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Molecular cloning and gene expression of *Cg-Foxl2* during the development and the adult gametogenetic cycle in the oyster *Crassostrea gigas*

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ABSTRACT

A *Foxl2* ortholog has been identified in a lophotrochozoa, the pacific oyster, which is a successive irregular hermaphrodite mollusc. Its cDNA has been called *Cg-Foxl2* (*Crassostrea gigas Foxl2*) and the deduced protein sequence is 367aa long. This sequence contains the conserved domain *Forkhead* box and its gene is devoid of intron at least in the first 926 bp of the cDNA, as found for Foxl2 factors. Real time PCR and *in situ* hybridization have shown a gonadic male and female *Cg-Foxl2* expression which increases during the adult gametogenetic cycle for both sexes, but with a significant increase occurring earlier in females than in males. In females this increase corresponds to the vitellogenetic stage. During development, a peak of *Cg-DMl* (a potential factor of the male gonadic differentiation) and *Oyvlg* (a germ cell marker) expression and a significant decrease of *Cg-Foxl2* expression were observed after metamorphosis in 1–1.5-month-old spats, a period of development when primordial germ cells may differentiate into germinal stem cells during the first gonadic establishment.

1. Introduction

Sex determination is the critical period preceding sexual differentiation, where a primary signal initiates the onset of a cascade of transcriptional or mRNA splicing factors, allowing the final differentiation of the gonads into testis or ovary. Three main systems of sex determination exist among phyla: a Genetic Sex Determination (GSD), an Environmental Sex Determination (ESD) and a mix of GSD and ESD (Valenzuela et al., 2003). In mammals where sexual differentiation has been most studied, the activation of such a cascade involves genes such as *Wt1*, *Sf1*, *Sry*, *Sox9*, *Gata4*, *Dmrt 1* and *MIH* in the male (Ferguson-Smith, 2007) and *Wnt4*, *RSpo1*, *Foxl2* and *Dax1* in the female (Wilhelm, 2007).

Foxl2 (*Forkhead box l2*) is one of the most conserved genes involved in the early events of the cascade leading to ovary differentiation, although its role is not well defined especially in invertebrates (Cocquet et al., 2002; Ottolenghi et al., 2005). The encoded protein belongs to the Forkhead/winged helix family of transcription factors (Kaestner et al., 2000). The members of this family are characterized by a conserved DNA-binding motif called *Forkhead* box domain and would be involved in the regulation of cell differentiation and proliferation (Lehmann et al., 2003). In many vertebrates examined to date which show different sex determination systems, such as mouse (Crisponi et al., 2001; Loffler et al., 2003), fish Nile tilapia (Wang et al., 2004), chicken (Govoroun et al., 2004), and red-eared slider turtle (Loffler et al., 2003), Foxl2 is expressed in embryonic somatic cells of ovaries and its expression is maintained in adult ovaries for most species (Uhlenhaut and Treier, 2006; Wotton et al., 2007). Knock-out mice for *Foxl2* (Ottolenghi et al., 2005) or double knock-out mice for *Foxl2* and *Wnt4* (Ottolenghi et al., 2007) showed respectively a failure of follicular formation or complete XX sex reversal with testis-like structures. All these results suggest an involvement of Foxl2, not only in female gonadal differentiation, but also in maintaining its differentiation throughout the adult life. This role of Foxl2 seems also conserved in hermaphrodite species such as the protogynous grouper, where the gene expression is significantly down-regulated during female-to-male sex change (Alam et al., 2008). In invertebrates, orthologs of *Foxl2* have also been characterized such as *NvFoxL2* in the cnidarian *Nematostella vectensis* (Magie et al., 2005), *SpFoxl2* in the echinid urchin *Strongylocentrotus purpuratus* which is highly expressed during the first 72 h of development (Tu et al., 2006) and *Sd-FoxL2* which shows an ubiquitous expression in the sponge *Suberites domuncula* (Adell and Muller, 2004).

The Pacific oyster *Crassostrea gigas* is a marine bivalve mollusc whose development occurs in three main stages (embryo, larvae,

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spats) (Galtsoff, 1964). Gonad establishment during organogenesis remains unknown. Only one specific marker of the primordial germ cells (PGCs), orthologous to *vasa* and called *Oyvlg*, was characterized in *C. gigas* (Fabioux et al., 2004a). Analysis of its expression by *in toto* hybridization suggests a migration of the PGCs to the anterior and the posterior sides of the body, around the digestive gland of later stage larvae and first post-larvae stage (Fabioux et al., 2004b). In juvenile oysters, PGCs would differentiate into two clusters of germinal stem cells (GSCs) which would proliferate intensively to form the gonadic tubules when the animals reach sexual maturity. The oyster is a successive and irregular protandric hermaphrodite with a diffuse and non-permanent gonad which evolves according to an annual reproductive cycle subdivided into four main stages (Heude-Berthelin et al., 2001). No sexual chromosomes have been characterized but a single-locus sex determinism has been suggested (Guo et al., 1998). A potential factor involved in the gonadic development or differentiation has also been characterized for the first time in lophotrochozoa and called *Cg-DMl* (*C. gigas DMRT-like*). Its expression studied by Real time PCR and *in situ* hybridization is observed (i) during the development where it significantly increases in 1.5-month-old spats; (ii) in adults of both sexes where it significantly increases only in male gonads at the end of the gametogenetic cycle (Naimi et al., 2009). However, the time window, factors and mechanisms involved in the adult sex change and in sex determination during the first gonadic development still remain unclear in *C. gigas*.

The objective of this work was therefore to identify a gene orthologous to *Foxl2* in order to have a marker of the ovarian differentiation in *C. gigas*. In this paper we report isolating and molecular characterization of *Cg-Foxl2* (*C. gigas Foxl2*) in the oyster. We also examined the gene expression and discussed the potential role of this factor in the sex determination of the oyster, in particular in association with the first establishment of the gonad during the development.

2. Materials and methods

2.1. Experimental animals

Animals (*C. gigas*, Bivalvia, Ostreidae) were purchased from a local oyster nursery (SATMAR, Gatteville, France). The developmental stages were obtained by mating adults from two families, one with a sexual imbalance rate in favour of males (14 and 32% female ratios in 2004 and 2005, respectively) and the other in favour of females (57% female ratio in 2006). The rearing conditions were as follows: for all stages, no particular photoperiod; larvae were kept in filtered and UV-treated water at 27 °C and fed with diatoms; spats were kept in 18 °C filtered and UV-treated water, with diatoms as food or under natural feeding and temperature conditions. The adult oysters we used in 2005 belonged to two families presenting sexual imbalance rates, with 2004 and 2005 female ratios of 80 and 63% for one and 14 and 32% for the other, respectively. Sampling was carried out by dissecting the gonadal area from the visceral mass in a region between the heart and labial palps. Histological observations of the sampled gonadal area allowed to determine the sex of the animals and to class them in four reproductive stages according to Heude-Berthelin et al. (2001) and Chavez-Villalba et al. (2003): stage 0 where gonads are restricted to dispersing small clusters of germ cells during the inactive stage; stage I corresponding to mitosis of spermatogonia or oogonia which, like the sex of the animal, are indiscernible at this stage; stage II, where all the stages of spermatogenesis and growing oocytes are visible in males and female gonadal tubules, respectively; stage III, corresponding to the mature reproductive stage.

