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Early gonadal differentiation is associated with the antagonistic action of *Foxl2* and *Dmrt1l* in the Pacific oyster

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ABSTRACT

As the second largest phylum in the zoological kingdom next to arthropods, the mechanism of gonadal differentiation in mollusca is quite complex. Currently, although much has been carried out on gonadal differentiation in the Pacific oyster, there is still unknown information that needs to be further explored. Here, analysis of the *Foxl2* and *Dmrt1l* expression in samples at different development periods of male and female gonads as well as in annual gonad samples revealed that Log_{10} (*Foxl2/Dmrt1l*) values were an effective method for sex identification in oysters. In differentiated gonadal tissue, Log_{10} (*Foxl2/Dmrt1l*) values greater than 2 were females and less than 1 for males. Subsequent sequential sampling of the same individuals verified that Log_{10} (*Foxl2/Dmrt1l*) values greater than 2 for resting gonads would develop as females and less than 1 would develop as males in the future. Relative expression analysis of *Foxl2* and *Dmrt1l* in the annual samples revealed a negative correlation between Log_{10} (*Foxl2*) and Log_{10} (*Dmrt1l*). Double fluorescence reporter validation results showed that DMRT1L protein was able to bind the *Foxl2* promoter and repress its activity with a weak dosage effect. Antagonism between *Dmrt1l* and *Foxl2* is therefore not restricted to vertebrates, and the competing regulatory networks are of great significance in the maintenance of gonadal sex in oysters after sexual differentiation. This study provides novel ideas and insights into the study of early gonadal differentiation in the adult oyster.

1. Introduction

Sex differentiation is conventionally conceived as the process by which an organism develops from embryonic gonadal selection to the ovary or testis (Weber and Capel, 2021). In contrast to the traditional genotypic sex determination (GSD) and environmental sex determination (ESD) (Hayes, 1998), the current view considers sex differentiation as a dynamic balance of gene expression in which pure GSD and ESD species are located at opposite ends of the scale, with possible intermediate combinations (Sarre et al., 2004). Among vertebrates, the vast majority of mammals exhibit a chromosomally inherited sex-determination pattern, referred to as GSD, with an XY male/XX female system. Birds also belong to the GSD system, but all birds are female heterogamety (Clinton and Haines, 1999). In addition, fish, frogs, and reptiles also have other sex determination patterns (Devlin and Nagahama, 2002; Ma and Veltsos, 2021; Nakamura et al., 1998; Sarre et al., 2004). In GSD species, a common belief is that sex is genetically controlled and fixed at the time of fertilized egg has been created (Sarre et al., 2004). For example, *Sry* (sex determining region Y) is a mammalian-specific sex marker and *Dmrt1* (doublesex and mab-3 related transcription factor 1) is a sex marker for most GSD species (Bachtrog et al., 2014; Graves, 2009; Wallis et al., 2008). In ESD species, sex determination is regulated both genetic and environmental factors, and sex discrimination is difficult especially for some species without sex chromosomes until gonads are differentiated (Weber and Capel, 2021). In some reptiles and fish, sex determination is regulated by DNA methylation, and thus DNA methylation can be applied as an epigenetic marker for sex prediction. For instance, in European seabass, DNA methylation is a valid marker for predicting sex in undeveloped gonads (Anastasiadi et al., 2018). Invertebrates exhibit a more complex and diverse sex determination system. Such as in the *Drosophila melanogaster* or *Caenorhabditis elegans*, sex is controlled by the ratio of X and autosomal chromosomes or by a single X chromosome (Bachtrog et al., 2014). Heteromorphic sex chromosomes (XX/XY) and sexual markers were identified in Pacific abalone, a gastropod mollusk (Luo et al., 2021). In addition, temperature (Siebert and Juliano, 2017), photoperiod (Walker, 2005) and food availability (Berec et al., 2005) can affect the sex of invertebrates.

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DMRT1 presents an apical dominant role in vertebrates and its role in mammals appears to be maintaining male fates (Matson et al., 2011). In invertebrates, members of *Dmrt* are involved in sex-specific development, and its homolog gene has been described as a master switch in *Daphnia* (Kato et al., 2011). In addition, *Dmrt* specifically initiates the male gonadal development in hermaphroditic planarians (Chong et al., 2013). In bivalves, the *Dmrt1l*, which evolved independently from the vertebrate *Dmrt*, shows sexually dimorphic expression (Evensen et al., 2022; Li et al., 2018; Yue et al., 2021). *Foxl2* is required for the differentiation of female supporting cells, but in most species, *Foxl2* expression exhibits sex differences rather than specific expression (Capel, 2017; Zhang et al., 2014). In mice, qChIP experiments demonstrated that DMRT1 protein can act directly on the *Foxl2* gene, and dual fluorescence reporter experiments found the same results in tilapia (Dai et al., 2021; Matson et al., 2011). Due to the antagonistic effect between these two genes, the Log_{10} (*Dmrt1l/Foxl2*) values were utilized to identify the sex of scallops and Log_{10} (*Dmrt1l/Foxl2*) values were lower than 0 in females and higher than 2 in males (Li et al., 2018).

The Pacific oyster (*Crassostrea gigas*) possesses a continuous hermaphrodite sex determination system without secondary sexual characteristics and constitutes one of the world's primary cultured shellfish (Broquard et al., 2020). Neither karyotype analysis nor genomics has revealed the presence of sex chromosomes. Although various hypotheses on the sex determination mode of oysters have been put forward, the conclusions are still inconclusive (Hedrick and Hedgecock, 2010; Yue et al., 2020). Early studies on sex differentiation in oysters were mainly related to the cloning of conserved genes (Naimi et al., 2009). Recently, some more conserved sex -related genes were also discovered in oysters with similar expression patterns by high-throughput sequencing technology (Broquard et al., 2021; Yue et al., 2018; Zhang et al., 2014). Transcriptome analysis of gonads unearthed several sex-related genes, such as *Foxl2* and *Dmrt1l*, which are evolutionarily ancient (Zhang et al., 2014). The expression of *Dmrt1l* is specific to the male gonads of *C. gigas* (Yue et al., 2021), while *Foxl2* is expressed in both male and female gonads, but differs between females and males (Naimi et al., 2009). Furthermore, both genes are localized in male germ cells (Yue et al., 2021). This provides evidence for the hypothesis that FOXL2 acts antagonistically with DMRT1 in the sex differentiation pathway of the oyster (Zhang et al., 2014).

