



## DNA methylation status of *cyp17-II* gene correlated with its expression pattern and reproductive endocrinology during ovarian development stages of Japanese flounder (*Paralichthys olivaceus*)

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### ABSTRACT

Cytochrome P450c17-II (*cyp17-II*, 17 $\alpha$ -hydroxylase) is responsible for the production of steroid hormones during oocyte maturation in vertebrates. The comparative expression pattern of *cyp17-II* gene during the gonadal development stages will provide important insights into its function of gonadal development. In addition, epigenetic modification especially DNA methylation plays a vital role in regulation of gene expression. The adult female Japanese flounder at different ovarian development stage (from stages II to V) was obtained in this experiment. The expression of *cyp17-II* gene in the ovary of Japanese flounder during the gonadal development stages was measured by quantitative PCR. Reproductive traits included gonadosomatic index (GSI), plasma estradiol-17 $\beta$  (E<sub>2</sub>) and testosterone (T) were also measured. Moreover, whole CpG dinucleotides methylation status of the two CpG rich regions in *cyp17-II* coding region was detected by bisulfate sequencing. In the ovary, the *cyp17-II* gene had the lowest mRNA expression at the early ovarian development stage, but then increased afterward. The variation trends of T and E<sub>2</sub> level were consistent with the *cyp17-II* expression pattern in ovary. In contrast, the whole methylation levels of each CpG rich region (exon 4 and 6) in *cyp17-II* coding region were declined from stages II to IV, then increased at stage V. The methylation levels of whole CpG sites in each CpG rich region were inversely correlated with the values of ovarian *cyp17-II* gene expression, T and E<sub>2</sub> level, and GSI. Based on the present study, we proposed that *cyp17-II* may regulate the level of steroid hormone, and then stimulate the oocyte growth and maturation. The *cyp17-II* gene transcriptional activity was possibly affected by the methylation level of CpG rich regions in coding region. These findings will help in the study of the molecular mechanism of fish reproduction and endocrine physiology.

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### 1. Introduction

Cytochrome P450c17 (CYP17) possessing 17 $\alpha$ -hydroxylase and 17, 20-lyase activities is a critical enzyme responsible for the pathways of steroid hormone synthesis in the gonads and head kidney of fish (Ko and Engel, 1993; Miller et al., 1997; Nagahama et al., 1994, 1997, 2000). Two forms of P450c17 were identified in some teleost species, they were P450c17-I and P450c17-II, encoded by *cyp17-I* and *cyp17-II* gene, respectively (Zhou et al., 2007a,b). P450c17-I possesses both hydroxylase and lyase activities, while P450c17-II only has the hydroxylase activity. In tilapia, P450c17-I is essential for the production of estradiol-17 $\beta$  (E<sub>2</sub>) during oocyte growth, while P450c17-II plays a key role in the production

of 17 $\alpha$ , 20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ , 20 $\beta$ -DP) during oocyte maturation (Zhou et al., 2007a,b). It was shown that only P450c17-II was involved in the cortisol production in the head kidney of tilapia (*Oreochromis niloticus*) and barfin flounder (*Verasper moseri*) (Jin et al., 2012; Zhou et al., 2007a,b). Nevertheless, so far, no data is available for the temporal profiles of *cyp17-II* gene expression in the ovary of Japanese flounder.

DNA methylation constitutes an important epigenetic factor in the control of genetic information. Gene expression is controlled by the chromatin configuration and the DNA methylation status of the specific genes besides by the presence of regulatory sequences (Marie et al., 2010). The placenta-specific methylation status of promoter I.1 of *Cyp19* was responsible for aromatase expression in buffalo placental cotyledons and coincided with the change of the expression of *Cyp19* gene in different stages of pregnancy (Ghai et al., 2010). In zebrafish, DNA methylation on cytosine serves to silence gene expression by interfering with the binding of certain transcription factors and through the recruitment of repressive chromatin machinery (Wu et al., 2011).

Abbreviations: GSI, Gonadosomatic index; E<sub>2</sub>, Plasma estradiol-17 $\beta$ ; T, Testosterone; *cyp17-II*, Cytochrome P450c17-II; RT, RNA extraction and reverse transcription; qPCR, Quantitative PCR; BS-PCR, Bisulfate PCR.