2.2. Reverse transcription, cloning and sequencing

Reverse transcription was carried out using oligo$(dT)_{15}$ (Promega, Charbonnières, France), 1 µg mRNA and 200 units of M-MLV RT (Promega). cDNAs were used as template for PCR amplification with two degenerate primers designed to anneal to the nucleotidic sequence encoding the conserved *Forkhead* domain of *Foxl2*. The sense primer Foxl2SN5 corresponding to the KNKKGWQN amino acid sequence was 5′-AAGAAYARRAARGGHTGGCARAA-3′, whereas the antisense primer Foxl2AS3 corresponding to the DMFEKGN amino acid sequence was 5'-AGTTBCCHTTYTCRAACATDTC-3′ (where Y is C or T, R is A or G, H is A, C, or T, B is C, G, or T, and D is A, G, or T). PCR was performed on a total volume of 50 µL with 40 ng of male and female gonadic area cDNA in 10 mM Tris/HCl, pH 9.0, containing 50 mM KCl, 0.1% Triton X-100, 0.2 mM of each dNTP, 1 µM of each primer, 1.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Promega). The reaction was cycled for 2 min at 96 °C, 5 cycles (10 s at 96 °C and 3 min at 65 °C), 5 cycles (10 s at 96 °C and 3 min at 60 °C), 35 cycles (30 s at 96 °C, 30 s at 50 °C and 1 min at 72 °C) followed by an extension step at 72 °C for 10 min.

A resulting 106 bp fragment was isolated and used to generate full-length cDNA by 5′ and 3′ RACE using the GeneRacer™ Kit (Invitrogen, Eragny sur Oise, France) using adaptor and gene specific primers Fox1S (5′-CAGTATTCGCCACAATTTGAGCTTAAA-3′), Fox1SN (5′-AAAGTTCCTCGTGAAGGTGGAGA-3′), Fox1AS (5′-CTCGAATGCCGGGTC-CAGGGTCCAGAA-3′) and Fox1ASN (5′-TCTCCACCTTCACGAGGAACTTT-3′. A sense primer Cg-FoxL2S5 (5'-CACGGACATTTTCGGTTCTCA-3′) and an antisense primer Cg-FoxL2AS3 (5′-TGTCTGTCAGTTCTTGGCGTCT-3′) were designed in the 5' and 3' cDNA ends to amplify full-length cDNA and genomic sequences of *Cg-Foxl2* by PCR in a total volume of 50 µL for 30 cycles (95 °C, 45 s, 56 °C, 45 s and 72 °C, 120 s), followed by an extension step at 72 °C for 10 min. Resulting fragments were purified and cloned into PCRII-Topo vector using a TA cloning kit (Promega) and sequenced using ABI cycle sequencing chemistry (GENOME express, Meylan, France).

2.3. Northern blot analysis

Total RNA was extracted from male and female gonads of adult oyster at stage II using Tri-Reagent (Sigma, Saint Quentin Fallavier, France) following the manufacturer's instructions. Poly(A^+) RNAs were purified using Dynabeads oligo-dT25 (Dynabeads® mRNA DIRECT™ KIT; Dynal A.S, Oslo, Norway). Around 3 µg of mRNA were run by electrophoresis on a 1% formaldehyde agarose gel and transferred onto a nylon membrane (Hybond-N^+, GE Healthcare, Orsay, France). Antisense digoxigenin-11-dUTP labelled *Cg-Foxl2* probe (base 9-1193 of *Cg-Foxl2* cDNA) was transcribed *in vitro* from *Cg-Foxl2* cDNA inserted in PCRII-Topo vector (Promega) using a DIG RNA labelling kit (Roche Diagnostics, Meylan, France). Hybridizations were performed during 16 h in 1% blocking reagent (Roche Diagnostics), 50% formamide, 750 mM NaCl, 5 mM EDTA, 0.1% Tween 20, 75 mM sodium citrate pH 7, 100 µg/mL Torula yeast RNA at 55 °C. Then, washes and signal analysis were performed following the manufacturer's instructions (Roche Diagnostics). After extensive washing with 2× SSC/0.1%SDS at room temperature (RT) and 0.5× SSC/0.1%SDS at 65 °C and blocking in 1.5% blocking reagent for 30 min, detection was performed by 30 min incubation with an alkaline phosphatase-conjugated anti-DIG antibody diluted 1:5000 in blocking solution, followed by overnight incubation with 2% NBT/BCIP solution in darkness at RT. All reagents were made following the manufacturer's instructions (Roche Diagnostics).

2.4. Phylogenetic analysis

A range of invertebrate and vertebrate protein sequences encoding Foxl2 homologues were aligned using the CLUSTALW software (Thompson et al., 1994). Phylogenetic analyses were performed by the neighbour-joining method (Saitou and Nei, 1987) using the *MEGA* software version 4 (Tamura et al., 2007).