In this research, to investigate the competing molecular regulatory mechanisms in the oyster and to discover molecular markers for differentiating sex in oysters with resting gonad development, patterns of Log_{10} (*Foxl2/Dmrt1l*) values in gonad development and annual gonads were described. Furthermore, the feasibility of Log_{10} (*Foxl2/Dmrt1l*) values as an early sex marker was verified by sampling the same individual twice at different stages of gonadal development. Finally, the antagonistic interaction between *Foxl2* and *Dmrt1l* was demonstrated by the dual fluorescence reporter assay. This study provides a theoretical basis for sex markers and sexual differentiation in sex-reversed shellfish.

2. Materials and methods

2.1. Oysters sampling

Set 1: Seven-month-old diploid Pacific oysters were collected in cages for culturing in Sanggou Bay, Weihai, Shandong, China. From January to December 2020, the gonadal tissues of oysters were sampled in the middle of each month. A portion was frozen with liquid nitrogen and kept at −80 °C for extended periods of time. Another part of the gonads was treated and used for histological identification of sex. The sample was immobilized in 4% paraformaldehyde for 12 h, dehydrated three times in 75% alcohol, and then stored at 75% alcohol for tissue embedding fixation. Detection of oyster ploidy using flow cytometry (Qin et al., 2021).

Set 2: To predict the direction of gonadal differentiation of oysters during the resting stage, oysters at 7 months old and 8 months old were selected in January and February 2021. Oysters were anesthetized with 5% magnesium chloride for 6 h and a small piece of gonadal tissue was taken and instantly frozen in the liquid nitrogen and kept at −80 °C. After sampling, oysters were labeled with labels and then collected in cages and put back in the sea area for culture until the gonadal maturity in May. Surviving oysters were dissected for sampling and fixing in 4% paraformaldehyde, and the sex was distinguished by histology. In addition, another 20 oyster gonads were immobilized in 4% paraformaldehyde to determine the development period of gonads in January and February.

2.2. Gonadal histology

Gonadal tissues preserved in 75% alcohol were treated with xylene, then embedded in paraffin and sliced to 5 μm thickness with a microtome. Following dewaxing and hydration, sections were stained with hematoxylin-eosin. The stained slides were observed with a microscope. According to the previous studies, the gonads were separated into resting, proliferating, growing, and maturing stages (Berthelin et al., 2010; Cherif-Feildel et al., 2019).

2.3. Detection of Foxl2 and Dmrt1l expression

To examine the expression variation of *Foxl2* and *Dmrt1l*, histologically staged samples were used to examine the relative expression levels of *Foxl2* and *Dmrt1l* at different periods of gonadal development. Oysters in set 1 were utilized to detect the relative expression of *Foxl2* and *Dmrt1l* in annual gonads. Oysters in set 2 were utilized to detect the relative expression of *Foxl2* and *Dmrt1l* in resting stage. The extraction of total RNA from the gonads was sampled from January to December 2020 and January and February 2021. Extraction of RNA and synthesis of cDNA templates were performed using TRIzol reagent and PrimeScriptTM RT kit and gDNA Eraser (TaKaRa, Japan) according to the instructions, respectively. The cDNA templates were first diluted 5-fold, and then real-time quantitative PCR (qPCR) experiments were performed with ChamQ SYBR Colour qPCR Master Mix (Vazyme, China). Three technical replicates were set up for each sample and then amplified on a LightCycler 480 instrument (Roche, UK). Relative expression levels were obtained with Elongation factor I (*Ef1*) (Renault et al., 2011) as an internal reference gene and computed with the $2^{-\Delta\Delta CT}$ method.

2.4. Antagonism of Foxl2 and Dmrt1l by luciferase reporter assay

The oyster *Foxl2* (NCBI NO. LOC105319597) promoter sequence (−2511, −1991, −1522 bp from translation start site) and *Dmrt1l* (NCBI NO. LOC105337844) promoter (−2654 bp from ATG) were amplified from gDNA with primers with enzyme cut sites (Table 1). After double digestion of the amplification product and the pGL3.0 plasmid, the amplification product was ligated to the pGL3.0 plasmid (Invitrogen, USA) with the T4 ligase (Takara, Japan) and named pGL3 − 2.5k*Foxl2*, pGL3 − 2.0k*Foxl2*, pGL3-1.5k*Foxl2* and pGL3 − 2.5k*Dmrt1l* respectively. The same method was utilized to obtain pcDNA3.1 plasmids with *Foxl2* and *Dmrt1l* open reading frame and named pcDNA3.1-*Foxl2* and pcDNA3.1-*Dmrt1l*, respectively. Predicting potential binding sites for transcription factors in the *Foxl2* promoter using the JASPAR program (http://jaspar.genereg.net/). Insecta was selected as taxonomic groups to predict transcription factor DMRT1L binding sites in *C. gigas* and the default parameters were filtered.

Lipofectamine 3000 (Invitrogen, USA) was utilized to transfect 500 μL of human embryonic kidney 293 T (HEK 293 T) cells. The pGL3-*Foxl2* was co-transfected with pcDNA3.1-*Dmrt1l* to explore the effect of transcription factors on promoter activation. A certain amount of empty vector was also co-transfected to make the total vector amount 550 ng. The *Foxl2* promoter was transfected as follows: 1) 200 ng of pGL3 − 2.5 k *Foxl2* plasmid and 0, 100, 200, and 300 ng pCDNA3.1-*Dmrt1l*; 2) 200 ng of pGL3-2.0 k *Foxl2* plasmid and 300 ng of pCDNA3.1-*Dmrt1l*; 3) 200

Table 1
Primers used in this study.