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Many previous studies have focused on the gene silencing effects of methylation of CpG islands and even scattered sites in promoter and exon 1 regions (Antequera and Bird, 1999). However, some report indicated that DNA methylation in the coding regions is also an important silencer and recent evidences demonstrated the silencing of human tissue-specific expression of PDHA2 gene by methylation of the coding region (Pinheiro et al., 2010). In addition, in our previous study, we have reported that the methylation pattern of specific CpG site in coding region had negatively regulated the gene expression of *cyp17-II* (Ding et al., 2012). However, nowadays, no data is available about the relationship between DNA methylation level of *cyp17-II* coding region and the gene expression during gonadal development stages of female Japanese flounder. The cDNA sequence of *cyp17-II* was obtained from Gene bank (FJ613529.2) and we have forecasted the CpG rich regions as previously described (Ding et al., 2012). It is supposed that DNA methylation pattern of the CpG sites within the coding region (gene body) might play an important role in regulating gene expression in different stages of ovarian development.

Our previous research mainly focused on the study of associations of SNPs in *cyp17-II* gene to the reproductive endocrine of Japanese flounder (Ding et al., 2012). But, the differences of methylation level of *cyp17-II* gene during ovarian development and the relationship between gene expression and DNA methylation is unclear. In the present study, we aimed to analyze the regulation mechanism of *cyp17-II* gene expression based on epigenetic. The samples of four ovarian development stages were obtained and the *cyp17-II* gene expression in ovary were examined by quantitative PCR, then the CpGs methylation map was determined by bisulfate sequencing in this experiment. In addition, reproductive traits: T level, E<sub>2</sub> level and GSI were measured. The amino acid encoded by *cyp17-II* gene analysis was performed, for the purpose to find whether these CpG sites studied were located in the proteins motifs which may play important role in enzymatic function.

## 2. Materials and methods

### 2.1. Experimental fish

The experimental female Japanese flounder were obtained from a local fish farmer every twenty days after reaching adulthood. They came from the same parents, and were reared in commercial fish pond under controlled conditions (20 ± 0.5 °C; ≥ 4 mg/L O<sub>2</sub>; 14:10 h light: dark cycle), and were fed on a commercially prepared diet. More than six individuals were randomly sampled each time. Sixty fish were obtained finally. All fish were anesthetized in tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO) prior to tissue sampling. The blood was extracted from the fish tail. Organs collected from fish were immediately frozen in liquid nitrogen and then stored at –80 °C until further processing. Body weight, viscera weight and gonad weight were measured in each sample fish to calculate gonadosomatic index (GSI = [gonad weight / (body weight-viscera weight)] × 100).

### 2.2. Histological analysis

Parts of the ovarian samples of all the sixty fish obtained were fixed in Bouin's solution for 24–48 h. Fixed gonad blocks were dehydrated in a 70%–100% ethanol series, embedded in paraffin wax, and 5–8 μm sections were cut by microtome (LEICA-RM2016), followed by hematoxylin and eosin (HE) staining to identify the ovarian developmental stage according to the criterion of Liu and Zhang (2003).

### 2.3. Steroid radioimmunoassay (RIA)

Blood samples from each fish obtained were clotted at 4 °C for 8 h, then centrifugated at 12,000 g for 10 min. The serum was obtained and transferred into 1.5 ml plastic microfuge tubes and then stored

at –40 °C. The serum concentrations of T and E<sub>2</sub> were detected by <sup>125</sup>I radioimmunoassay of the samples in ovarian development stages II to V (Three fish were randomly selected from each development stage), using the method of Wen et al. (2006).

### 2.4. RNA extraction and reverse transcription (RT)

Total RNA of ovary tissue was extracted using RNAiso reagent (TaKaRa, Japan) following manufacturer's instructions. The ovary tissues were obtained from three different individuals of each ovary stage. RNA concentration of each sample was quantified in UV spectrophotometer (Ultrospec-2100Pro, Amersham), and an agarose gel was applied to check RNA integrity. Complimentary DNA was synthesized with PrimeScript® RT reagent Kit (TaKaRa). Total RNA (500 ng) was reverse transcribed in a total volume of 10 μl using 2 μl 5 × PrimeScript® Buffer (for Real Time PCR), 0.5 μl PrimeScript® RT Enzyme Mix I, 0.5 μl OligodT Primer (50 μM), 0.5 μl Random 6 mers (100 μM) and RNase Free dH<sub>2</sub>O. Reaction conditions were at 37 °C for 15 min, then at 85 °C for 5 s.

