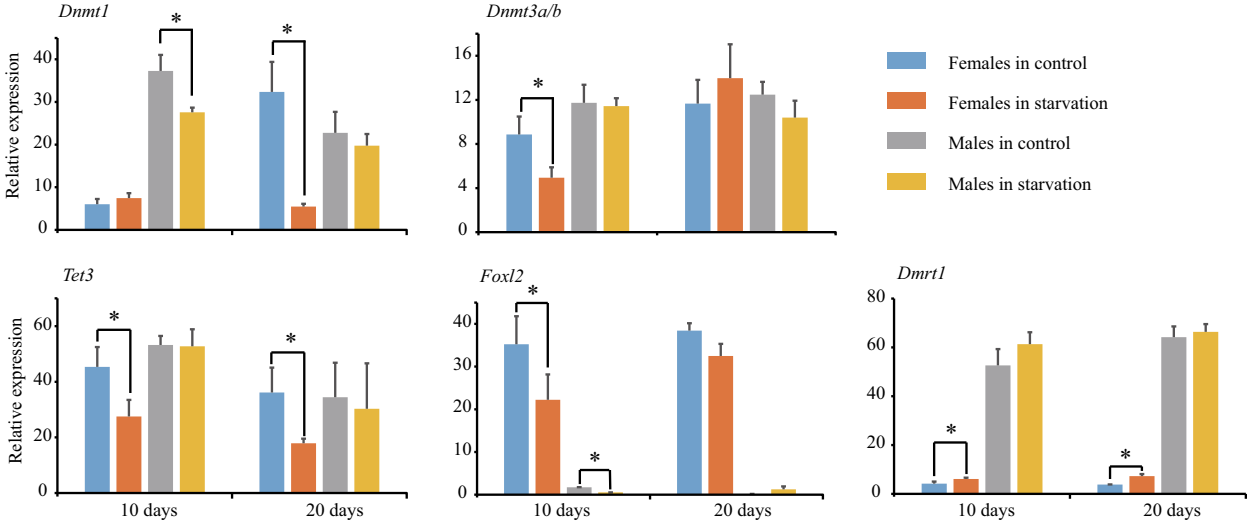

Fig. 3. Gene expression levels were altered under starvation stress. Relative expression levels of *Dnmt1*, *Dnmt3a/b*, *Tet3*, *Foxl2* and *Dmrt1* in male and female oysters treated with starvation for 10 and 20 days (control $n = 3$, starved n = 3). An asterisk indicates a significant difference ($P < 0.05$).

demethylation state in both female and male gonads (Fig. 4A). Similarly, the 15 CG sites of *Dmrt1* presented lower methylation levels between 2.67% ± 1.02% and 13.33% ± 3.50% (Fig. 4B). The methylation levels of *Foxl2* did not differ between the sexes, and also did not differ significantly during gonadal development at various times ($P > 0.05$). Although the methylation levels of *Dmrt1* were higher in females than in males, they did not differ significantly from each other ($P > 0.05$). Previous studies have shown that *Dnmt1*, *Dnmt3a/b* and *Tet3* are in hypermethylated state. We then analyzed their methylation status during gonadal development by McrBC-PCR. The relative methylation levels of *Dnmt1* were significantly higher ($P < 0.05$) in females than in males in the resting phase, while no significant difference was observed between males and females in the proliferative phase. However, in the maturation phase, the methylation levels were significantly higher ($P < 0.05$) in males than in females (Fig. 4C). The relative methylation levels of *Dnmt3a/b* were significantly higher ($P < 0.05$) in males than in females in the proliferative and maturation phases, respectively (Fig. 4D), while there were no differences during the resting phase. *Tet3* exhibited similar methylation patterns to *Dnmt3a/b* (Fig. 4E).