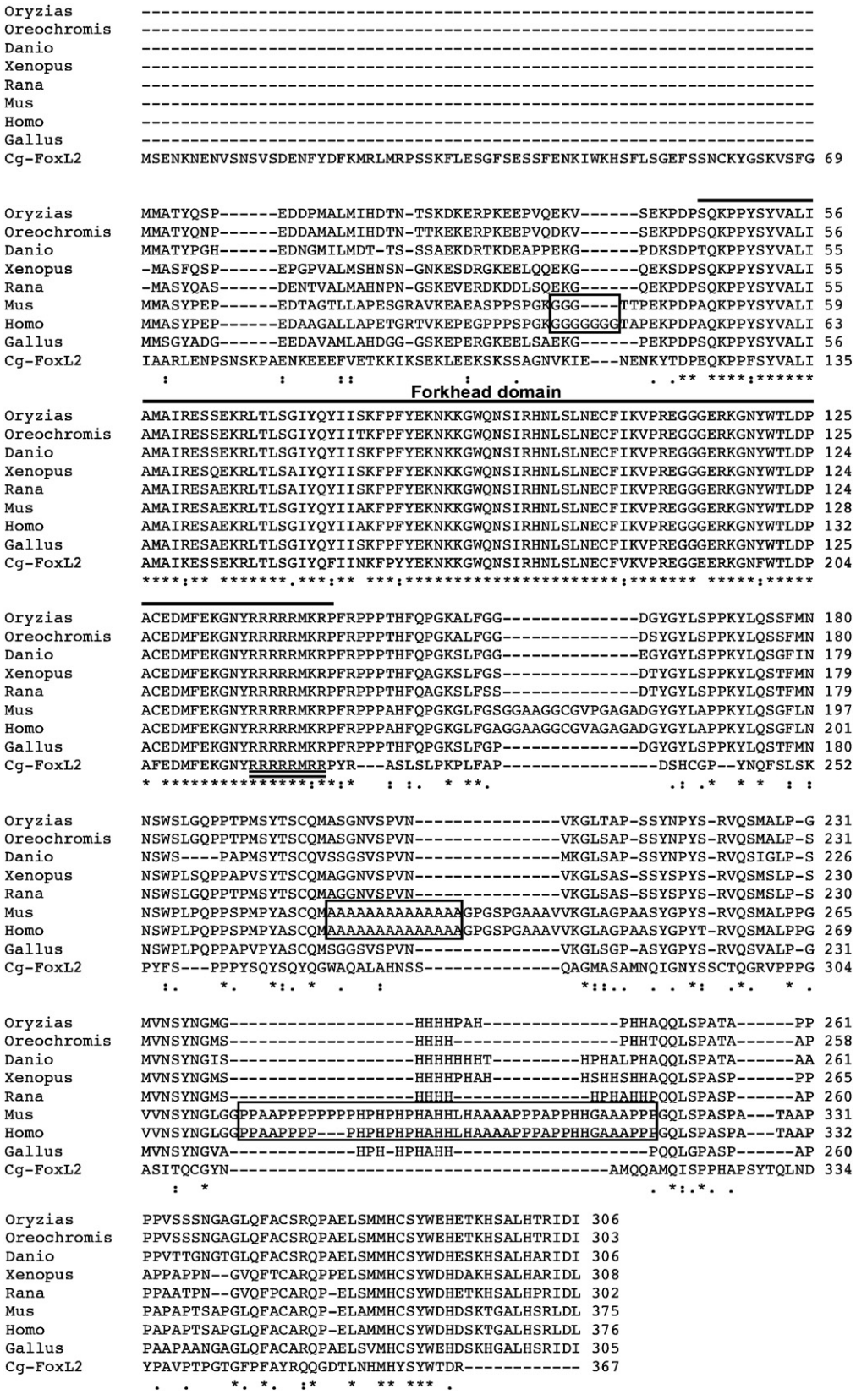

Fig. 1. Alignment of amino acid sequences of oyster Cg-Foxl2 with Foxl2 proteins of some vertebrates. Identical amino acids are indicated by asterisks. Amino acids with conserved similarities are indicated by dots or colons. The solid bar indicates the *Forkhead* domain. The putative NLS sequence is double underlined. The polyalanine tract (A), glycine rich repeats (G), and proline repeats of mammals are boxed. Alignments were generated using CLUSTALW. The numbers of the amino acid residues are highlighted. (GenBank accession no. medaka (*Oryzias latipes*: AB252055), tilapia (*Oreochromis niloticus*: AY554172), zebrafish (*Danio rerio*: BC116585), xenopus (*Xenopus laevis*: AB372218), frog (*Rana rugosa*: AB372103), mouse (*Mus musculus*: AF522275), human (*Homo sapiens*: AF301906), chicken (*Gallus gallus*: AY4871 65), oyster (*Crassostrea gigas*: FJ68956).

2.5. Real time quantitative PCR

Quantitative RT-PCR analysis was performed using the iCycler apparatus (BioRad, Marnes-La-Coquette, France). Total RNA was isolated from adult tissues and from developmental stages using Tri-Reagent (Sigma) following the manufacturer's instructions. After DNase I ROI (Promega) treatment of 15 min at RT, 1 µg of total RNA was reverse transcribed using 200 units of M-MLV reverse transcriptase (Promega) during 90 min at 37 °C. The iQ™ SYBR Green supermix PCR kit (BioRad) was used for real time monitoring of 5 ng template cDNA amplification for 40 cycles (15 s/95 °C, 15 s/60 °C). Accurate amplification of the target amplicon was assessed by constructing a melting curve. Amplification of cDNA was performed using primers specific to *Cg-Foxl2* (qFoxl2S2 5′-AATATCAGGGATGGGCACAA-3′, qFoxl2AS1 5′-GTCCTTGGGTGCAGGAACTA-3′), to *Cg-DMl* (DMS 5′-CCATGGAGTGGTGTCCGC-3′, DMASB 5′-GTTGCCTTTCTGCTATCAGGG-3′) (Naimi et al., 2009) and to *Oyvlg* (VS-S9 5′-TCCCCGAGGAGATT-CAGA-3′, VS-AS9 5′-ACGTCAGTGCAAGCACCA) (Fabioux et al., 2004a). A parallel amplification of the oyster elongation factor 1α (*EF1α*, GenBank accession no. BQ426516) reference transcript was performed using qfElongI (5′-ACCACCCTGGTGAGATCAAG-3′) and qrElongI (5′-ACGACGATCGCATTTCTCTT-3′) primers. The relative mRNA levels were normalized to *EF1α* transcripts using the following formula $N = 10 \times 2^{(\mathrm{Ct}EF1\alpha - \mathrm{Ct}Cg\text{-}gene)}$ (Lelong et al., 2007). Statistical analyses were performed using two tailed Mann–Whitney *U*-test.

2.6. Histology

Unshelled spats of 3, 3.5 and 4 months of age were fixed in Davidson's fixative for 48 h. Sections were stained with a Prenant–Gabe trichrome according to a classical protocol (Gabe, 1968). The slides were therefore examined and pictures were taken with a Nikon Eclipse 80i microscope coupled to a Nikon DXM1200-C camera (Nikon, Champigny-Sur-Marne, France).

2.7. In situ hybridization

One-cm slices of adult oysters were fixed in Davidson's fixative. Some sections were stained with a Prenant–Gabe trichrome as mentioned above to illustrate the structure of the gonads. For *in situ* hybridization experiments, 5 µm-slices were treated with 5% proteinase K in TE buffer for 30 min at 37 °C. After pre-hybridization for 2.5 h at RT and 30 min at 52 °C, hybridization was performed overnight at 54 °C with DIG-labelled sense (control) or antisense riboprobe (400 ng/mL) in hybridization buffer. Probes were synthesized using a DIG RNA labelling kit (Roche Diagnostics) and with the *Cg-Foxl2* cDNA as a template. After extensive washing (2× SSC at RT and 0.5× SSC at 65 °C) and blocking in 1.5% blocking reagent for 1 h, detection was performed by 2 h incubation with an alkaline phosphatase-conjugated anti-DIG antibody diluted 1:500 in blocking solution, followed by 3 h incubation with 2% NBT/BCIP solution in darkness at RT. All reagents were made following the manufacturer's instructions (Roche Diagnostics). Sections were examined and pictures were taken with a Nikon Eclipse 80i microscope coupled to a Nikon DXM1200-C camera (Nikon, Champigny-Sur-Marne, France).

