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In vivo RNA interference in oyster –*vasa* silencing inhibits germ cell development

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Abstract:

This study investigated the potential of RNA interference, which is technically challenging in bivalve mollusc species, to assess gene function in the oyster Crassostrea gigas. We designed dsRNA targeting the oyster vasa-like gene (Oyvlg), specifically expressed in oyster germ cells. In vivo injection of oyvl-dsRNA into the gonad provokes a knockdown phenotype corresponding to germ cell underproliferation and prematurely arrested meiosis througout the organ. The most severe phenotype observed is sterile. This knockdown phenotype is associated with a decrease in Oyvlg mRNA level of between 39% and 87%, and a strong reduction in OYVLG protein, to an undetectable level. Therefore, Oyvlg appears to be essential for germ cell development in Crassostrea gigas, particularly for mitotic proliferation and early meiosis. Our results demonstrate for the first time that in vivo RNA interference works efficiently in a bivalve species, opening major perspectives for functional genetic studies.

Keywords: marine bivalve, *Crassostrea gigas*, RNAi, vasa, germline

1. Introduction

The oyster *Crassostrea gigas* has stimulated a great deal of biological research as it represents a major economic resource for aquaculture (production: 4.2 million metric tons; FAO 2005), it plays a sentinel role in estuarine and coastal marine habitats [1], and it belongs to the Lophotrochozoa, a vast and diverse branch of bilaterian animals that have been little studied with respect to genomics. The recent emergence of bivalve genomics with substantial characterization of genome-wide expression sequences, especially for *C. gigas* [1,2], argues for the rapid development of methodologies to unravel gene function in these species.
Classic functional genetic approaches such as mutagenesis are not yet available for bivalve molluscs. A powerful alternative method for reverse genetics is RNA interference (RNAi), which can be a quick and efficient technique for determining the loss-of-function phenotype of a gene [3]. The RNAi revolution was started by evidence that double-stranded RNA (dsRNA) could knock-down the expression of specific genes [4]. The ~25-nucleotide small interfering RNA (siRNA) fragments generated by processing long dsRNA are reported to be the mediators of RNAi [For review 5]. Small interfering RNA provide sequence specificity to the RNA-induced silencing complex (RISC) that inhibits the corresponding mRNA, thereby silencing the targeted gene [6]. RNA interference has been widely used *in vitro* and *in vivo* in vertebrate and invertebrate species [4,7-10]. Conversely, RNAi studies are scarce in molluscs. RNAi has been used, for example, in gastropods to explore gene functions in the nervous system [11] and in the cephalopod *Sepia officinalis* to analyse the role of Muscle Regulatory Factor in tentacle muscle differentiation [12]. In bivalve molluscs, RNAi remains a technical challenge. To document *in vivo* gene silencing by RNAi in oyster, we injected dsRNA targeting the oyster *vasa*-like gene (*Oyvlg*). In *Drosophila* and *Caenorhabditis vasa* plays a key role in germ cell differentiation, as clearly demonstrated by functional analysis of mutation or inactivation of the gene, which in the most striking cases can lead to total sterility [13,14]. In the oyster *C. gigas*, *Oyvlg* is specifically expressed in germ cells and was thought to play a role in germline development [15,16]. In this study, the oyster *vasa*-like gene was chosen to develop *in vivo* RNAi in the oyster, not only to assess the function of *Oyvlg* in germline formation but also to investigate the potency of this methodology to serve as a routine means for gene function assignment in bivalve molluscs.

2. Results and discussion

Validation of OYVLG specific antibody

As demonstrated by immunodetection on western blot against total protein extracts from oyster tissues (mantle, gills, muscle, labial palps, digestive gland, gonad), the synthetic polyclonal antibodies (Millegen, Labege, France) targeting two peptides specific to OYVLG recognized a unique band of apparent molecular weight of 79kDa corresponding to the predicted size for OYVLG (Figure 1). The distribution of the antigenic protein appeared restricted to gonadic tissue in both sexes with a higher quantity of protein in female than in male mature gonad, in accordance with the *oyvlg* mRNA expression pattern [16]. As a result, the antibodies (Fab1+Fab2) were used in this study to detect and quantify OYVLG protein amount.