Since neither the starved nor the control oysters were in resting phase on days 10 and 20, we selected methylation genes (*Dnmt3a/b* and *Tet3*) with differences between males and females in both proliferation and maturation phases for analysis under starvation stress. Unexpectedly, starvation stress for 10 and 20 days resulted in 71% ($P < 0.05$) and 85% ($P < 0.05$) decrease in the relative methylation levels of *Dnmt3a/b in* male oysters, respectively. Meanwhile *Tet3* also underwent demethylation, with relative methylation levels of 84% ($P < 0.05$) and 80% ($P < 0.05$) as compared to the control, respectively.

3.4. The expression and DNA methylation of Dgkd under starvation stress

The response of *Dgkd*, which is differentially methylated between males and females, was next examined under starvation conditions. At 10 days of starvation stress, the expression level of *Dgkd* in the female gonads was significantly downregulated by 72% ($P < 0.05$) (Fig. 5A), and the corresponding methylation signal of the female *Dgkd* promoter was significantly increased by 25% ($P < 0.05$) (Fig. 5B). At 20 days of starvation stress, the expression level of *Dgkd* in female gonads was reduced, but the difference compared to the control was not so notable (Fig. 5A). The expression levels of *Dgkd* remained unchanged throughout the stress period in male oysters (Fig. 5A). But, the methylation signal of the *Dgkd* promoter was reduced by 87% ($P < 0.05$) in male gonads, reflecting the decrease in DNA methylation levels (Fig. 5B). These results suggested that starvation stress exerts but different degrees of effects on gene expression and methylation in females and males, indicating that DNA methylation was involved in the interaction between genetics and environment.