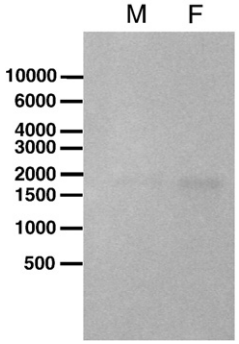

Fig. 2. Northern blot analysis of *Cg-Foxl2* mRNA in male (M) and female (F) gonads of adults of *C. gigas* showing the presence of only one *Cg-Foxl2* mRNA transcript between 1500 and 2000 b in both sexes. Three point 8 µg of male gonads at stage II and 2. 9 µg of female gonads at stage II were spotted. *Oyvlg* was taken as a control of gonads mRNA quality (results not shown).

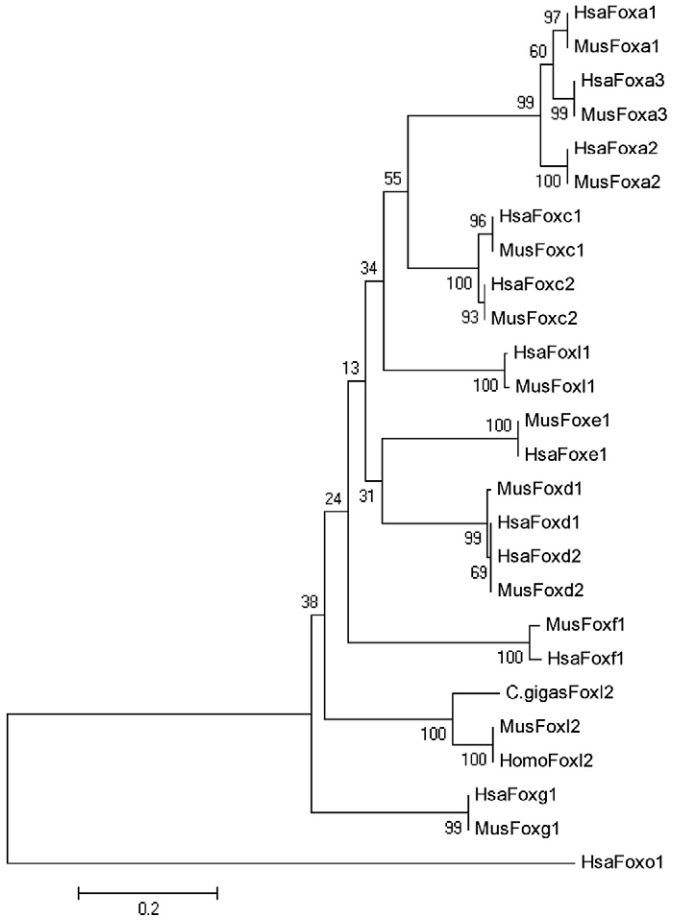

Fig. 3. Phylogenetic tree of the Fox proteins showing that Cg-Foxl2 is clustered together with Foxl2. Phylogenetic analysis was performed by neighbour-joining method (Saitou and Nei, 1987) using *MEGA4* software (Tamura et al., 2007). Numbers in the branches represent the bootstrap values (%) from 100 replicates (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in units of number of amino acid substitutions per site. (GenBank accession no. BC033890, BC096524, EU275778, BC037083, EU402966, ***NM_010446***, BC052011, ***NM_001453***, BC113437, ***NM_013519***, AF315075, ***NM_008024***, ***NM_183298***, ***NM_004473***, ***NM_008242***, ***NM_004472***, ***NM_004474***, ***NM_008593***, ***NM_010426***, ***NM_001451***, FJ68956, AF522275, AF301906, ***NM_005249***, ***NM_008241***, ***NM_002015***).

3. Results

3.1. Cg-Foxl2 cloning and sequence analysis

A complete *Cg-Foxl2*-like sequence of 1357 bp (results not shown) (GenBank accession no FJ68956) shows a 5′ untranslated region (UTR) of 31 bp, an open reading frame (ORF) of 1104 bp with the ATG codon at position 32 bp and a TAA stop codon at position 1133 bp, and a 3′ untranslated region of 222 bp including a single poly(A) signal (AATAAA) at position 1314 bp, 21 bp upstream of the poly(A) tail. The

full length, continuity and sequence of this cDNA were confirmed on several male and female cDNA samples amplified by PCR with Cg-FoxL2S5 and Cg-FoxL2AS3 primers and sequenced. The deduced amino acid sequence (Fig. 1) is 367aa long, contains the *Forkhead* domain consensus sequence (from aa 124 to 224), also known as "winged helix" domain, characteristic of the FOX protein family. However, the Cg-Foxl2 protein is devoid of polyalanine tract (A), glycine-rich (G) and proline (P) repeats. PCR amplification of the genomic DNA performed in the same conditions and with the same primer as those used to amplify full-length cDNA revealed the presence of only one band corresponding to the first 926 bp of the Cg-Foxl2 cDNA sequence (results not shown). Northern blot analysis (Fig. 2) revealed the presence of only one *Cg-Foxl2* mRNA transcript between 1500 and 2000 b in both sexes, although its expression was very faint in male gonads at stage II. The quality of the mRNA was evidenced by the appearance of a band with *Oyvlg* probes (results not shown).

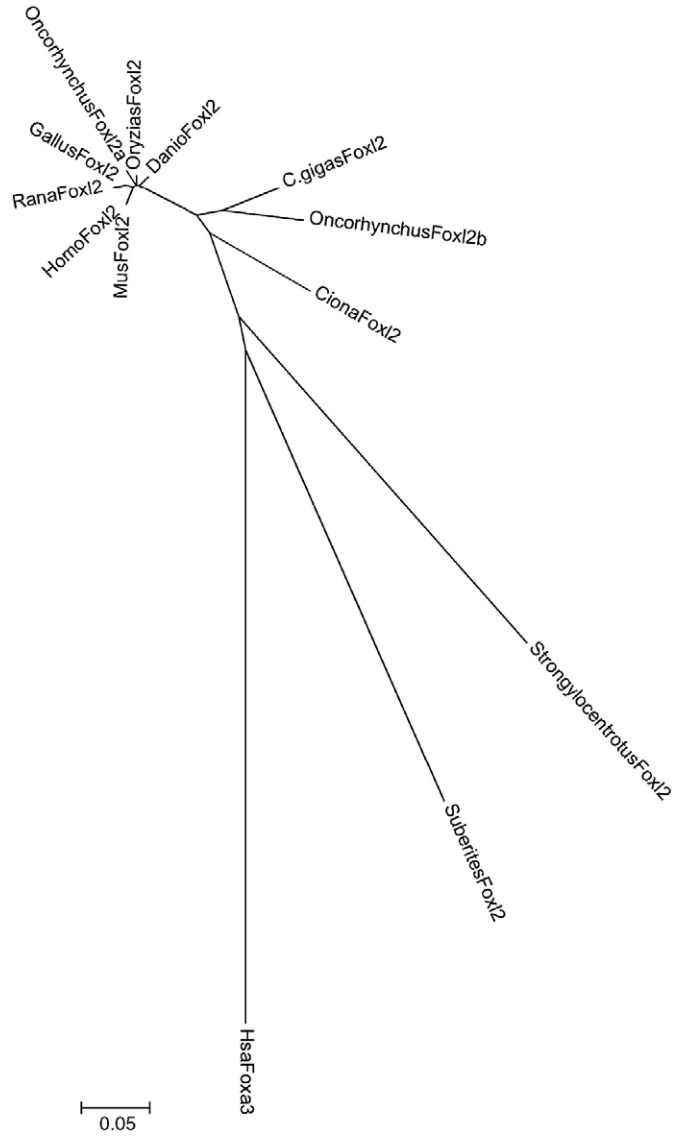

Fig. 4. Phylogenetic tree of the Foxl2 proteins showing that Cg-Foxl2 forms an out-group with the trout Foxl2b and ciona Foxl2. Human Foxa3 was used as an out group. Phylogenetic analysis was performed by neighbour-joining method (Saitou and Nei, 1987) using *MEGA4* software (Tamura et al., 2007). The bootstrap consensus tree inferred from 100 replicates (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in units of number of amino acid substitutions per site. (GenBank accession no FJ68956).