### 2.5. Quantitative PCR (qPCR)

Quantitative PCR was performed using an Eppendorf iCycler iQ Multicolor Real-Time PCR Detection System (Eppendorf, Hamburg, USA) and the iQ™ SYBR Green Supermix (Takara, Japan) according to the manufacturer's protocol. QPCR primers for *cyp17-II* and 18S ribosomal RNA were listed in Table 1. The 25 μl mixture of PCR consisted of 12.5 μl SYBR Green supermix, 9.5 μl RNase-free water, 0.5 μl of each primer and 2 μl reverse transcribed product. PCR program was as follows: 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 20 s, 64 °C for 15 s and 72 °C for 15 s. At the end of PCR run, a melt curve analysis was performed to ensure that only a single product was amplified. The experiment was repeated in triplicate. Meanwhile, as an internal control, 18S ribosomal RNA was amplified in the same manner using primers specific for Japanese flounder. All other PCR reagents were used but without cDNA added for a PCR negative control.

All target gene expression was normalized against the 18S rRNA expression calculated threshold-cycle (Ct) level. The samples from stage II with serial dilutions of total cDNA were used as calibrators in this experiment. The result was analyzed according to the  $2^{-\Delta\Delta C_t}$ , where  $\Delta\Delta C_t = (C_{t, cyp17-II} - C_{t, 18S})_{\text{time } 0} - (C_{t, cyp17-II} - C_{t, 18S})_{\text{time } 0}$ , time 0 represents the stage II, according to the method described by Kenneth and Thomas (2001).

### 2.6. Bisulphate modification of genomic DNA and forecasting the CpG rich region

Genomic DNA was isolated from ovary tissues of three individuals (the same ovary tissues were used for RNA isolating) with each reproductive stage using Marine Animals DNA Kit (CW BIO). The DNA purity and concentration were measured using an UV spectrophotometer (Amersham, American), and their integrity was evaluated by agarose gel electrophoresis. Then the genomic DNA (200 μg) was bisulfate-modified by the Methylamp™ DNA Modification Kit (TIANGEN, China) according to the manufacturer's instructions. Finally, bisulfite treated DNA was eluted by 16 μl of elution buffer, as previous described (Ding et al., 2012).

**Table 1**  
Primers used for quantitative PCR.

Primer name	Sequence (5' to 3')	Product size (bp)	Tm (°C)	Accession no.
<i>cyp17-II</i>	F:GAGCCGAGCAGTGAGCGTGTC R:ACGAGTCCCAGACCGACAG	159	62	FJ613529.2
18S	F:ATTGACGGGAAGGGCACCAC R:ATGCACCACCCACAGA	134	62	EF126037.1

**Table 2**  
Primers used for bisulphate PCR (BS-PCR).

Primer name	Sequence (5' to 3')	Product size (bp)	Tm (°C)	Accession no.
cyp17-II-e4	F:TTGTTTACTGGGGTTATGGTTT R:CCTTCATCCAAAATAAATATCCAC	196	56	FJ613529.2
cyp17-II-e6	F:TTTGTGTAAGGTAAGATGGATA R:CAACAAATAAACCAAAATCCACAAC	149	54	

The CpG rich regions of *cyp17-II* were forecasted using online MethPrimer design software (<http://www.urogene.org/methprimer/index1.html>).