Design of RNAi experiment in oyster

The oyster *vasa*-like gene was chosen for the development of an RNAi method in oyster for several important reasons. (a) The determining of *Oyvlg* role in *C. gigas* is of major interest for our physiological research into oyster reproduction. (b) The spatiotemporal expression of *oyvlg* mRNA is clearly characterized in oyster [16] showing a specific expression in germ cells. (c) inactivation of the *vasa* gene has been successful for several species [13,14,17] leading to a clear phenotypic effect that is easily measurable (*i.e* partial or total sterility); and (d) specific antibodies are now available against OYVLG to measure the effect of *oyvl*-dsRNA administration at the protein level, in addition to real-time PCR for the mRNA level [15].
Because long dsRNA have been demonstrated to perform efficient gene silencing in invertebrates [3], we synthesised two long dsRNAs, *oyvl4*-dsRNA and *oyvl5*-dsRNA, by *in vitro* transcription. Designing two targets is recommended and commonly called a "redundancy experiment" to avoid false positives [18]. Both dsRNAs were designed to contain *vasa* specific domains, and to be outside the sequence amplified by real-time PCR primers so as to avoid any bias from the injected dsRNA when quantifying o*yvlg* mRNA. In our preliminary experiments, no differences were observed in response to injection of *oyvl4*-dsRNA alone, *oyvl5*-dsRNA alone or a mixture of both dsRNAs (data not shown). All the experiments presented in this paper were, therefore, done with a mix of *oyvl4*- and *oyvl5*-dsRNAs, called "*oyvl*-dsRNA".
To validate the *in vivo* dsRNA injection method in oyster, we used an original technique consisting in monitoring, by *in situ* hybridization, the administration of DIG-labelled *oyvl*-dsRNA into the target organ. The DIG-labelled *oyvl*-dsRNA have been observed in a large part of the gonad around the injection point showing the efficiency of the administration of the dsRNA into the gonad (Figure 2). Direct injection in the target organ is, therefore, an efficient method for administrating dsRNA to oyster tissues. The DIG-labelled dsRNA developed in the present study represents important technical progress for examining the first crucial step for *in vivo* RNAi success: the administration of dsRNA in animal tissues.

Oyvl-*dsRNA* in vivo *injection provokes abnormal germ cell development*

One month post-injection, 44% of oysters injected with 20µg *oyvl*-dsRNA and 80% of oysters injected with 100µg *oyvl*-dsRNA presented defects in germ cell development affecting all the gonadic area, both in females and males. Upon histological examination of 20µg dsRNA-injected gonads, there were fewer germ cells and development was prematurely curtailed compared with control gonads (Figure 3). Females with the abnormal phenotype halted their gametogenesis at prophase I of meiosis before the vitellogenesis, whereas vitellogenic oocytes were observed in all control females. In males with the abnormal phenotype, germ cells developed no further than the spermatocyte stage. Conversely, spermatids and spermatozoids were observed in all control males (Figure 3). Moreover, in oysters showing abnormal phenotype, apoptotic germ cells were visible with a significant number of haemocytes invading the gonadic tubules likely translating an active resorption of degenerating germ cells (Figure 3).
Defects in gonad development appeared even stronger in females and males injected with 100µg *oyvl*-dsRNA. The gonadic tubules appeared almost fully regressed in all the gonadic area. They contained scarce germ cells, all blocked at early stages of gametogenesis while the gonads of control oysters are fully mature (Figure 3). Haemocyte infiltration was also observed in the gonadic area of oysters injected with 100µg *oyvl*-dsRNA. This suggests that gonadic tubules had stopped developing and started to degenerate. This most severe defect is clearly similar to sterile phenotypes described in mouse and *Drosophila vasa* mutants. Tanaka *et al*. [19] demonstrated that male mice homozygous for a mutation of *vasa* exhibited reproductive deficiency. The premeiotic male germ cells ceased their differentiation before pachytene spermatocyte stage and underwent apoptosis. In *Drosophila*, ovaries of null *vasa*-mutants contained fewer developing cysts than ovaries of wild-type *Drosophila* [20]. No non-specific