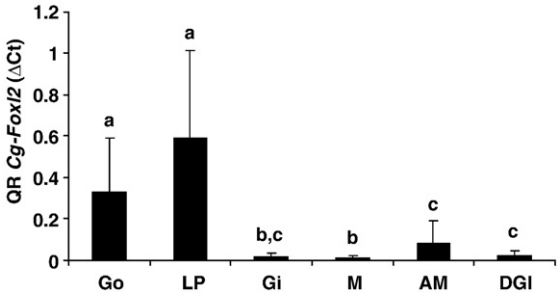

Fig. 5. Real-time RT-PCR *Cg-Foxl2* transcripts quantification relative to *EF1α* in different tissues of *C. gigas*. Total RNA were isolated from male and female gonads at various stages (Go), labial palps (LP), gills (Gi), mantle (M), adductor muscle (AM) and digestive glands (DGI). QR: Relative quantity, Arbitrary units. Values are mean ± SEM of triplicates. Different letters (a–c) indicate significant differences ($P<0.05$) between tissues. This graph shows mRNA levels significantly higher in gonads and labial palps compared to other tissues. Number of animals ($n=10$).

3.2. Phylogenetic analysis

Phylogenetic analysis was performed to investigate the relationship between Cg-Foxl2 and members of the Fox family. A complete protein sequence alignment of Cg-Foxl2 and members of the Fox family from both protostomes and deuterostomes (Fig. 1) indicated that Cg-Foxl2 shared the highest amino acid identity rates with the Foxl2 of the human (33%), mouse (31%), zebrafish, tilapia and chicken (40% each), medaka (39%), frog (42%) and xenopus (38%). The alignment also revealed that Foxl2 protein sequence is conserved amongst species, especially in the *Forkhead* domain where putative NLS sequences with basic amino acids have been identified at the C-terminal end (RRRRRMRR). In contrast, the polyalanine tract and glycine and proline repeats are only found in mammals. The corresponding phylogenetic tree confirmed that Cg-Foxl2 was clustered with Foxl2 subfamily. Cg-Foxl2 showed the highest sequence identities with Foxl2 of zebrafish, tilapia and medaka (89% each), human, mouse and chicken (88% each), and frog and xenopus (both 87%) (Results not shown). The phylogenetic tree generated using the *Forkhead* domain of oyster, human and mouse *Fox* (Fig. 3) provided evidence that Cg-Foxl2 is grouped with other Foxl2 with high bootstrap support (100). Within the Foxl2 subfamily in both protostomes and deuterostomes (Fig. 4), *Cg-Foxl2* presented an out-group (97 bootstrap support) with Foxl2b of the rainbow trout and Foxl2 of ciona.

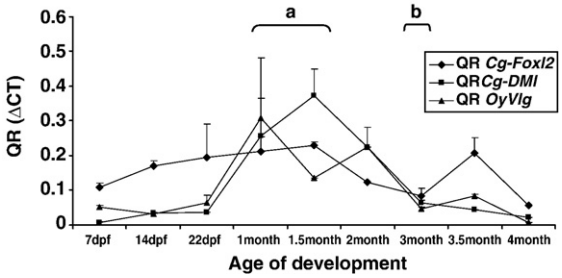

Fig. 6. Real time RT-PCR transcripts quantification relative to EF1α in developmental stages of *C. gigas* with *Cg-Foxl2*, *Cg-DMl* and *Oyvlg* primers. Total RNAs were isolated from veliger larvae 7 and 14 days post-fertilization (7 and 14 dpf), post-metamorphosis larvae (22 dpf) and 1 to 4-month-old spat (1, 1.5, 2, 3, 3.5 and 4 months). QR: Relative Quantity, arbitrary units. Values are mean ± SEM of triplicates of pools of different biological samples (each pool containing 100 mg of tissue). This graph shows significantly higher *Cg-DMl* and *Oyvlg* mRNA levels [Different letters (a and b) indicate significant differences ($P<0.05$)] in spats between 1–1.5 and 3 months of age while *Cg-Foxl2* transcript levels significantly decreased from 1–1.5 to 3 months old.

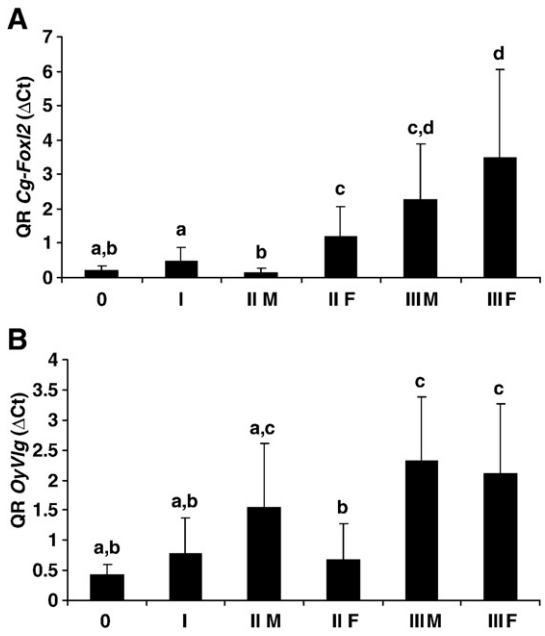

Fig. 7. (A) Real time RT-PCR *Cg-Foxl2* transcripts quantification relative to *EF1α* in the gonads of adults of *C. gigas* during the gametogenetic cycle. This graph shows mRNA levels increasing in both sexes during the gametogenetic cycle, with a significant drop from stage II in females and stage III in males. (B) Real time RT-PCR *Oyvlg* transcripts quantification relative to *EF1α* in the gonads of adults of *C. gigas* during the gametogenetic cycle. Stage 0: undifferentiated gonad ($n=5$); Stage I: gonia mitosis and sex indiscernible ($n=19$); Stage II: active spermatogenesis (IIM, $n=18$) and growing oocytes (IIF, $n=36$); Stage III: male (IIIM, $n=7$) and female (IIIF, $n=6$) mature stage. QR: Relative Quantity, arbitrary units. Different letters (a–d) indicate significant differences ($P<0.05$) between tissues. This graph shows *Oyvlg* mRNA expression increasing during the adult gametogenetic cycle for both sexes but with significant differences between sexes at stage II.