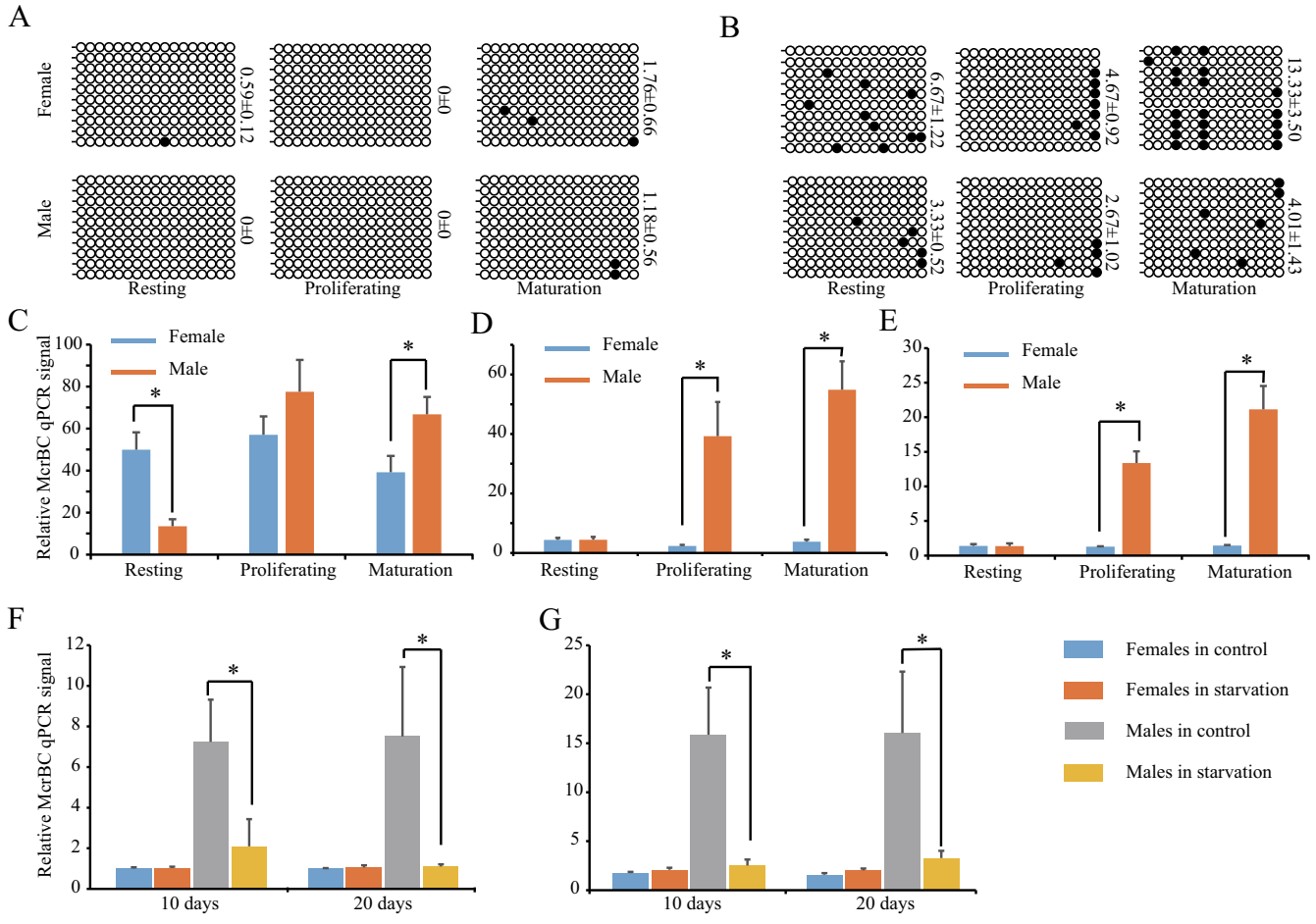

Fig. 4. DNA methylation patterns under starvation stress. DNA methylation patterns of *Foxl2* (A) and *Dmrt1* (B) in resting, proliferating and maturation stage of female and male gonads. Solid circles indicated CG methylated sites and empty circles indicated unmethylated. Methylation levels were calculated based on the CG sites that underwent methylation divided by the total CG sites and expressed as mean ± standard deviation. The relative DNA methylation level of *Dnmt1* (C), *Dnmt3a/b* (D) and *Tet3* (E) in resting, proliferating and maturation stage of female and male gonads. The relative DNA methylation level of *Dnmt3a/b* (F) and *Tet3* (G) at 10 and 20 days of starvation treatment relative to the control (control n = 3, starved n = 3). The McrBC enzyme was able to digest the methylated sites, using the undigested DNA as an internal reference; therefore, the relative methylation level was calculated using the $2^{\Delta\Delta Ct}$ method, and the signal intensity was proportional to the methylation level. An asterisk indicates a significant difference ($P < 0.05$).