### 2.7. Bisulphate PCR (BS-PCR) and efficiency of bisulfite modification

BS-PCR primers were designed on oligo 6 software based on the sense strand of the bisulfate-modified DNA (Table 2). PCR was carried out in a final volume of 25  $\mu$ l containing 50 ng bisulfate-treated DNA, 10 pmol each primer, 0.3 mM each dNTP, 0.125  $\mu$ l TaKaRa EpiTaq™ HS (for bisulfate-treated DNA) (5 U/ $\mu$ l). PCR cycling conditions were as follows: 4 min at 95 °C; 40 cycles of 30 s at 94 °C, 30 s at 56 °C for cyp17-II-e4 or 30 s at 54 °C for cyp17-II-e6, and 1 min at 72 °C; and finally ended with 10 min at 72 °C for extension. Three independent PCR amplifications were carried out for each sample. Genomic region corresponding to the CpG rich regions (i.e. exon 4 and 6) were also amplified using untreated genomic DNA as template for comparison purpose. All the PCR products were separated on 1.5% agarose gel, purified using the TIAN gel midi Purification Kit (TIAGEN, China) and then cloned into the PGM-T vector (TIAGEN, China), and transferred into Trans-5a Chemically Competent Cell (Beijing TransGen Biotech Co, Ltd). At least three positive recombinant colonies of each product were sequenced using the ABI3730XL sequencer (ABI, USA).

Bisulfate sequencing is the gold standard in determining DNA methylation. The method is based on the selective deamination of cytosine to uracil by treatment with bisulfite and the sequencing of subsequently generated PCR products. In contrast to cytosine, 5-methylcytosine does not react with bisulfite and can therefore be distinguished (Grunau et al., 2001). So in order to value the rate of chemical conversion of the region of exon 4 and 6, the efficiency of the bisulfite modification was

calculated by the number of non-converted cytosine pre the total number of cytosine which was not in the context of a CpG dinucleotide.

### 2.8. Analyzing the amino acid sequence encoded by open reading frame of *cyp17-II* gene

The ORF (open reading frame) of *cyp17-II* gene was searched by ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/orf.cgi>). The conserved P450c17-specific region was identified in *cyp17-II* gene of Japanese flounder by compare its amino acid residues with Korean rockfish (*Sebastes schlegeli*) (JN165364), barfin flounder (*Verasper moseri*) (FJ168471), half-smooth tongue sole (*Cynoglossus semilaevis*) (EU732518.2), tilapia (*Oreochromis niloticus*) (XM\_003454670.1), three-spined stickleback (*Gasterosteus aculeatus*) (NM\_001267655.1) and Fugu rubripes (*Takifugu rubripes*) (NM\_001105221.1). Transcription factors (TFs) binding sites were searched by TFSEARCH software (<http://www.cbrc.jp/research/db/TFSEARCH.html>).

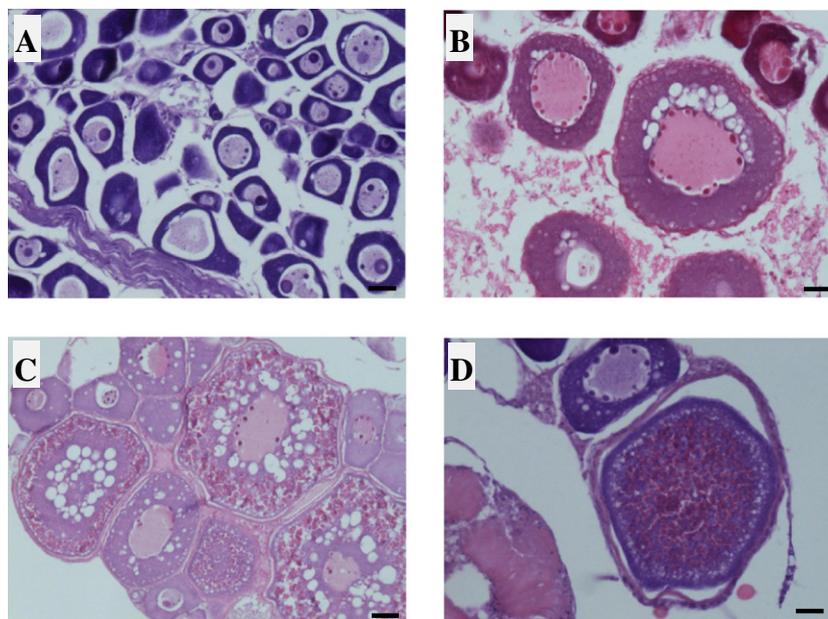
### 2.9. Statistics

The relevant values in this study were analyzed using SPSS for one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests and *T*-test. Statistical significance was considered as  $P < 0.05$ .