3.3. Cg-Foxl2 expression in adult tissues

Cg-Foxl2 mRNA, measured by real-time quantitative RT-PCR, were detected in all tissues tested (Fig. 5) with significantly higher levels in gonads (mix at various gametogenetic stages; mean ± SEM: 0.33 ± 0.26) and labial palps (mean ± SEM: 0.58 ± 0.42) than in gills (mean ± SEM: 0.01 ± 0.01), mantle (mean ± SEM: 0.01 ± 0.01) adductor muscle (mean ± SEM: 0.08 ± 0.10) and digestive glands (mean ± SEM: 0.02 ± 0.02).

3.4. Cg-Foxl2 expression during the development and in the adult gonads

During the oyster development, Real time RT-PCR experiments (Fig. 6) showed that, after a period of slight non-significant increase of expression between 7-days post-fertilization (dpf) larvae and 1.5-month-old spat, *Cg-Foxl2* mRNA levels were then significantly decreased except in the 3.5-month-old spats which showed a non-significant increase. *Cg-Foxl2* mRNA levels were also analysed in parallel with those of *Cg-Dmls* and *Oyvlg* (Fig. 6). Although *Oyvlg* expression was irregular, both factors showed a significant peak of expression around 1–1. 5 month compared to 3-month-old spats.

The analysis of *Cg-Foxl2* mRNA expression in the gonads of adults at different stages of the gametogenetic cycle (Fig. 7A) revealed a general increase for both sexes. More specifically, mRNA levels increased significantly in stage II females (mean ± SEM: 1.17 ± 0.90) as compared to stage II males (mean ± SEM: 0.14 ± 0.12) or to earlier stages (mean ± SEM: 0.21 ± 0.14; stage 0 and mean ± SEM: 0.48 ± 0.40; stage I). At stage III, this increase continues in females (mean ± SEM: 3.50 ± 2.56) which show a level not significantly different from males of the same stage (mean ± SEM: 2.25 ± 1.62). *Oyvlg* expression measured in parallel (Fig. 7B) showed, as *Cg-Foxl2*, an increase during the adult gametogenetic cycle for both sexes. This increase is

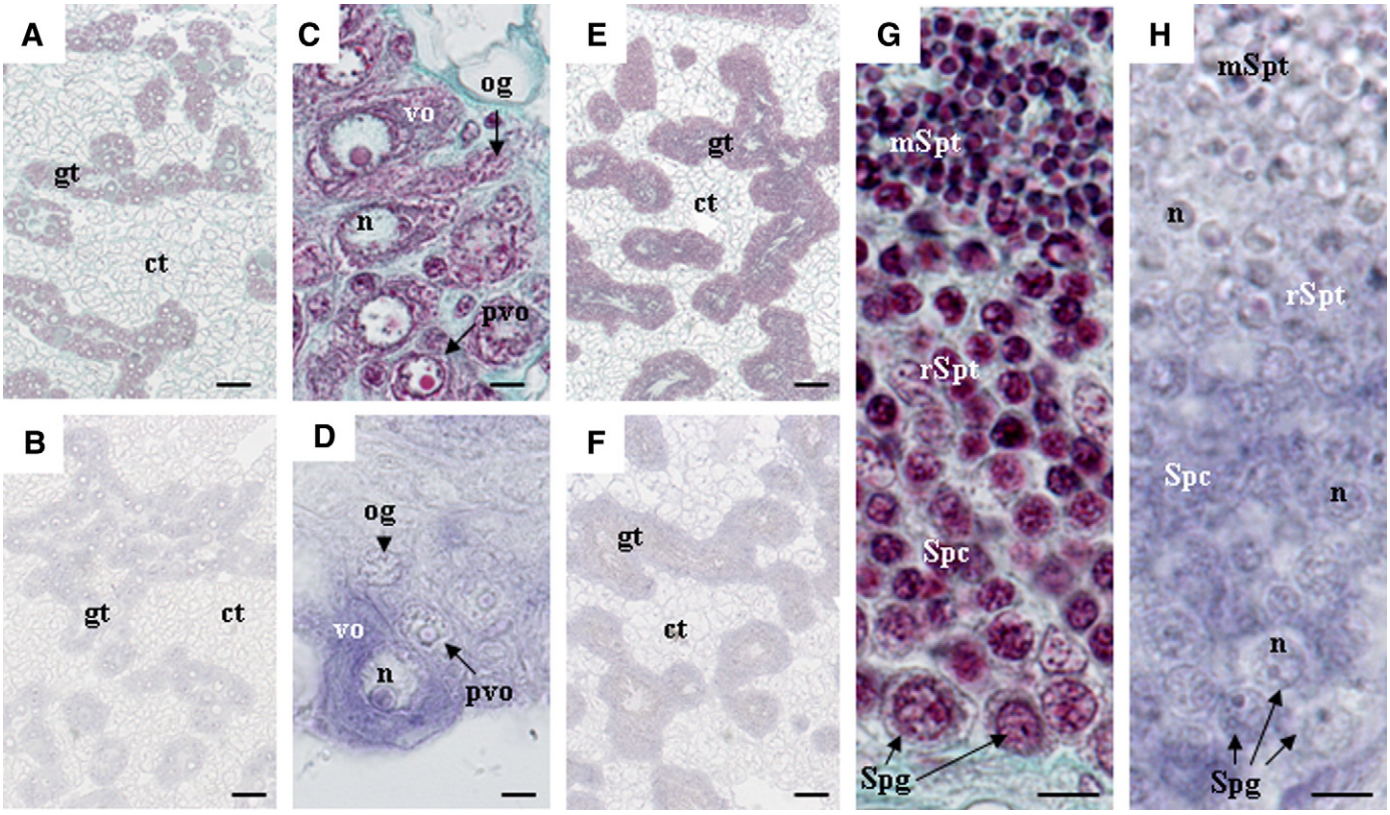

Fig. 8. Histological sections of gonads of stage II female (A, C) and male (E, G) stained with a Prenant–Gabe trichrome — *Cg-Foxl2* mRNA expression pattern by *in situ* hybridization in the gonadic tubules with antisense riboprobe (B, D, F and H) and sense riboprobe (results not shown). Female (A–D) and male (E–H) gonads with a cytoplasmic mRNA staining, faint in the oogonia and pre-vitellogenic oocytes and more intense in vitellogenic oocytes (C, D) and in the germinal cells from spermatogonia to mature spermatids and/or the surrounding somatic cells (G, H). mSpt: mature spermatids, n: nucleus, og: oogonia, pvo: pre-vitellogenic oocyte, rSpt: round spermatids, Spc: spermatocytes, Spg: spermatogonia, vo: vitellogenic oocyte. Bars: 5 µm.