defects were observed in gonads of oysters injected with *oyvl*-dsRNA and no oyster mortality was recorded during RNAi experiments, indicating that dsRNAs were not toxic for oysters.
We demonstrated here that the *oyvl*-dsRNA injection into oyster gonads provoked partial or total sterility, probably associated with *oyvlg* gene product deficiency. The knock-down phenotype was observed throughout the gonad, although we injected *oyvl*-dsRNA at only one point. This pattern confirmed a systemic spread of dsRNA though the gonad as demonstrated in other species [21]. This systemic spread of dsRNA could not be followed using DIG-labelled dsRNA since it likely was the result of new synthesized *oyvl*-dsRNA issued from the injected *oyvl*-dsRNA. The severity of the knock-down phenotypes appeared dsRNA-dose-dependent and resulted in complete sterility, translated by the complete regression of the gonadic tubules and the degeneration of germ cells, at the highest dose (100µg). Moreover, the knock-down phenotype appeared more severe one month post injection than after 9 days, when only 40% of the oysters injected with 100µg *oyvl*-dsRNA displayed a knock-down phenotype, probably because it was too soon to visualize alterations of cellular processes occurring during germ cell development.

Knock-down of Oyvlg *mRNA and protein expression*

A 70% inhibition of mRNA level after dsRNA treatment was considered as a threshold for effective RNA interference [22]. In our data, a ≥ 70% reduction of *oyvlg* mRNA level compared with the control was obtained for 3 out of 21 oysters injected with 20µg dsRNA (14%) and for 4 out of 10 oysters injected with 100µg dsRNA (40%) (Figure 4). Nevertheless, the knock-down phenotype visible at one month post-injection was already clearly observed with only a 39% inhibition of *oyvlg* mRNA, for 4 out of 9 oysters injected with 20µg dsRNA (44%) and for 4 out of 5 oysters injected with 100µg dsRNA (80%) (Figure 4). The injection of *oyvl*-dsRNA clearly triggered an RNA interference mechanism, and a threshold around 40% for mRNA level reduction appeared enough to obtain the knock-down phenotype. The mRNA level reduction was greater for oysters injected with 100µg than with 20µg *oyvl*-dsRNA (Figure 4) and was correlated with the most severe knock-down phenotype, confirming the dose-dependant effect of RNAi discussed previously. The quantity of 100µg dsRNA, corresponding to a mean concentration of 20µg dsRNA/g oyster body weight, is within the range of dsRNA quantity injected into other adult invertebrates to obtain RNA interference: around 50µg dsRNA/g was used in honeybee and 15µg dsRNA/g was used in shrimp [9,23]. The level 20µg dsRNA/g of body weight could be therefore considered as an optimal quantity of dsRNA for *in vivo* RNAi experiments in adult oysters. The inhibition rates of *oyvlg* mRNA levels were similar 9 days and 1 month post-injection, indicating no decrease of RNAi effect during this time. These results suggest the existence of a dsRNA amplification process in oyster cells, as was demonstrated in organisms such as *Drosophila* and *Caenorhabditis* [24,25].
Whereas significant reduction of *oyvlg* mRNA level was observed as early as 9 days post-injection, no reduction of mRNA level was observed for two other gonad-specific genes; the specificity of the dsRNA effect is therefore clearly shown. Mean relative levels of *og-TGFβ* mRNA, specifically expressed in auxiliary cells of the germ cells [26], were 0.54±0.20 for controls, 0.69±0.30 and 0.59±0.17 for oysters injected with 20µg and 100µg *oyvl*-dsRNA, respectively. Furthermore, relative levels of a neuropeptide Y related receptor, specifically expressed in *C. gigas* germ cells (Genbank accession n°**AM856249**, unpublished data) were also statistically similar in the three tested conditions: 1.98±1.28, 1.81±0.96 and 3.90±2.05 for controls, oysters injected with 20µg and 100µg *oyvl*-dsRNA, respectively. These assays were not repeated at one month post-injection because the defects in the gonad were already so strong that most of gonad-specific genes would be consequently affected.
Oysters showing reduction of *oyvlg* mRNA level after dsRNA treatment also displayed dramatic reduction of OYVLG protein level (Figure 5). Nine days post-injection, when the mRNA decrease