Name	Primers (5′-3′)	Purpose
*Dmrt1l*_CDS F	ATGTCTGGTAACATTATTATCGATA	
*Dmrt1l*_CDS R	TTAATTTGTGTCGCGATACATAGCC	
*Foxl2*_CDS F	ATGTCGGAGAACAAAAACGAAAATG	
*Foxl2*_CDS R	TTACCTGTCAGTCCAGTACGAGTAA	
-2.5k_*Dmrt1l* F	AACAGGAAGTTCCTTCTACTCCTAA	
-2.5k_*Dmrt1l R*	CACTTCAAAAACTAATTTTTCAGCA	dual fluorescence reporter assay
-2.5k_*Foxl2* F	AATTATTTTGTTAACTATCTATGAG	
-2.5k_*Foxl2* R	GCTAATGAAGCATTTATATATTTGT	
-2k_*Foxl2* F	ATAGAAAGTCTACGGGGATGTTG	
-2k_*Foxl2* R	CATTTATATATTTGTTACAAGATAA	
-1.5k_*Foxl2* F	GAAGCTAAAATACGACTGAAAAACG	
-1.5k_*Foxl2* R	GCTAATGAAGCATTTATATATTTGT	
Dmrt1l RT F	ACCTGTGGGTCCTTGCCTT	
Dmrt1l RT R	GCTCTTGATTGGTGCTCTATGG	qPCR
Foxl2 RT F	CACAGTGTGGTTACAACGCAATGC	
Foxl2 RT R	CCTGTCAGTCCAGTACGAGTAATGC	
Mut1-*Foxl2* F	ATT**GGCTG*AAGTTGTGGCCCTGGGTCAGGAGCTCTG	
Mut1-*Foxl2* R	CCACAACTTCAGCCAATCCTTGCTCACTATAATCATGTCATTG	site-directed mutagenesis
Mut2-*Foxl2* F	TAGATACGTTGTTCCTCAAACTACGTTCATCCACTAATATGAAAA	
Mut2-*Foxl2* R	GAG**GAACAACGTATCTAAGACTATCGAAATGTCAGTCAAAAAGTG*	
ChIP-*Foxl2* F	GACTGACATTTCGATAGTCTTA	CHiP-PCR
ChIP-*Foxl2* R	AATAGAAAGTCTACGGGGATGTT	

* Italicized letters indicate mutant bases.

ng of pGL3 − 1.5 k *Foxl2* plasmid and 300 ng of pCDNA3.1-*Dmrt1l*. The *Dmrt1l* promoter was transfected as follows: 200 ng of pGL3-2.5 k *Dmrt1l* plasmid and 0, 100, 200, and 300 ng pCDNA3.1-*Foxl2*. And then, the 50 ng of pRL-TK (Promega, USA) was transfected to assess transfection efficiency and as an internal control. All transfections were performed three times, with three replicates each. After transfection for 48 h, the treated cells were washed with 1 × PBS and lysed in 100 μL lysis solution. Lysates were subjected to Luc activity analysis utilizing the luciferase assay kit (Promega, USA) in the Synergy NEO2 instrument. The results were normalized relative to the renilla luciferase activity.

2.5. Site-directed mutagenesis of Foxl2 promoter elements and chromatin immunoprecipitation assays

To verify whether the transcription factor DMRT1L can bind to the promoter region of *Foxl2* and reduce *Foxl2* expression, the point mutation was performed in *Foxl2* promoter based on previous studies (Min et al., 2022; Dai et al., 2021). Based on the potential binding sites (−2258 ATTTTACAAAGTTGTGG and − 2075 GAGCAAAAATGTATTTA) of DMRT1L at the *Foxl2* promoter predicted by the JASPAR program, the mutant site primers (Mut1-Foxl2F/R and Mut2-Foxl2F/R) were designed separately with the online software (http://www.vazyme.com) (Table 1). The mutant fragments were produced using the pGL3-2.5k*Foxl2* plasmid as the template and with the high-fidelity enzyme (Vazyme, China). After digestion of the plasmid template by Dpn1 endonuclease (NEB, USA), the point mutant plasmids were obtained by recombination with Exnase II (Vazyme, China). The target mutant plasmids were identified by Sanger sequencing and named Mut1-*Foxl2* and Mut2-*Foxl2*, respectively. The mutation of the *Foxl2* promoter was transfected as follows: I) 200 ng of pGL3-2.5 k *Foxl2* plasmids and 300 ng pCDNA3.1; II) 200 ng of pGL3-2.5 k *Foxl2* plasmids and 300 ng of pCDNA3.1-*Dmrt1l*; III) 200 ng of Mut1-*Foxl2* plasmids and 300 ng of pCDNA3.1; IV) 200 ng of Mut1-*Foxl2* plasmids and 300 ng of pCDNA3.1-*Dmrt1l*; V) 200 ng of Mut2-*Foxl2* plasmids and 300 ng of pCDNA3.1; VI) 200 ng of Mut2-*Foxl2* plasmids and 300 ng of pCDNA3.1-*Dmrt1l*. Each group was co-transfected with 50 ng of the pRL-TK plasmid. All transfections were performed three times, with three replicates each. Detection of luciferase activity was performed 48 h later.

To confirm that the transcription factor DMRT1L can bind to the *Foxl2* promoter, chromatin immunoprecipitation PCR (ChIP-PCR) assays were performed. Firstly, the ORF region of *Dmrt1l* and the pCMV-Myc-C plasmid were doubled-cleaved by *Eco*RI and Kpn1 endonuclease (NEB, USA), and then the two fragments were ligated with T4 ligase to construct the pCMV-DMRT1L expression plasmid with Myc tag. And finally, the 4 μg pGL3-2.5kFoxl2 plasmid, 6 μg pCMV-DMRT1L plasmid, and 1 μg pRL-TK plasmid were transfected into 10 mL HEK 293 T cells with transfection reagent Lipofectamine 300. Latterly, the ChIP Assay Kit (Beyotime, China) was utilized for performing chromatin immunoprecipitation experiments according to the instructions. Briefly, cells were first incubated with 1% formaldehyde to cross-link the target protein and the corresponding genomic DNA, followed by lysis and ultrasonic treatment of the cells. Next, 20 μL of supernatant liquid was aspirated out as cellular extract (positive control) to check plasmid transfection into the cells. The remaining supernatant liquid was incubated overnight with 1 μg of anti-Myc- tagged antibody and 1 μg of non-specific antibody IgG (negative control) that did not bind to the labeled protein, respectively. Finally, the DNA obtained was amplified with specific primers (Table 1) and detected using a 1% agarose gel.