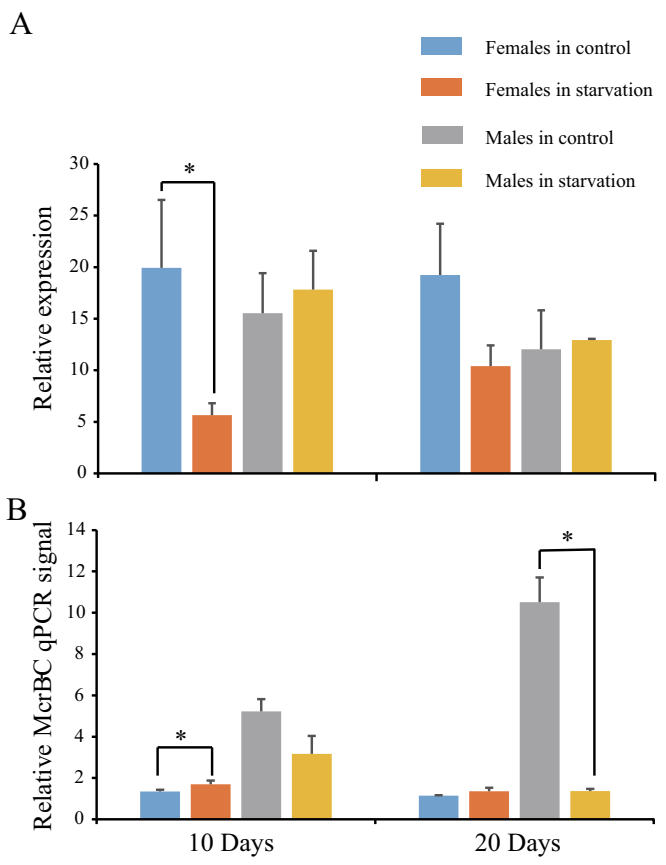

Fig. 5. Starvation treatment led to altered expression and methylation levels of *Dgkd* genes. (A) Relative expression levels of *Dgkd* at 10 and 20 days of starvation treatment (control n = 3, starved n = 3). (B) The methylation levels of *Dgkd* at 10 and 20 days of starvation treatment relative to the control (control n = 3, starved n = 3). The McrBC enzyme was able to digest the methylated sites, using the undigested DNA as an internal reference; therefore, the relative methylation level was calculated using the $2^{\Delta\Delta Ct}$ method, and the signal intensity was proportional to the methylation level. An asterisk indicates a significant difference ($P < 0.05$).

3.5. The DNA methylation and expression patterns of Dgkd during gonad development

DNA methylation was the mediator between gene expression and starvation stress. So, the association between gene expression and DNA methylation was explored by understanding the DNA methylation and gene expression levels of the *Dgkd* promoter at different times of gonad development. Results showed that the methylation levels of *Dgkd* promoter was more than twice as high in males (90.33% - 92.85%) as in females (13.76% - 37.85%) in proliferating and mature stage, suggesting sex-specific differences in the genes under consideration (Fig. 6A). In addition, the expression level of *Dgkd* gene was significantly higher in females than in males at maturity, while showing a negative correlation with DNA methylation (Fig. 6B). The promoter methylation and expression were weakly negatively correlated throughout the period of gonadal development (rho = −0.42, $P > 0.05$). When only the maturity stage was considered, promoter methylation levels and expression levels showed a strong negative correlation (rho = −0.90, $P < 0.05$).

3.6. The relationship between Dgkd promoter methylation and gene expression

The effect of DNA methylation on gene expression was verified with the dual fluorescence reporter. *Dgkd* promoter 1 and 2 methylated plasmids significantly reduced the luciferase activity to 21% ($P < 0.05$) and 18% ($P < 0.05$) of the control, respectively (Fig. 7), indicating that the gene expression was inhibited by the DNA methylation in vitro.

4. Discussion

4.1. Starvation hinders oyster gonad development and alters the sex ratio

The effects of environmental stress on the reproductive system in fish can be manifested in follicle development and oocyte number and size (Kime, 1999). In the present study, gonad development was retarded under prolonged starvation stress, along with a reduction in follicle number, suggesting an effect of starvation on the reproductive capacity of the oyster, in line with the previous reports in oysters (Liu et al., 2010). Under starvation conditions, animals are forced to trade off survival and reproduction, but ultimately sacrifice reproduction for the opportunity to live (Lynn et al., 2015). Previous work has shown that starved oysters rapidly deplete glycogen, display decreased protein and lipid content, and exhibit a reduced RNA/DNA ratio (Liu et al., 2010). These suggests that starving oysters in this study spend energy that would otherwise be allocated for reproduction on basic metabolism, which results in delayed or undeveloped gonads, especially in the female gonads. Predictably, the sex ratio was significantly skewed toward males at the end of the starvation stress, consistent with the outcome of starvation stress in other animals (Sakae et al., 2020; Sakae and Tanaka, 2021; Stenyakina et al., 2010). In some mollusks, females require about 1.5 times more energy to produce eggs than males need to produce sperm (Russell-Hunter, 1979). Thus, sex reversal occurs as a survival strategy for oysters under starvation conditions, spending energy on the sex that is most conducive to survival. In practical production, adequate food supply contributes positively to the development of the gonads and the increase in the proportion of female oysters.