reached 70%, OYVLG protein was totally absent from gonadic tissue (Figure 4). One month post-injection, decrease of OYVLG protein had reached 83%, but appeared weaker overall compared with mRNA level reduction (except in one oyster n°100.6, Figure 5). Post Transcriptional Gene Silencing triggered by RNAi stems from degradation of target mRNAs. The OYVLG protein detected probably results from the progressive accumulation of translated "residual" *oyvlg* mRNA escaping from RNAi machinery. In our data, "residual" *oyvlg* mRNA varied from 13% to 48%.
A high variability in RNAi response was observed between individuals (Figures 4 and 5). Variation in the amount of dsRNA actually penetrating into the germ cells probably contributed, to a large extent, to the variability in RNAi response. Direct injection of dsRNA solution into the circulatory system, through the adductor muscle or in the pericardic region, would probably improve the delivery of dsRNA into the cells of the target organ, since hemolymph efficiently reaches all the organs of the oyster.

The role of the oyster* vasa*-like gene in germ cell development

In previous studies, we demonstrated that *Oyvlg* is specifically expressed in germ cells of both male and female oysters, and we hypothesized *Oyvlg* to have a role in germ cell formation [16]. However, the function of *Oyvl*g in germline development had never been demonstrated since no functional genetic tools were available in oyster. In this study, *in vivo oyvl*-dsRNA injection was realized in the gonad of oysters at the initiation of the reproduction, when gonadic tubules are filled with germ stem cells and some gonia at the start of the proliferation. The *oyvl*-dsRNA injection was clearly associated with defective germ cell development, particularly visible one month later when control oysters reached maturity. The number of germ cells was reduced and their development arrested at the first step of meiosis. The most severe phenotype showed total sterility translated by the complete degeneration of germ cells and the regression of gonadic tubules in the whole gonadic area (Figure 3). Our results demonstrate that O*yvlg* has an essential role in germ cell (germ stem cells and gonia) proliferation and is probably implicated in oocyte and spermatocyte differentiation. Conversely, *Oyvlg* would not be essential in the last step of gametogenesis, vitellogenesis or spermiogenesis, since RNAi experiments realized according to the same protocol in maturing oysters showed no visible phenotype (data not shown). In *Drosophila*, *vasa* appeared to have an essential function in female gametogenesis but not in male gametogenesis. In mouse, however, the *Mvh* gene appeared necessary for spermatogenesis completion but not for oogenesis. In oysters, we observed defects both in male and female germ cell development in *oyvl*-dsRNA-treated gonads. A similar molecular regulation of early gametogenesis is suggested in both sexes, probably due to the alternative hermaphrodite status of oysters as observed in *Caenorhabditis* [13].

3. Material and methods

Biological material

Oysters were obtained from Marennes-Oléron (France) cultured stocks and transferred to the Ifremer Laboratory in Argenton (France). They were acclimated for one week and with optimal conditions for germ cell maturation [details in 27].

dsRNA synthesis

Two fragments from positions 495 to 1020 (*oyvl4*) and 29 to 906 (*oyvl5*) of *oyvlg* cDNA (GenBank accession no. **AY423380**) were amplified by RT-PCR using total RNA extracted from gonad as template. PCR fragments were sub-cloned into pCR4-TOPO vector (Invitrogen, Paisley, UK) and sequenced. Recombinant plasmids were purified by using the Plasmid midi kit (Qiagen), linearised either with NotI or SpeI (Promega, Madison, WI) enzymes (4h at 37°C using 5U/µg plasmid), phenol-chloroform extracted and finally ethanol-precipitated and suspended in RNAse-free water. The purified plasmids were transcribed *in vitro* on both strands using T7 and T3 MEGAscript® Kit (Ambion, Austin, TX, USA) to produce *oyvl4* and *oyvl5* sense and antisense single stranded RNA (ssRNA). The ssRNA were phenol-chloroform extracted, ethanol-precipitated and suspended in RNAse free saline solution (10mM Tris/10mM NaCl) to a final concentration of 0.5µg/µL after quantification by spectrophotometry (Nanodrop, Thermo Scientific). Equimolar amounts of sense and antisense ssRNA were heated at 100°C for 1min and left to cool at room temperature for 10 hours for annealing. Each dsRNA (1µg) was analysed by 1% agarose gel electrophoresis to ensure that it existed as a single band of 525bp (*oyvl4*) or 877bp (*oyvl5*).