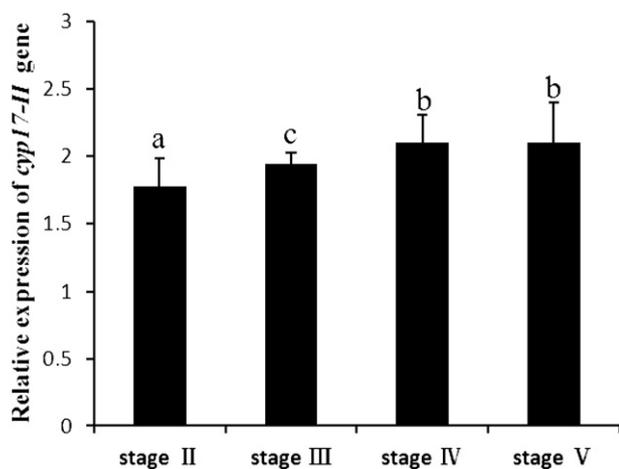
## 3. Results

### 3.1. Histological analysis of the gonad of Japanese flounder

Gonadal histological analysis was conducted in sixty Japanese flounder we obtained in this experiment. The result showed that forty-five of them were female. Their ovarian development stage was from II to V



**Fig. 1.** Histological photomicrographs of the ovary of Japanese flounder. A: The ovary in the stage II, bar = 75  $\mu$ m; B: The ovary in the stage III, bar = 75  $\mu$ m; C: The ovary in the stage IV, bar = 150  $\mu$ m; D: The ovary in the stage V, bar = 75  $\mu$ m.



**Fig. 2.** Ovarian mRNA expression of *cyp17-II* in of Japanese flounder at ovarian development stages II to V. Each histogram represents the mean of three determinations. Values are expressed as mean  $\pm$  standard error of mean. Different letters indicate significant difference ( $P < 0.05$ , one-way ANOVA, followed by Duncan's test).

(Fig. 1), and more than four fish were at each development stage. Three fish were randomly selected from each development stage were used for experiment.

### 3.2. Expression of *cyp17-II* in ovary during the ovarian development stage

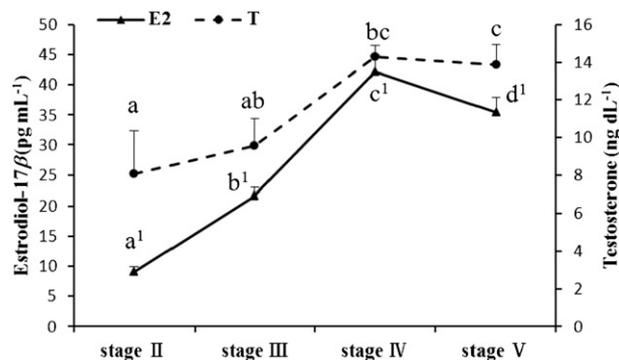
The variation of *cyp17-II* expression during the ovarian development stage of Japanese flounder is shown in Fig. 2. The relative mRNA expression of *cyp17-II* continuously increased from stages II to IV, with highest expression level observed in stage IV ( $P < 0.05$ ). There was a moderately decline in *cyp17-II* expression at stage V.