2.6. Statistical analyses

In this study, data with biological replicates were presented as the mean ± SD. Before analysis of variance, data were checked for normality and homogeneity of variance. Differences in expression levels of *Foxl2* and *Dmrt1l* at different periods of gonadal development and dual luciferase reporter results were analyzed using a one-way analysis of variance (ANOVA) and Tukey's test.

3. Results

3.1. Gene expression of the oyster gonads

For the purpose of studying the expression patterns of *Foxl2* and *Dmrt1l* in *C.gigas*, an assay was performed to examine these two genes' relative expression levels in different developmental stages of the gonads. The results showed that *Foxl2* expression levels were significantly higher than those of *Dmrt1l* in resting (Fig. 1A), females proliferating (Fig. 1B), females growing (Fig. 1C) and females maturation stages (Fig. 1D). The expression of *Foxl2* was more than 100-fold higher than that of *Dmrt1l* in the last three stages (Fig. 1B-D). Among the three stages of the male gonad (Fig. 1E-G), the expression of *Dmrt1l* was higher than *Foxl2* in the growing and maturation stages (Fig. 1F). Then, to further

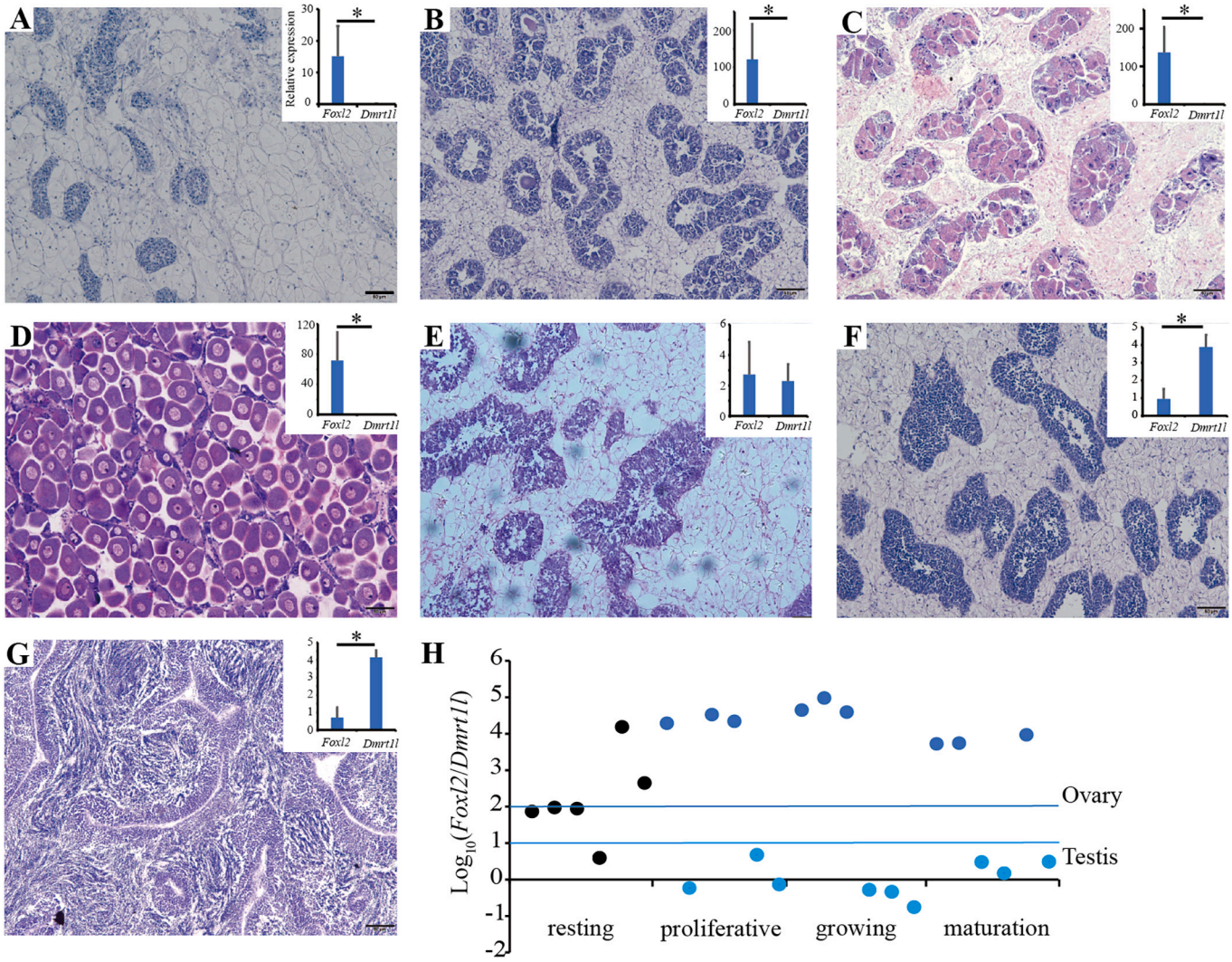

Fig. 1. Gene expression of the oyster gonads. *Foxl2* and *Dmrt1l* expression in resting stage (A), female proliferating stage (B), female growing stage (C), female mature stage (D), male proliferative stage (E), male growing stage (F), male mature stage (G). H The patterns of Log_{10} (*Foxl2/Dmrt1l*) values in the four stages. A-G Three oyster samples were utilized for each period, H each dot represents one oyster sample. Dark blue dots indicate female oysters, light blue dots indicate male oysters, and black dots indicate oysters that cannot distinguish between the sexes. An asterisk indicates statistical differences ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

explore whether there was a relationship between *Foxl2* expression and *Dmrt1l* expression that could be used for sex identification, the Log_{10} (*Foxl2/Dmrt1l*) values were calculated for the four reproductive stages in females and males. The results showed during the proliferative, growth, and maturation stages, values below 1 were for male gonads and above 2 for female gonads. Whereas, the Log_{10} (*Foxl2/Dmrt1l*) values were greater than 2, less than 1, or between 1 and 2 in the resting stage (Fig. 1H).