DIG-labelled dsRNA synthesis

Recombinant plasmids (*oyvl4* and *oyvl5*) were synthesized and linearised as described above. Single stranded RNAs were synthesized and DIG-labelled using T3 or T7 RNA-polymerase (20U/µg plasmid) and DIG RNA-labelling mix (Roche). Sense and antisense DIG-labelled-ssRNA were annealed as described above and dsRNA were stored at -80°C.

dsRNA administration and sampling

Oysters were anesthetized in $MgCl_2$ solution (3/5 fresh water, 2/5 seawater and 50g/L $MgCl_2$) for 3 hours. Anesthetized oysters were injected in the gonad with 100µL saline solution containing dsRNA or saline solution for the control. After dsRNA injection, oysters were maintained in raceways in conditions allowing optimal gonad maturation.
Oysters were injected at T0 (initiation of reproduction), T7 (7 days) and T14, with 20µg (N=24) or 100µg (N=10) *oyvl*-dsRNA (a mixture of *oyvl4* dsRNA and *oyvl5* dsRNA in equal amounts) or with the same volume of saline solution (control, N=24).
At T9 and T30, 12 oysters injected with 20µg *oyvl*-dsRNA, 5 oysters injected with 100µg *oyvl*-dsRNA and 12 control oysters were sampled. Their gonads were immediately dissected: a large transversal section of all the gonadic area was realised for histological examination and the rest of the gonad was placed in total RNA and protein extraction solution.
For dsRNA tracking, 10 oysters were injected with 20µg DIG-labelled-dsRNA and sampled 9 days after injection for histological and *in situ* hybridization examinations.

Histology, in situ hybridisation (ISH) and real-time RT-PCR analysis

The gonadic development was assayed on histological slides of a transversal section of all the gonadic area according to Fabioux *et al.* [27] for dsRNA-injected and control oysters at T0, T9 and T30. The DIG-labeled *oyvl*-dsRNA sampled were analysed by ISH using *Oyvlg* DNA probes according to Fabioux et al. [16].
Total RNA was isolated from the gonad of treated and control oysters, using Extract All (Gibco BRL). Samples were then treated with DNAse I (1 U/µg total RNA, Sigma) to prevent DNA contamination. RNA concentrations were measured as described above and RNA quality was

checked by using the Bioanalyser 2100 (Agilent). From 2µg of total RNA, RT-PCR amplifications were carried out as described in Fabioux et al. [15] using specific primers for Oyster *vasa*-like gene (*Oyvlg*) [15], Oyster-gonadal-TGFß-like (og-TGFß) [26] and, Neuropeptide Y related-receptor like (NPY-receptor) (Forward: 5' GTGGCTTGTGGGCTTATTGT 3', Reverse: 5' CTGAAATCCGAATGGACGAC 3'). The calculation of relative mRNA levels of target genes was based on the comparative Ct method (see Fabioux et al [15] for $\Delta\Delta$Ct formulae) and was normalized to *Elongation Factor I* (*EFI*) as no significant differences of Ct values were observed for *EFI* between control and injected oysters (Kruskall-Wallis test = 3.74; *P*= 0.15, CV= 3.6%). The relative mRNA levels are expressed in "number of copies of target gene per copies of *EFI*".

Antibodies and Western blot analysis

Polyclonal antibodies (Fab1 and Fab2) against two peptides (126-GSKNDGESSGFGGG-139, 165-EEGHFARECPEPRK-178, respectively) coded in the *Oyvlg* cDNA sequence were produced in rabbits by MilleGen (France).
Total protein extractions were realized from gonadic tissue of mature female and mature male, mantle, gills, muscle, labial palps and digestive gland according to Corporeau and Auffret [28]. Before denaturation of protein samples, total protein extracts were quantified using DC protein assay (Bio-Rad, CA, USA) and adjusted to a final concentration of 1mg/mL. Twelve micrograms of each protein extract were loaded onto SDS-polyacrylamide gel to ensure identical amount of protein between samples.
Western blot was performed as described in Corporeau and Auffret [28] using the anti-OYVLG polyclonal antibody produced in this study (dilution 1/5000). Blots were revealed using an Immun-star AP detection kit (Bio-Rad, CA, USA). The amount of OYVLG protein was quantified using the Multi-analyst software (Biorad, CA, USA) with the background signal removed. The obtained value is expressed in OD/mm^2 and represents the spot intensity expressed as mean count per pixel, multiplied by the spot surface. After revelation and signal quantification, membranes were de-hybridized for 1h at room temperature in de-hybridizing buffer (100mM glycine, 100mM NaCl, pH 3.2) and re-hybridized with an anti-histone H3 antibody (# 9715, Cell Signaling Technology, Danvers, MA, USA; dilution 1/5000) to control for identical amount of total protein between samples