### 3.3. Serum T and $E_2$ levels during the ovarian development stage

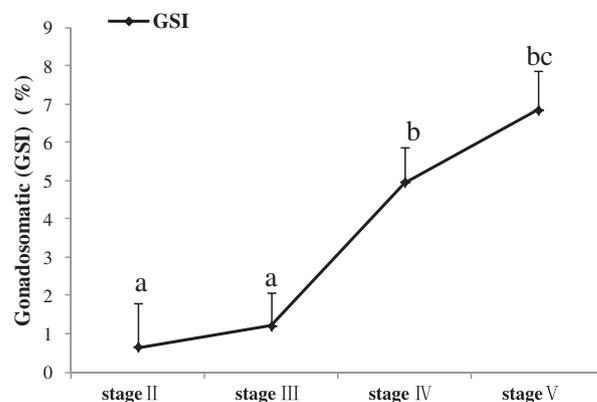
The changes of serum T and  $E_2$  levels corresponding with ovarian development stage are shown in Fig. 3. The serum T level continuously increased from stages II to IV, and T content at stage V slightly decreased. The  $E_2$  level, which averaged  $9.07 \pm 0.95$   $\text{pg mL}^{-1}$  in stage II, moderately increased to  $14.28 \pm 1.13$   $\text{pg mL}^{-1}$  in stage III, then subsequently reached to  $42.09 \pm 1.81$   $\text{pg mL}^{-1}$  in stage IV ( $P < 0.05$ ), finally rapidly decreased to  $5.35 \pm 1.26$   $\text{pg mL}^{-1}$  in stage V. The average level of GSI was increased from stages II to V, and peaked in stage V (Fig. 4).

### 3.4. Identification of ORF and conserved P450c17-specific region

The ORF (open reading frame) of *cyp17-II* gene was found at 34 nt to 1608 nt (relative to the cDNA sequence obtained from NCBI). The



**Fig. 3.** Serum testosterone (T) and 17 $\beta$ -estradiol ( $E_2$ ) levels at different stages of ovarian development. Statistically significant differences are indicated with different letters above the bars ( $P < 0.05$ , one-way ANOVA, followed by Duncan's multiple).



**Fig. 4.** Gonadosomatic index (GSI) of female Japanese flounder at different stage. Values are expressed as mean  $\pm$  standard error of mean. Different letters indicate significant difference ( $P < 0.05$ , one-way ANOVA, followed by Duncan's multiple).

conserved P450c17-specific region was at 994 nt to 1062 nt (Fig. 5). The four CpG sites at 1005 nt, 1016 nt, 1019 nt and 1025 nt (relative to the cDNA sequence obtained from NCBI) in exon 6 were in the conserved P450c17-specific region.

### 3.5. Forecast of TFs binding sites in exon 4 and 6

The TFs binding sites in exon 4 and exon 6 were forecasted using TFSEARCH software. Putative transcription factor: GATA-1, GATA-2, CDP CR, CP2, MZF1 and Cdx A were in exon 4 sequence (from 586 bp to 781 bp) (Fig. 6); GATA-1 and E2F were searched in exon 6, also in the conserved P450c17-specific region (Fig. 5).

### 3.6. Efficiency of bisulfite modification

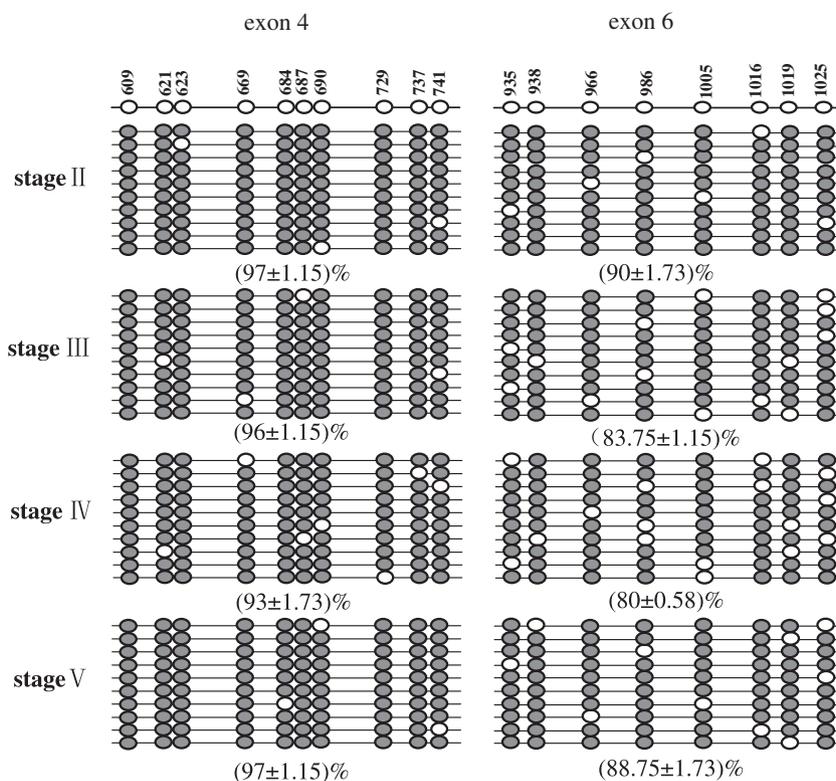
In order to evaluate the efficiency of the bisulfite modification step, the rate of conversion of 43 (exon 4) or 29 (exon 6) cytosines, which were not in the context of a CpG dinucleotide, was examined. The sequencing results showed that only thirteen non-converted C were found in all copies of twelve different samples of two CpG dinucleotide sequence (exon 4 and 6). The conversion efficiency was above 99.5%. It indicated that almost all cytosine had been chemically converted to uracil by the bisulfite modification. This proved that the bisulfite treatment procedure was very efficient and should not produce considerable artifacts due to incomplete conversion.