With the increase of oyster farming density, an insufficient supply of bait in the farming sea area could lead to a biased male ratio between farmed and wild oysters. This could cause a series of ecological problems, such as: 1. the nutritional structure of the farmed sea area will change due to the difference in the food structure of male and female oysters (Baghurst and Mitchell, 2002); 2. long-term male bias will lead to a reduction in the genetic diversity of the population, which is not conducive to the population's ability to cope with complex and variable environments; 3. starvation stress will pass on the male phenotype to offspring through epigenetic inheritance, causing bias in the sex ratio of offspring and further increasing the population's sex differences. Therefore, the rational development and utilization of natural marine areas is of great significance for biodiversity, oyster genetic diversity and sustainable development of the industry.

4.2. Altered expression levels of sex-associated genes and DNA methylation-associated genes under starvation stress

In nature, oysters are a species with a high sex reversal rate of up to 50% (Broquard et al., 2020). During sexual reversal, the genomic DNA sequence is not altered, but the phenotype was changed, suggesting epigenetic involvement in the sexual reversal process. In this study, the increase in the number of male oysters under starvation conditions involved epigenetic regulation, mainly manifested as the decrease in the expression of DNA methyltransferase (*Dnmt1*, *Dnmt3a/b*) and demethylase (*Tet3*). The expression levels of *Dnmt3a/b* and *Tet3* were unchanged in males under starvation conditions, indicating that DNA methylation and demethylation are always in dynamic equilibrium. The decreased expression of *Dnmt1* in males and females on days 10 and 20 of starvation stress, respectively, may be related to the reduced number of reproductive cells, as this gene plays a role in cell proliferation in mammals (Bestor, 2000). Previous studies have shown that DNA methylation levels were higher in males than in females (Sun et al., 2022a). In starvation-stressed female oysters, *Tet3* expression levels were reduced earlier than *Dnmt1*, which caused a temporary increase in