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Figures

Figure 1

Figure 1: Western blot probed with anti-OYVLG antibodies to analyse the level of OYVLG protein in oyster tissues: mantle (lane 1), gills (lane 2), muscle (lane 3), labial palps (Lane 4), digestive gland (lane 5), male gonad (lane 6), female gonad (lane 7). Twelve micrograms of total protein extract from each tissue were loaded into the gel. A single band of about 79 kDa was detected in female and male gonad.

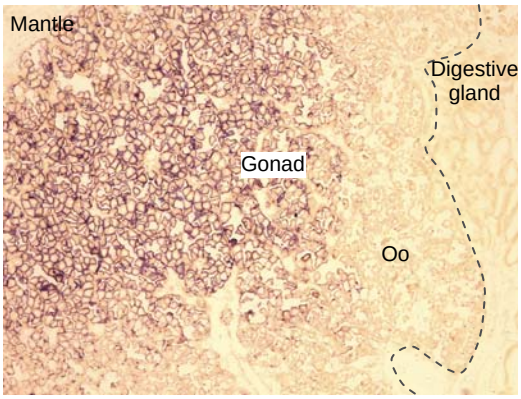

Figure 2

Figure 2: *In vivo* dispersion of DIG-labelled *oyvl*-dsRNA injected into oyster gonad. DIG-labelled dsRNA, stained in dark blue, appeared to have dispersed in a large part of the gonad. Oo: oocyte. Magnification 100X. Scale bar represents 100µm.