### 3.7. The correlation between methylation levels of CpG dinucleotides of two CpG rich regions and gene expression

Using bisulfite conversion and subsequent DNA sequencing, we were able to characterize the DNA methylation pattern of *cyp17-II* gene in ovary. An extremely high level of DNA methylation was observed in CpG dinucleotide sequence of all ovarian development stages. The average methylation level of exon 4 in stage II was approximately 90%, continuously decreased from stages II to IV, and with the lowest level observed in stage IV (80%), finally, sharply increased to 88.75% in stage V. Meanwhile, the average methylation level of exon 6 was lower than that of exon 4, but the variation trend was consistent with exon 4 during the ovarian development (Fig. 7).

The relationship between DNA methylation level of each CpG rich region and gene expression was given in Fig. 8. Methylation level of the four CpG dinucleotides (in exon 6) which were in conserved P450c17-specific region showed strong negative correlation with gene expression; they were high in stage II and declined in stage III or IV, finally increased in stage V (Fig. 9).





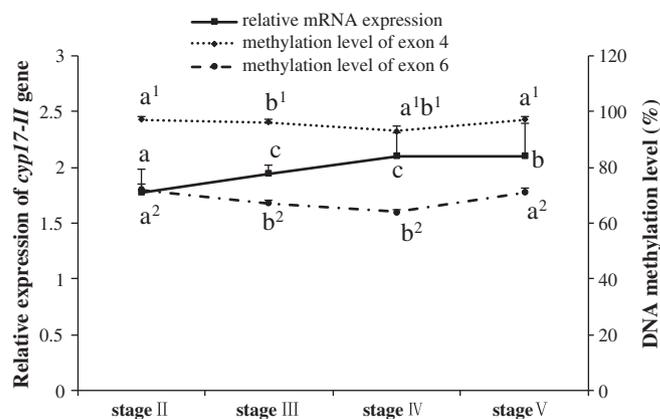
**Fig. 7.** DNA methylation patterns of exon 4 and 6 in *cyp17-II*. ○ represents an unmethylated CpG, and ● represents a methylated CpG. Each line represents the methylation status of all CG sites in a clone. The first line indicates the localizations of studied CpG sites related to the sequence of *cyp17-II* coding region. The percentage indicates the average methylation level (under the line) of all clones of each development stage, calculated as the number of methylated CpG sites per total number of CpG sites in each stage, data as mean ± SEM.

gross quantitative indicator of gonad condition (Zhou et al., 2007a,b), the GSI gradually increased from stages II to V. So, we speculated that the high relationship between variations of *cyp17-II* expression and serum levels of T and E<sub>2</sub> demonstrated that P450c17s might regulate the level of androgen and estrogen which can promote the oocyte growth and maturation.