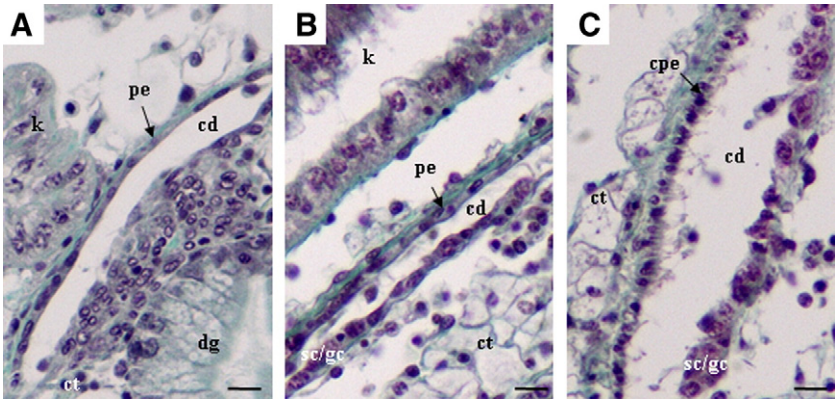

Fig. 9. Histological sections of the gonadic area of spats of 3 months (A), 3. 5 months (B) and 4 months old (C) stained with a Prenant–Gabe trichrome. Collecting ducts can be observed in spats from 3 months of age at the periphery of the gonadic area. They are limited on one side by a thin pavement epithelium at 3 and 3. 5 months of age (A and B) and by a ciliated prismatic epithelium at 4 months of age (C). On the other side, where the gametogenesis takes place, some undistinguishable somatic and germinal cells appear progressively between the age of 3.5 and 4 months (B and C). cd: collecting duct; cpe: ciliated prismatic epithelium; ct: connective tissue; dg: digestive gland; k: kidney; pe: pavement epithelium; sc/gc: somatic and germinal cells. Bars: 10 µm.

progressive in males and significantly higher in stage II males (mean ± SEM: 1.54 ± 1.07) compared to stage II females (mean ± SEM: 0.67 ± 0.60) while it is more abrupt in females, where it is significant in stage III (mean ± SEM: 2.10 ± 1.17).

The cell localization of *Cg-Foxl2* mRNA expression in male and female stage II gonads can be observed by *in situ* hybridization in parallel with histological sections stained with a Prenant–Gabe trichrome (Fig. 8). At this stage, the gonadal tubules which are invaginated in a connective tissue, develop and show all the types of germinal cells (Fig. 8C and G) are specifically stained with the antisense probe (Fig. 8A, B, E and F). In the female gonads (Fig. 8A–D), a cytoplasmic mRNA staining was observed in germ cells, although it was faint in oogonia and pre-vitellogenic oocytes and more intense in the vitellogenic ones (Fig. 8C and D). In male gonads (Fig. 8E–H), a cytoplasmic mRNA staining was detected in the germinal cells from spermatogonia to mature spermatids, although it was difficult to exclude a staining in the somatic cells surrounding them in the gonadal tubules (Fig. 8G and H). Negative controls with the sense riboprobe gave the same signal as the antisense probe (results not shown).

3.5. Histology of the gonad in spats

The structure of the gonadic area of spats of 3, 3.5 and 4 months of age is illustrated on Fig. 9. Collecting ducts can be observed at the periphery of the gonadic area from 3 months of age. At 3 months of age, these ducts are limited on one side by a thin pavement epithelium, which seems to start to be ciliated at 3.5 months of age and replaced by a ciliated prismatic epithelium when the animal is 4 months old. On the other side, where the gametogenesis takes place, some somatic and/or germinal cells appear progressively between 3.5 and 4 months. Whatever the age, no gonadic tubules are seen. In our conditions we were unable to distinguish the gonadal area of younger spats because of (i) the difficulty of processing them due to the fact that they are too small to be unshelled and (ii) gonads probably not being formed and consequently the gametogenesis not having started yet."

4. Discussion

We report here the molecular characterization of *Cg-Foxl2*, which is the first cDNA sequence of a forkhead factor identified in the protostomia lophotrochozoa phylum. The genomic sequence of *Cg-Foxl2*, identical to the cDNA sequence, revealed the absence of intron, at least in the first 926 bp of the gene, like *Foxl2* genes identified in vertebrates (Cocquet et al., 2002; Govoroun et al., 2004; Nakamoto et al., 2006). Phylogenetic analyses of Cg-FoxL2 with members of the Fox family from both protostomes and deuterostomes provided evidence that Cg-Foxl2 is grouped with the Foxl2 subfamily with high bootstrap support (100). The deduced amino acid sequence presents a high conserved *Forkhead* domain characteristic of the Fox protein family (Weigel and Jackle, 1990; Katoh and Katoh, 2004). Putative NLS sequences characterized by the presence of basic amino acids have also been identified in Cg-Foxl2 at the C-terminal end of the *Forkhead* box as in other Fox factors (Carlsson and Mahlapuu, 2002). However, this factor, as Foxl2 in invertebrates and non-mammalian vertebrates, contained neither a polyalanine tract (A) nor glycine (G) nor proline (P) repeats which are only found in mammals (Cocquet et al., 2003; Wang et al., 2004; Liu et al., 2007). When the phylogenetic analysis was restricted to the Foxl2 subfamily of protostomes and deuterostomes, Cg-Foxl2 appeared as an out-group (97 bootstrap support) with Foxl2b of the rainbow trout and Foxl2 of invertebrates such as the urochordata ciona, which is in agreement with the general phylogenetic classification. Because teleosts have highly-polymorphic genomes due to a relatively recent lineage specific polyploidisation (Wittbrodt et al., 1998; Steinke et al., 2006), at least two *Foxl2* paralogs, *Foxl2a* and *Foxl2b*, have been characterized so far (Baron et al., 2004). According to our phylogenetic analysis, *Foxl2b* could be the ancestral form. In the oyster a single transcript of *Cg-Foxl2* was detected by northern blot in the gonads of both sexes.

Real time PCR analysis of the *Cg-Foxl2* mRNA showed that its expression is significantly higher in gonads and labial palps than in other tissues. This gonadal expression is in agreement with a role in the differentiation of the gonad in the oyster as it has been shown in several vertebrates (Cocquet et al., 2002, 2003), although Foxl2 is also expressed in other tissues such as the eyelids and the brain and/or the pituitary gland (Govoroun et al., 2004; Wang et al., 2004; Nakamoto et al., 2006; Alam et al., 2008; Oshima et al., 2008) depending on species. In the oyster, high levels of expression of *Cg-Foxl2* observed in the labial palps, organs which are rich in storage tissue (Heude-Berthelin et al., 2000) like the gonadic area but physically independent from it, are not inconsistent. In adult oysters, the gonad is a diffuse organ made of numerous gonadal tubules invaginated in a storage tissue made of vesicular cells, the whole of these constituting the gonadic area (Franco et al., 2008). It is well known that gonadal differentiation is indirectly linked to growth since reserves from the storage tissue around the tubules are shared out between these two physiological processes. In the same manner, although it has not been explored yet, a relationship between the labial palps (in particular their storage cells) and the oyster gonadal differentiation could be

hypothesized. Besides, a positive correlation between the weight of the animal and sex in favour of the females has already been evidenced in the sea-bass, a species with a polygenic sex determination (Vandeputte et al., 2007).