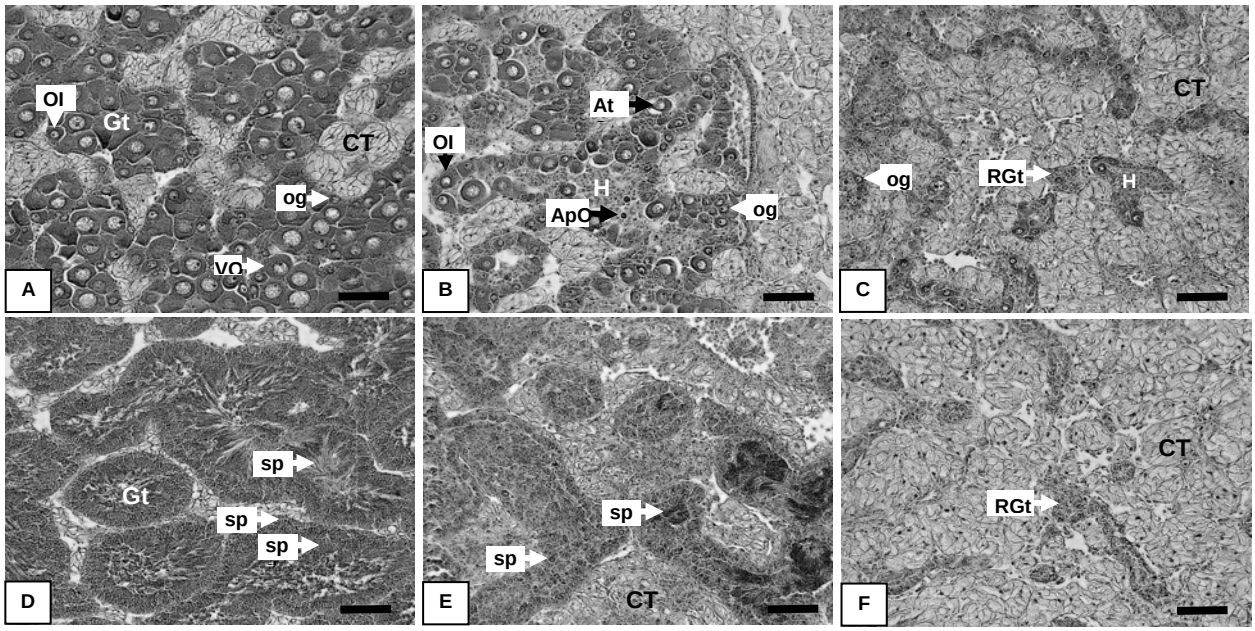

Figure 3

Figure 3: Effects of *in vivo oyvl*-dsRNA injection on germ cell development in oyster, one month post-injection.
(A) Female control, injected with saline solution. Oocytes are in vitellogenesis. (B) Female injected with 20µg *oyvl*-dsRNA (n°20.19). Gonadic tubules are constituted by oogonia, Oocytes I and atretic oocytes phagocytized by haemocytes. (C) Female injected with 100µg *oyvl*-dsRNA (n°100.10). Gonadic tubules are mostly degenerated. (D) Male control injected with saline solution. Germ cells are in active gametogenesis. (E) Male injected with 20µg *oyvl*-dsRNA (n°20.20). Gonadic tubules are filled with a limited number of germ cells, spermatogonia and spermatocytes. (F) Male injected with 100µg *oyvl*-dsRNA (n°100.8). Gonadic tubules are

degenerated. Gt: Gonadic Tubule; CT: Conjunctive Tissue; H: Haemocytes; og: oogonia; OI: Oocyte I; VO: Vitellogenic Oocyte; AtO: Atretic Oocyte; ApO: Apoptotic Oocyte; RGt: Residual Gonadic tubule; Spg: Spermatogonia; Spc: Spermatocytes; Spd: Spermatids; Spz: Spermatozoïds. Magnification 400X. Scale bars represent 100µm.

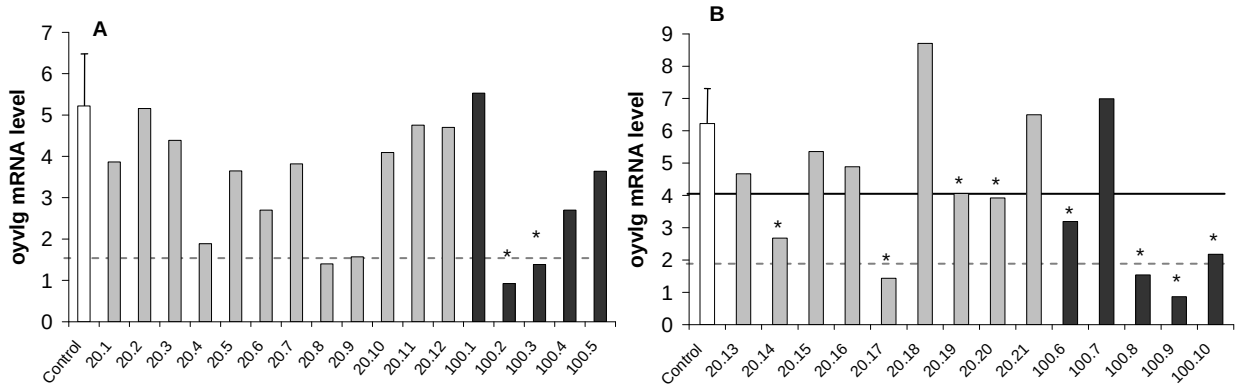

Figure 4

Figure 4: Levels of *oyvlg* transcripts relative to *EFI* transcripts analyzed by real-time PCR and expressed in "number of copies of *Oyvlg* per copies of EFI" for control, oysters injected with 20µg (N=12 at T9 and N=9 at T30) (light grey) and 100µg (N=5 at T9 and T30) (dark grey) *oyvl*-dsRNA. The control is the mean of *oyvlg* mRNA levels of all control oysters (N=12 at T9 and T30). Bar represents confidence interval at 5% level. Asterisks (*) indicate oysters showing knock-down phenotype. (A) Nine days post-injection. (B) One month post-injection. Horizontal black line indicates the threshold of 39% inhibition of *oyvlg* mRNA level compared with control at one month post-injection. Grey dotted line indicates the threshold of 70% inhibition of *oyvlg* mRNA level compared with control, considered as the threshold for effective RNAi [22].