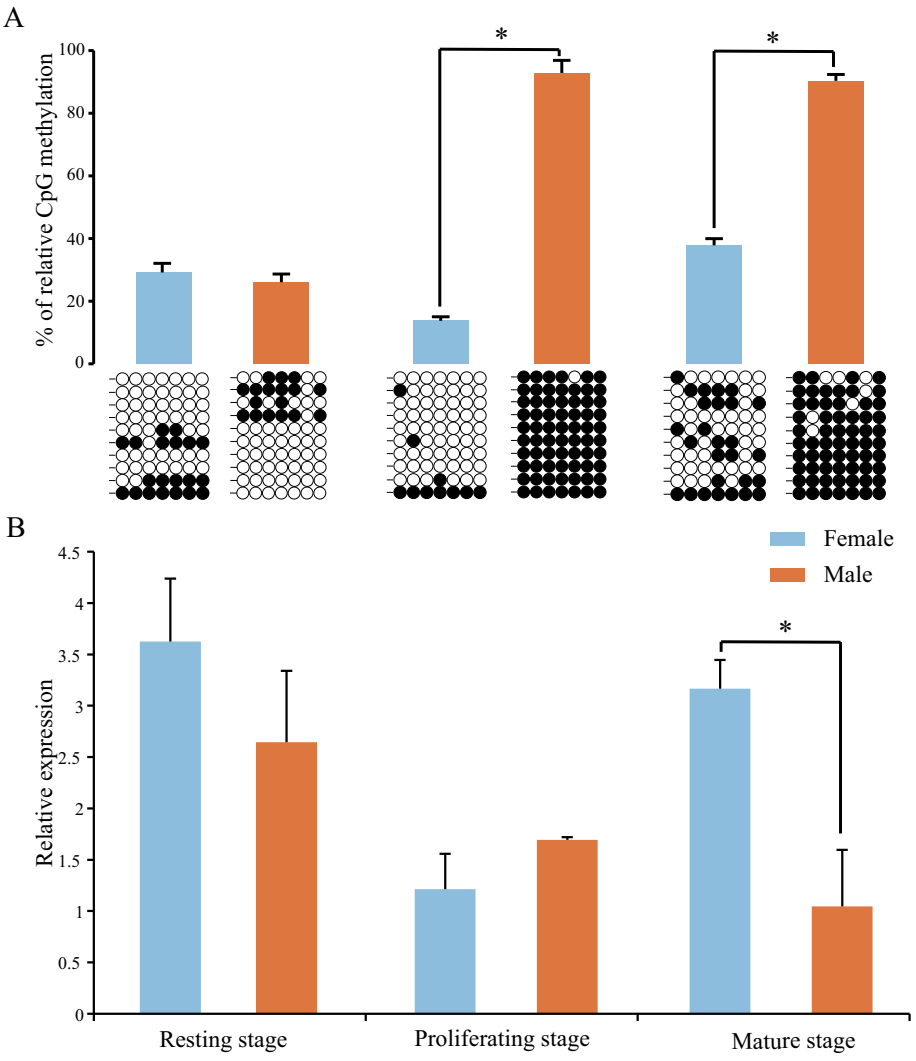

Fig. 6. DNA methylation was negatively correlated with *Dgkd* expression. (A) The DNA methylation patterns of *Dgkd* in resting, proliferating and maturation stage of female and male gonads. Solid circles indicated CG methylated sites and empty circle indicated unmethylated. Methylation levels were calculated based on the CG sites that underwent methylation divided by the total CG sites and expressed as mean ± standard deviation (n = 3). (B) The relative expression of *Dgkd* in resting, proliferating and maturation stage of female and male gonads. An asterisk indicates a significant difference.

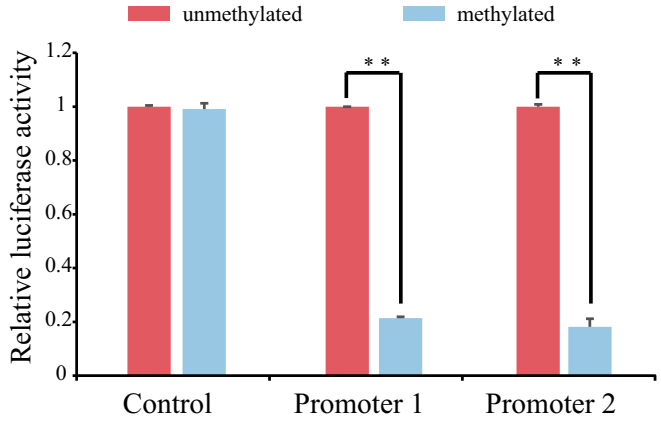

Fig. 7. The expression of *Dgkd* was regulated by DNA methylation. Controls indicated pGL3-basic null plasmids ($n = 6$) and promoter1 (n = 6) and 2 (n = 6) indicated pGL3 plasmids constructed with *Dgkd* upstream sequences. The relative fluorescence activity of the methylated group was normalized to the relative luciferase activity of the unmethylated group. Double asterisks indicate a highly significant difference ($P < 0.01$).

DNA methylation, followed by a reduction in *Dnmt1* expression levels that allowed methylation levels to remain relatively stable. The temporary process might lead to the activation of some male sex-related genes, such as *Foxl2* and *Dmrt1*.

The resting period serves as a critical window for sex determination (Cherif-Feildel et al., 2019), during which oysters in this period will initiate a new female or male sex differentiation driven by sex-determining genes (Broquard et al., 2021; Zhang et al., 2014). In this study, starvation stress resulted in decreased expression levels of the female sex-linked gene *Foxl2* and increased expression of the male sex-linked gene *Dmrt1* in female oysters. These results were not limited to oysters, but similar results were also demonstrated in medaka (Sakae et al., 2020). Therefore, starvation stress on oysters in resting phase in this study resulted in low expression of *Foxl2* and high expression of *Dmrt1* in some individuals, which likely contributed to a male-biased sex ratio.