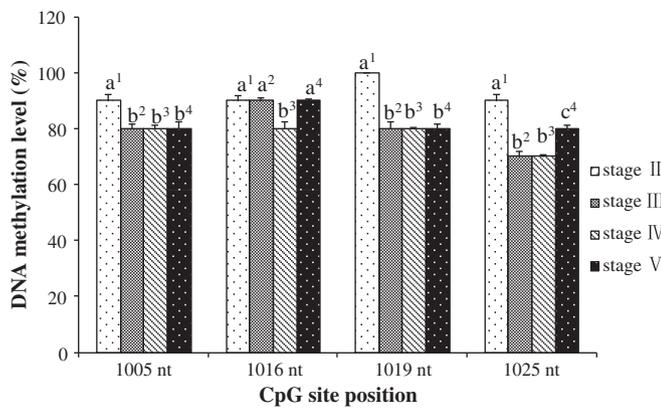
DNA methylation emerged as an important epigenetic modification by regulating gene expression. In general, DNA methylation represses transcription, and loss of methylation is associated with gene activation (Nan et al., 1998). In addition, recent evidence indicated that DNA methylation of the coding region was more powerful in suppressing gene expression than that of the promoter region (Hsieh, 1997; Irvine et al., 2002). Hence, in order to elucidate the mechanism behind the regulation of *cyp17-II* gene expression in different ovarian development stage of Japanese flounder, methylation analysis of the CpG dinucleotides of two CpG rich regions in *cyp17-II* coding region were carried out in our study. The average DNA methylation level of exon 4 was higher than that of exon 6 in the four ovarian development stages. Moreover, the two CpG methylation pattern were inversely correlated with *cyp17-II* mRNA levels and serum levels of T and E<sub>2</sub>. Coincidentally, the DNA methylation level of exon 6 at stage II was 90%, which was similar to that of female Japanese flounder with GG genotype (90.7%), in our previous study (Ding et al., 2012). So it suggested that methylation at these sites might account for the decreased expression of this gene.

Transcriptional activity suppression has been proposed to occur through decreased binding of TFs to mCpG (Kazeto et al., 2000; Mori et al., 2003). Initially, CpG methylation was thought to be a general repressive epigenetic mark in vertebrate genomes. But recent studies revealed a new role of CpG methylation, as an activation mark that creates transcription factor binding sites, suggesting a more complex role for CpG methylation (Chatterjee and Vinson, 2012). It is reported that GATA-4/6 were responsible for activating P450c17 gene expression at early stage of mouse embryonic development (Shi et al., 2009) and, GATA-6 is recruited to the promoter during activating transcription of

human CYP17 (Sewer and Jagarlapudi, 2009). In addition, six GATA family transcription factor members (GATA-1 to GATA-6) were exhibiting similar DNA-binding properties (Ko and Engel, 1993; Merika and Orkin, 1993). But, no report was available about E2F, CDP CR, CP2 and MZF Cdx A transcription factors action on P450c17 gene so far. The gene expression was initiated by transcription factor binding to genetic regulatory elements. However, chromatin structure surrounding the regulatory elements must also be in a configuration permissive to transcription factor binding. Here, we proposed that the DNA methylation might alter the chromatin structure, where allow these transcription factors to binding to. Thus, the differential of *cyp17-II* gene expression during ovarian development stage maybe affect by DNA methylation status. However the conclusion we inferred requires a further investigation.



**Fig. 8.** The correlation between gene expression and CpG methylation level of *cyp17-II* gene at different ovarian development stage. Values as mean ± SEM. Different letters indicate significant difference ( $P < 0.05$ , one-way ANOVA, followed by Duncan's test).



**Fig. 9.** The DNA methylation level of the four CpGs (at position of 1005 nt, 1016 nt, 1019 nt and 1025 nt in exon 6) of *cyp17-II* at different ovarian development stage. The value was calculated as the number of methylated CpG sites per total number of CpG sites of the sequenced copies in each stage. Values as mean  $\pm$  SEM. Different letters indicate significant difference ( $P < 0.05$ , one-way ANOVA, followed by Duncan's test).

## 5. Conclusions

This study showed that the T and E<sub>2</sub> level were consistent with the trend of *cyp17-II* expression at different ovarian development. While the whole methylation levels of the two CpG rich regions was inversely correlated with the gene expression. These findings indicated that *cyp17-II* may regulate the levels of estrogen and androgen in female fish. Furthermore, the ovarian *cyp17-II* gene expression may be regulated by the methylation level of CpG site in the coding region by interfering in the TFs binding to the regulatory elements. Our work may help us to elucidate the molecular mechanism of endocrine physiology in fish from the epigenetic point of view.

## Conflict of interest

The authors have no conflict of interest to declare.

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