The analysis of the *Cg-Foxl2* mRNA expression along an adult gametogenetic cycle in *C. gigas* showed an increase matching the seasonal development of the gonad. Indeed, in adults, the gonad is a non-permanent organ evolving according to an annual reproductive cycle. The gametogenesis is initiated each year from clusters of germ cells scattered in the storage tissue (stage 0). Then these germ cells proliferate (stage I), differentiate and mature (stages II and III) within the gonadal tubules. As the gonadal tubules grow and ramify, they spread through the surrounding connective tissue which regresses (Heude-Berthelin et al., 2001; Fabioux et al., 2004a). Moreover, intrinsic characteristics of the oyster gametogenesis lead to difference between males and females and between stage II and III, illustrated by an increase of the number of germ cells during spermatogenesis and the increase of the oocyte volume during the folliculogenesis (Lango-Reynoso et al., 2000; Li et al., 2000; Heude-Berthelin et al., 2001; Franco et al., 2008). This is indirectly illustrated by the expression of *Oyvlg*, a germ cell specific marker, which increases linearly during spermatogenesis and abruptly at stage III in females, reflecting in the latter the maternal mRNA storage process during the oocyte vitellogenesis (Li et al., 2000). In contrast, the increase of *Cg-Foxl2* expression is linear in both sexes although it starts to be significant in female at stage II, a time of the gametogenetic cycle corresponding to the start of the vitellogenesis. These results suggest a role of Cg-Foxl2 in the vitellogenesis in the oyster which is in agreement with the role of Foxl2 in the folliculogenesis hypothesized in adults of many species amongst mammals (Crisponi et al., 2001; Cocquet et al., 2003), birds (Loffler et al., 2003; Govoroun et al., 2004) and fish such as the Nile tilapia and the medaka (Wang et al., 2004; Nakamoto et al., 2006). Such a role of Cg-Foxl2 in the oyster's vitellogenesis can also be hypothesized from our *in situ* hybridization results which show an mRNA expression in pre- and vitellogenic oocytes in stage II female gonads. This Foxl2 expression in germinal cells, although in contrast with the follicular somatic cell expression mentioned for most species (Crisponi et al., 2001; Govoroun et al., 2004; Nakamoto et al., 2006; Uhlenhaut and Treier, 2006), has also been found in mice and chicken developing and mature ovaries (Cocquet et al., 2002; Govoroun et al., 2004). However, to give credit to our *in situ* hybridization results an important point has to be clarified. Indeed, we mentioned that *Cg-Foxl2* staining observed in the gonads appeared not only with the antisense but also with the sense probe. These results may suggest the presence of natural antisense mRNAs (NATs) of *Cg-Foxl2* in the oyster. Very little is known about these NATs, especially what relates to their cell expression. Interestingly, they have been mentioned for *Wt1*, a transcription factor involved in sex determination (Dallosso et al., 2007) but also and above all for *FoxL2* in mice (Cocquet et al., 2005). In this case, the Foxl2 mRNA expression studied by *in situ* fluorescence hybridization (FISH) appeared co-localized with the NAT expression in the cytoplasm of the somatic cells of the mature and developing ovaries, suggesting therefore a regulation of the gene by its NAT (Cocquet et al., 2005). Finally a dimorphic gonadal expression of *Cg-Foxl2* was observed at stage II, where meiosis and early vitellogenesis take place respectively in males and females, but not at stage III which corresponds to ripe gonads. *In situ* hybridization, although the stage II male is not at the stage where *Cg-Foxl2* mRNA is the most strongly expressed by real-time PCR, illustrates best the cell localization of the mRNA expression as it shows all types of germinal cells from spermatogonia to mature spermatids. Thus, in these animals a staining was observed in the cytoplasm of germinal cells such as spermatogonia and/or in the somatic cells surrounding them although it is impossible at this microscopic level to differentiate the cell limits. Such a male expression and its increase observed at stage III is in disagreement with the results observed in other species including hermaphrodites, where *Foxl2* expression is about two to one hundred times higher in adult ovaries than in adult testes (Govoroun et al., 2004; Alam et al., 2008; Oshima et al., 2008). All these results taken together may indicate a new function of Foxl2 in the male gonadal maturation of the oyster.

The establishment of the gonad during the development of the oyster remains unknown. However, the analysis of the *Oyvlg* mRNA expression by Fabioux et al. (2004b) has allowed to precise the development and the location of primordial germ cells (PGCs) during the development of the oyster. From the veliger larvae stage, PGCs migrate to two areas on both sides of the dorso-ventral axis which correspond to the future gonad location around the digestive gland in late larvae stages. After metamorphosis, PGC start to differentiate into two groups of germinal stem cells which would later proliferate intensively to form the gonadic tubules when juveniles reach sexual maturity (Fabioux et al., 2004b). *Oyvlg* mRNA is of maternal origin and its content gradually decreases during early development until the last stage analysed, the trocophorae stage (18 h post-fertilization) (Fabioux et al., 2004b), probably reflecting the decrease of the relative proportion of germ cells precursors to the total number of cells. In our study, *Oyvlg* mRNA levels started to increase significantly after metamorphosis, reaching a peak in 1–1.5-month-old spats and then decreasing in older spats (aged 3 to 4 months), reflecting again the decrease of relative number of germ cells per total number of cells. A *Cg-DMl* mRNA level increase was observed concomitantly to that of *Oyvlg* and the kinetic of expression of both appears in agreement with the establishment in the pericardic region of the two primary gonads symmetrically to the digestive gland (Fabioux et al., 2004b). In contrast with the *Oyvlg* and *Cg-DMl* mRNAs profiles, *Cg-Foxl2* mRNA relative levels slowly increased in 7 dpf larvae to 1. 5 month old spats and then decreased until spats were 3–3.5 months old, a stage at which our histological observations showed the presence of distinguishable gonadal collecting ducts and visible gametogenesis initiation. From these results and due to the impossibility to differentiate sexes before an advanced stage of gametogenesis in the oyster, it is difficult to specify whether Cg-FoxL2 is an early female sex differentiation marker as proposed in vertebrates (Loffler et al., 2003; Govoroun et al., 2004; Wang et al., 2004; Nakamoto et al., 2006; Yamaguchi et al., 2007). Nevertheless, because the oyster is generally male for the first year of its life, we could conceive that such a candidate ovary-determining gene is under-expressed in a pool of spats developing into a majority of males.

To evidence a sexual dimorphic gene expression in the oyster, especially during development, it would be necessary to work on monosexual oyster populations which are actually unavailable. However, by specifying the window of sexual determinism, biotic or abiotic factors could be applied at this time to influence the gonadal differentiation.

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