3.2. Determining timing of molecular sex differentiation

Subsequently, to further determine whether Log_{10} (*Foxl2/Dmrt1l*) value could be utilized as a stable marker of sex discrimination, we investigated the variation of its values in the annual gonads. The results showed that in months from March to October sex can be distinguished based on histological observations (data not shown). Though the Log_{10} (*Foxl2/Dmrt1l*) values fluctuated, the values greater than 2 were considered females and less than 1 were considered males. Unsurprisingly, the Log_{10} (*Foxl2/Dmrt1l*) values showed the same trends as in the resting stage in the months (January, February, November and December) when the sex was difficult to distinguish (Fig. 2).

3.3. Sexual prediction of the resting stage

Based on the results of our annual survey shown in Fig. 2, Log_{10} (*Foxl2/Dmrt1l*) values >2 were always correctly identified as females, and < 1 were always correctly identified as males. However, in January and February when oysters were in the resting stage, there were three possible values (> 2, <1 or ≥ 1, and ≤ 2). During these months it is not clear whether these values serve to forecast the sex of *C. gigas* in the adult early gonadal stage when the sex was difficult to distinguish by histology. Therefore, we sampled the same individual twice (see materials and methods), the first time to calculate Log_{10} (*Foxl2/Dmrt1l*) values, and the second time to identify sex by histology. The sex of the gonads sampled in January (Fig. 3A) and February (Fig. 3B) could not be determined by histological analysis; however, several oysters had Log_{10} (*Foxl2/Dmrt1l*) values >2 or < 1, and these oysters developed into females and males respectively. This demonstrates that even in the resting stage, the *Foxl2/Dmrt1l* assay can predict the sex of mussels if the Log_{10} (*Foxl2/Dmrt1l*) values are >2 or < 1. However, individuals with Log_{10}

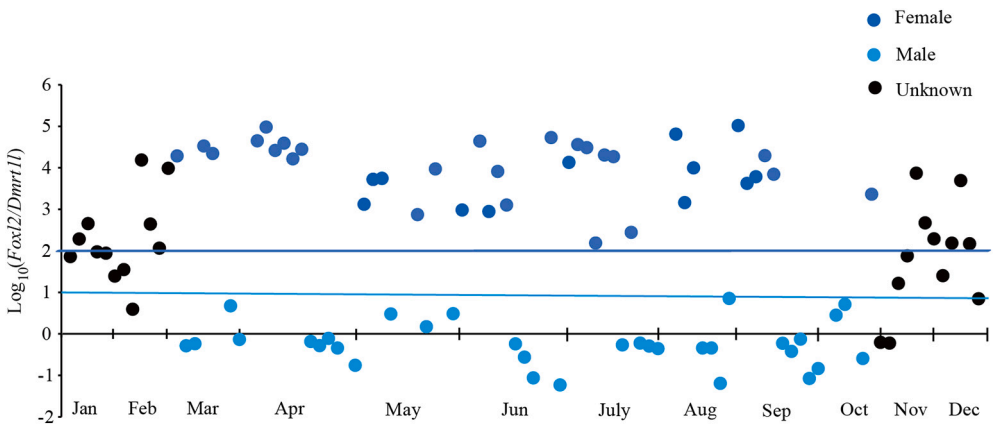

Fig. 2. Variation of Log_{10} (*Foxl2/Dmrt1l*) values in the annual gonads. Each dot represents one oyster sample, dark blue dots indicate female oysters, light blue dots indicate male oysters, and black dots indicate oysters that cannot distinguish between the sexes (Unknown). A total of 92 oyster samples were used in set 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