Alterations in gene expression levels induced by environmental factors were associated with epigenetic regulatory mechanisms. In fish, temperature altered the methylation levels of *cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a), *Foxl2* and *Dmrt1* promoters, thereby causing sex reversal (Anastasiadi et al., 2018; Navarro-Martin et al., 2011). In addition, starvation also reduced DNA methylation levels of genes related to gonadal development in zebrafish (Fan et al., 2019). However, the altered gene expression levels of *Foxl2* and *Dmrt1* under starvation stress were not directly regulated by DNA

methylation in this study, which confirms previous hypotheses on the regulation of sex determination by DNA methylation in oysters (Sun et al., 2022a).

4.3. The methylation of Dgkd is associated with sex

Unlike vertebrates, invertebrates exhibit relatively low DNA methylation levels, and the hypermethylated genes are mainly widely expressed genes, while the hypomethylated genes are specifically expressed genes, which may be beneficial for increasing the adaptive capacity of oysters to the environment (Keller et al., 2016; Riviere, 2014; Roberts and Gavery, 2012). The promoter of *Dgkd* exhibited methylation differences between males and females in oysters (Sun et al., 2022a). In this study, the methylation pattern of *Dgkd* at different periods of gonadal development demonstrated that the methylation levels of *Dgkd* promoter differed by sex throughout gonad development. In the starving environment, animals alter their metabolism to maximize survival and metabolic changes are accompanied by alterations in metabolites (Lynn et al., 2015). Starvation stress caused alterations in *Dgkd* methylation levels that were not always accompanied by alterations in gene expression, which was consistent with our studies on DNA methylation and expression of *Dgkd* genes during proliferation. Remarkably, the activity of the induced hypermethylated *Dgkd* promoter was significantly inhibited in vitro, which strongly suggested that DNA methylation regulated the expression of *Dgkd*. As a central switch in the second messenger-activated signaling pathway, diacylglycerol kinase converts DAG (diacylglycerol) to phosphatidic acid (PA) and regulates the respective levels of these two bioactive lipids. DGKD plays a key role in development by controlling diacylglycerol levels and regulating the signaling pathways of PKC (protein kinase C) and EGF (epidermal growth factor) receptors (Sakane et al., 2002; Sato et al., 2013). In arthropods, PKC signaling is associated with male sex determination (Toyota et al., 2021; Toyota et al., 2017). In addition, studies in human embryonic cancer cells have found that PKC upregulated *Dmrt1* gene expression (Koji et al., 2006). All in all, our findings suggested that the DGKD-PKC-DMRT1 cascade signaling pathway is part of the male sex determination process in Pacific oyster.

Although a conserved sex determination pathway was found in oysters (Zhang et al., 2014), the effect of DNA methylation on this pathway was dramatically distinct from that in fish (Shao et al., 2014). It is likely that hypermethylation of widely expressed genes in lower animals is a way of coping with environmental changes, in addition to maintaining genetic stability during evolution. This would allow limited DNA methylation to take full advantage of phenotypic plasticity in response to adverse environments. The ability of adult oysters to undergo sex reversal is due to the presence of germinal stem cells in the gonads (Cavelier et al., 2017; Cherif-Feildel et al., 2019), and it was the proliferation rate of one gametocyte relative to another (oogenic versus spermatogonial) that ultimately determines sex in oysters (Lucas, 1975). Thus starvation-induced alterations in DNA methylation appear to activate the male gametogenesis pathway, and may lead to a male-biased sex ratio in oysters.

5. Conclusions

To investigate the effect of food factors on the sex ratio of oysters, four full sibling families were subjected to starvation stress. Adult oysters subjected to chronic starvation stress sex ratios underwent male bias and slow gonad development. The underlying mechanism was that starvation directly or indirectly regulates gene expression associated with gonad development and sex determination by affecting DNA methylation. Our results provide a theoretical basis for the long-term observation of a female bias in nutrient-rich marine areas (Quayle, 1988).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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