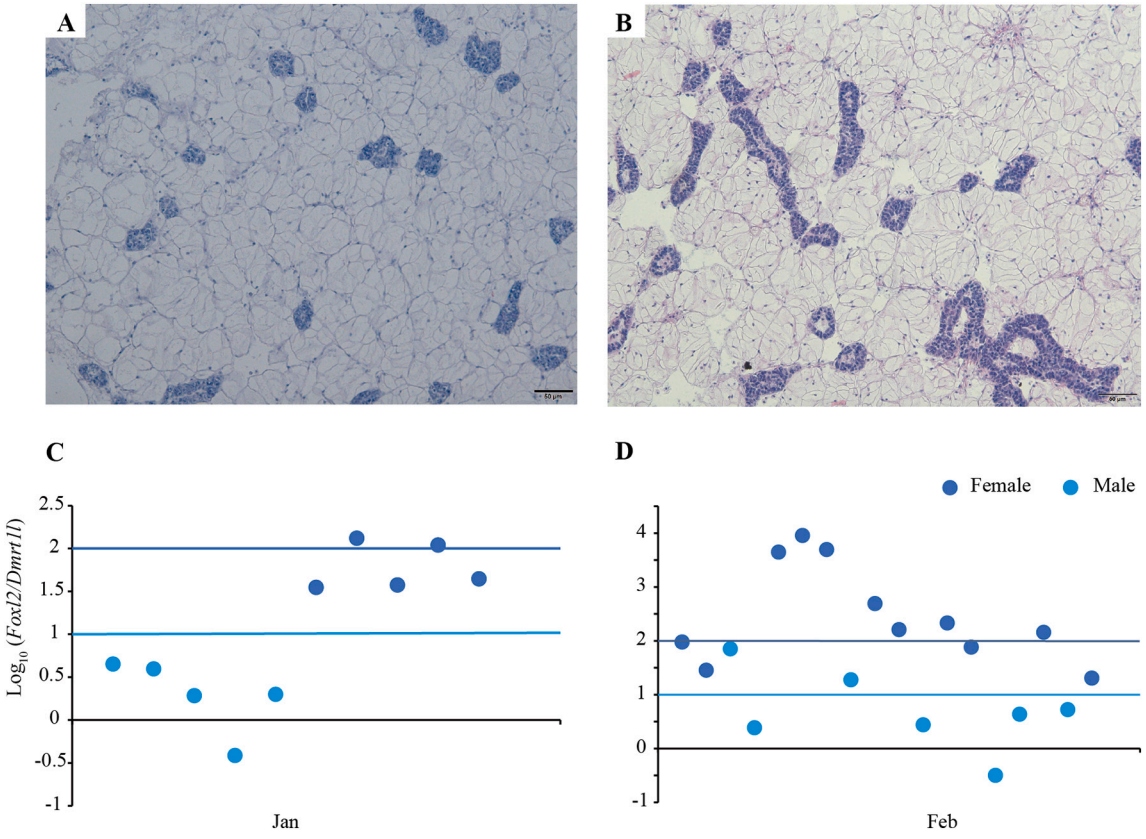

Fig. 3. Sexual prediction of resting stage. The histological analysis of gonads in January (A) and February (B). Log_{10} (*Foxl2/Dmr1*) values in January (Jan, C) and February (Feb, D). Each dot represents one oyster sample, dark blue dots indicate female oysters and light blue dots indicate male oysters. A total of 10 oyster samples (C) and 18 oyster samples (D) from set 2 were utilized. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(*Foxl2/Dmrt1l*) values between 1 and 2 developed as either females or males in the future and their sex could not be predicted (Fig. 3C and D). Therefore, we were not able to predict the sex of individuals with Log_{10} (*Foxl2/Dmrt1l*) values between 1 and 2.

3.4. Co-expression pattern of these two genes in oyster gonads

The co-expression pattern of *Foxl2* and *Dmrt1l* was described using log values of their expression levels in annual samples and correlation analysis was performed. Two major co-expression patterns existed between *Foxl2* and *Dmrt1l* during the 12 months investigated: (i) a significant negative correlation ($r = -0.75$, $P < 0.01$) for the 8 sexual differentiated months, with a regression equation of $y = -0.9118\times$-0.0776 (Fig. 4A); (ii) a no significant negative correlation ($r = -0.23$, $P > 0.05$) for the other months that the sexual undifferentiated, with a regression equation of $y = -0.4729\times$-0.5538 (Fig. 4B).

3.5. Antagonistic action of Foxl2 and Dmrt1l in oysters

The luciferase assay showed that DMRT1L significantly inhibited the

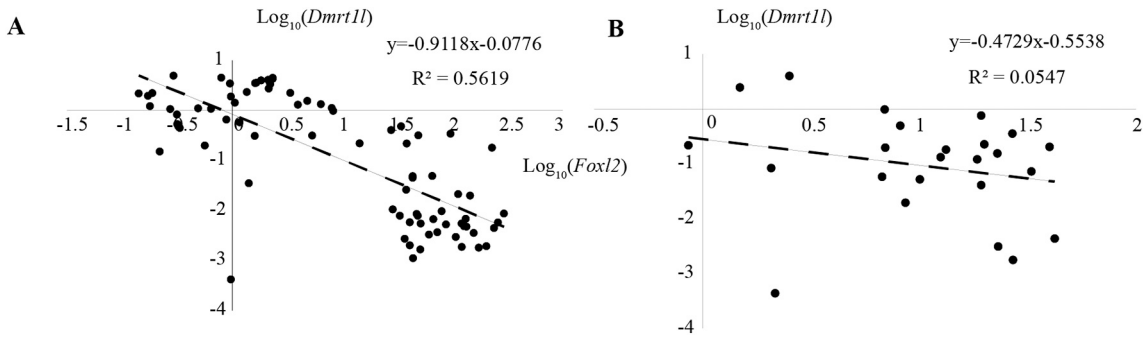

Fig. 4. Correlation analysis between *Foxl2* and *Dmrt1l*. (A) Correlation analysis between Log_{10} (*Foxl2*) and Log_{10} (*Dmrt1l*) from March to October. (B) Correlation analysis between Log_{10} (*Foxl2*) and Log_{10} (*Dmrt1l*) from November to February.

Foxl2 promoter activity in HEK293 cells. (Fig. 5A). Activity of the 2.5 kb promoter region of *Folx2* was inhibited by the transcription factor DMRT1L, which reduced the activity of the *Foxl2* promoter to 66.2%. However, in the promoter regions 2.0 kb and 1.5 kb, DMRT1L had no significant effect mediated on *Foxl2* expression, which was 0.98-fold and 1.09-fold of that without DMRT1L, respectively (Fig. 5B). Three potential DMRT1L binding sites (site1-3: −2258 to −2242 bp, −2075 to −2057 bp and − 41 to −25 bp) were predicted from the starting codon ATG of *Foxl2* (Fig. 5B).

Based on the analysis of the above results, it was obtained that DMRT1L may bind the *Foxl2* promoter and repress its expression. Therefore, the potential DMRT1L binding sites in *Foxl2* promoter were mutated and then subjected to the dual fluorescence reporter assay. Interestingly, the results showed that the relative fluorescence activity of the mutated experimental groups (III and V) was significantly higher compared to the blank control group I and unmutated group (II) (Fig. 5C). This implies that the mutated promoter region has higher transcriptional activity than the unmutated promoter region. The relative fluorescence activity of mutation 1 was significantly reduced by the addition of transcription factor DMRT1L (IV), but was not significantly

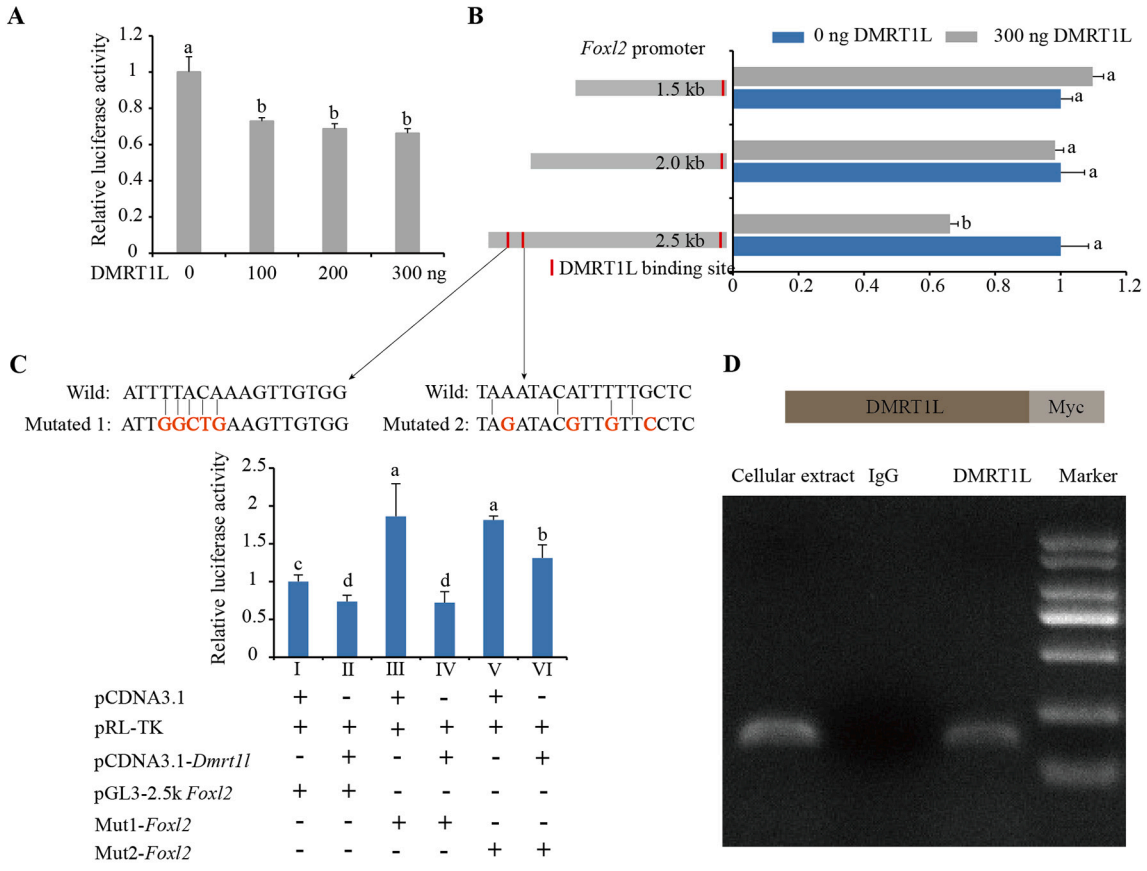

Fig. 5. Influence of DMRT on the *Foxl2* promoter. (A) DMRT1L overexpression represses the promoter activity of *Foxl2* in HEK293 cells. (B) 5′-deletion mapping of the DMRT1L-binding region on the oyster *Foxl2* promoter. Red boxes indicate predicted DMRT1L binding sites. (C) Enzyme activity measurement of point mutation of *Foxl2* promoter. pCDNA3.1 represents the empty vector; pRL-TK represents the internal control vector; mut1-*Foxl2* and mut2-*Foxl2* represent the vectors for the mutation Foxl2 promoter, respectively. "+" and "-" denote whether the plasmid was transfected into the HEK-293 T or not. Data are mean ± S.D. (D) ChIP-PCR assay in HEK-293 T with pCMV-DMRT1L. Cell extracts were used as a positive control and IgG was used as a negative control to detect the presence of non-specific proteins. Marker denotes a 2000 bp marker. Different letters indicate statistical differences ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

different from the unmutated group (II) with DMRT1L. Mutation 2 (VI) showed significantly lower relative fluorescence activity compared to the control group (V) but was significantly higher than the unmutated group (II) and mutation 1 (IV). Although luciferase activity was reduced in both mutation 1 and mutation 2 compared to the control, luciferase activity was increased in mutation 2 (VI) compared to mutation 1 (IV). So, the transcription factor DMRT1L may inhibit *Foxl2* gene expression in combination with the binding site 2.

To further verify the binding of DMRT1L to *Foxl2* promoter binding site 2, we performed a ChIP-PCR experiment. The results showed that the specific primers were able to amplify bands in the positive control (cellular extract) and DMRT1L groups, while the absence of bands in the negative control IgG group (Fig. 5D). In conclusion, the DMRT1L was able to bind to the *Foxl2* promoter region and repress *Foxl2* gene expression.

4. Discussion

4.1. Log_{10} (*Foxl2/Dmrt1l*) values as an efficient method for sex identification of C. gigas

FOXL2 and DMRT1L are considered to be the second players in the sex-determining downstream cascade pathway, whose expression levels are monitored both during the gametogenic cycle and the early stages of adult gonadal differentiation (Broquard et al., 2021; Naimi et al., 2009; Santerre et al., 2013). *Foxl2* was expressed in gonads of both sexes, whereas *Dmrt1l* was expressed only in male gonads and its expression level showed sexual dimorphism, in line with the previous studies (Naimi et al., 2009). In other animals the presence of many important ovarian-specific factors (β-Catenin, Follicle suppressor, FOXL2, R-spondin, and WNT4), are required for ovarian development (Biason-Lauber, 2012; Chassot et al., 2014). Therefore, the hypothesis that ovarian formation is the "default" gonadal developmental pathway due to the absence of SRY expression, seems to be disproven in many species (Huang et al., 2017). In the oysters, whether the female gonad is the "default" gonadal development pathway is not clear, as *Foxl2* is expressed in small amounts in the resting stage relative to the proliferative and mature phases, but the expression of *Dmrt1l* is associated with the production of male oysters. In the scallops, the expression level of *Dmrt1l* was significantly higher than *Foxl2* (Li et al., 2018), while the opposite was true in oysters. Therefore, in order not to introduce negative numbers, we used Log_{10} (*Foxl2/Dmrt1l*) values for the identification of oysters' sex. By analyzing Log_{10} (*Foxl2/Dmrt1l*) values for four stages of gonadal development, the values were always below 1 in male gonads and above 2 in female gonads, which indicated that in oysters, (i) *Foxl2* and *Dmrt1l* had essential effects for sex maintenance and gametogenesis, and (ii) Log_{10} (*Foxl2/Dmrt1l*) values served as a valid tool for identification of sex.

4.2. Log_{10} (*Foxl2/Dmrt1l*) values can predict the direction of resting gonad development

The dynamics of the Log_{10} (*Foxl2/Dmrt1l*) values in the annual samples matched the gonad development stage. January, February, November, and December correspond to the resting stage (sex is not determinable), and the sex can be determined by histological observation in the other eight months, in line with former work on the annual development of the gonads (Kim et al., 2010). Even though most oysters have entered the resting stage in November, the presence of sperm or eggs in individual oysters can still be observed in histological analyses due to interindividual variation. The dynamic changes of Log_{10} (*Foxl2/Dmrt1l*) values combined with histological observations indicated that molecular sex differentiation precedes histological sex differentiation, in agreement with previous studies on the scallops (Li et al., 2018). In the scallops, sexual reversal does not occur once sexual differentiation occurs (Li et al., 2018), but can occur in oysters. The time window for early gonadal differentiation in adult oysters is defined as the termination of a cycle when the animal matures and spawns. At the beginning of a new cycle, germinal stem/progenitor cells emerge (Cherif-Feildel et al., 2019) whose appearance is driven by sex-determination genes that trigger male or female differentiation (Broquard et al., 2021). The window for sex reversal in the oyster is the resting stage, during which extensive molecular genetics and cellular processes facilitate sex differentiation, suggesting that is a cumulative process. The developmental window for sex is prolonged, extending over several weeks (Weber and Capel, 2021). The asynchrony of gonadal development due to differences between individuals explains the presence of Log_{10} (*Foxl2/Dmrt1l*) values in three forms in the resting stage of gonadal development. The Log_{10} (*Foxl2/Dmrt1l*) values in sex determined/differentiated individuals were between 1–2 and in the other two cases, the gonads had initiated the pathway of female or male sex differentiation.

The resting stage is a critical period for sex differentiation in oysters. The recent paper has reportedly linked genes associated with resting sex differentiation to future sex (Broquard et al., 2021), while other studies related to sex differentiation in the oyster have focused on the comparison of differences of both sexes after the resting stages (Dheilly et al., 2012; Yue et al., 2018; Zhang et al., 2014). This is mainly caused by the fact that the oysters are continuous hermaphrodites thus leading to uncertainty of sex in the resting stage. In this study, resting oysters were sampled under anesthesia and then temporarily reared at sea until they developed to the discernible sex, to determine if we could predict resting sex by future phenotype. Analysis of gonad samples at different stages of development showed that Log_{10} (*Foxl2/Dmrt1l*) values can be used as a molecular evaluation method to assess early gonadal differentiation in some oysters at resting stage if their Log_{10} (*Foxl2/Dmrt1l*) values are >2 or < 1. Thus, this marker can be utilized to identify the sex of oysters with resting gonads, followed by multi-omics analysis to discover key genes in the early gonadal stages of sexual differentiation.

4.3. Inhibitory mechanism of gonad differentiation in C. gigas

Sex differentiation is the accumulation of molecular events that tip the sex scales in favor of one fate until a threshold is reached that is sufficient to maintain and stabilize one pathway while inhibiting the other (Weber and Capel, 2021). Once gametogenesis begins, individuals remain in an undifferentiated state of gonad until after spawning. Some individuals keep the same sex for several years, while others may undergo multiple sexual reversals during their lifetime (Berthelin et al., 2010; Broquard et al., 2020). The resting period is an essential time window for gonadal differentiation in oysters, during which sex-related genes, including *Foxl2* and *Dmrt1l*, are involved (Broquard et al., 2021). The co-expression patterns of these two genes varied during gonadal differentiation and undifferentiation, which resulted in a significant negative correlation between *Dmrt1l* and *Foxl2* from March to October, but a nonsignificant negative correlation in other months. The expression of genes associated with early sexual differentiation can influence the future sex phenotype of oysters (Broquard et al., 2021). This indeterminate sex may be related to a combination of genetic and environmental factors (Yue et al., 2020). The accumulation of *Dmrt1l* expression suppresses the expression *Foxl2*, resulting in male oysters. Thus, the future direction of sexual differentiation in oysters is influenced by the early expression of *Foxl2* and *Dmrt1l*. Once sex is determined, *Foxl2* and *Dmrt1l* expression is maintained at a relatively stable level to maintain sex differentiation or gonadal development.

In normal conditions, gonadal cell fate transition occurs as determined by *Dmrt1l* (Matson and Zarkower, 2012). In vertebrates, the binding of DMRT1L to the *Foxl2* promoter leads to a reduction in *Foxl2* gene expression levels (Dai et al., 2021; Matson et al., 2011). In vitro experiments likewise confirmed the ability of DMRT1L to bind to and inhibit the activity of the oyster *Foxl2* promoter, which may be related to the fact that DMRT1L in oysters shares a similar function with DMRT1 in vertebrates (Zhang et al., 2014; Yue et al., 2021). Unlike tilapia (Dai

et al., 2021), the dual fluorescence reporter results did not show a repressive effect of FOXL2 on *Dmrt1l* gene expression (Supplemental Fig. 1), suggesting that FOXL2 may indirectly regulate *Dmrt1l* gene expression through other network pathways in oysters. The resting phase is a critical period in oyster gonad development during which two distinct regulatory networks direct the differentiation of germinal stem cells toward male or female gonads and may lead to sexual reversal (Cherif-Feildel et al., 2019). For the formation of either sex to be ensured, both signaling networks must work in a mutually exclusive manner. In males, the expression of genes involved in male differentiation (*Dmrt1l*, *SoxH*) leads to not only enhanced activation of the male sex differentiation pathway and also repression of key genes (*Foxl2*) for female sex differentiation (Li et al., 2018; Sun et al., 2022). Likewise, activation of female signaling molecules might negatively affected male gene expression (Zhang et al., 2014). Apparently, this mutually exclusive regulatory signaling is essential for both the initial establishment of sex and the maintenance of gonadal differentiation in adulthood. Like Yin and Yang, *Foxl2* and *Dmrt1l* maintain female and male gonadal phenotypes by activating their own sex differentiation pathways and blocking each other's signaling networks, respectively (Huang et al., 2017). Therefore, antagonism of these sex-specific transcriptional regulators is essential for the stability of gonadal phenotypes in both sexes throughout reproduction.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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