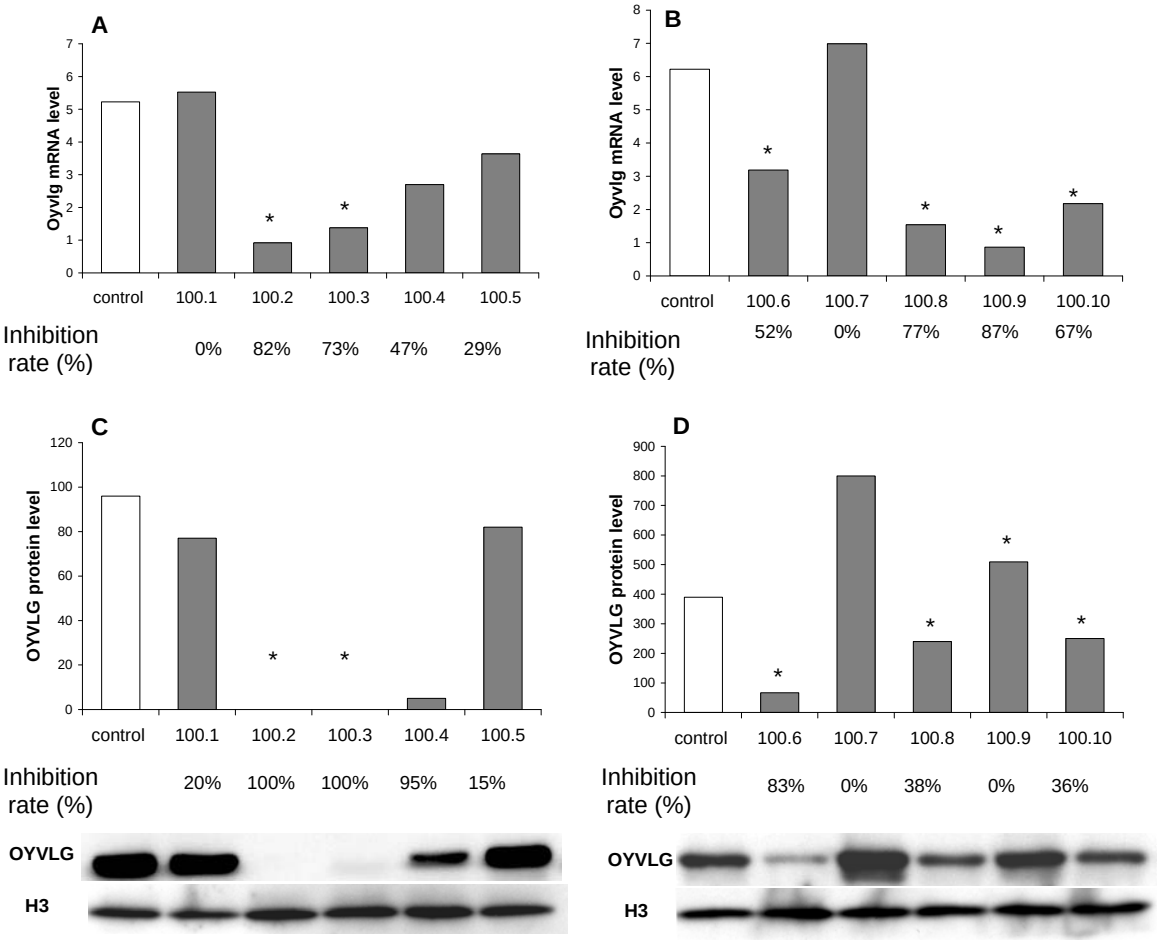

Figure 5

Figure 5: Levels of both *oyvlg* transcripts relative to *EFI* transcripts measured by real-time PCR (expressed in "number of copies of *Oyvlg* per copies of *EFI*"), and OYVLG protein quantified on western blot (expressed in OD/mm^2) for oysters injected with 100µg *oyvl*-dsRNA (N=5 at T9 and T30).
Bars represent confidence interval at 5% level. (A) mRNA levels 9 days post-injection. The inhibition of *oyvlg* mRNA level ranged from 0 to 82%. (B) mRNA levels 1 month post-injection. The inhibition of *oyvlg* mRNA level ranged from 0 to 87%. The control used for mRNA level measurement is the mean of *oyvlg* mRNA levels of all control oysters (N=12 at T9 and T30 (C) OYVLG protein level 9 days post-injection (OD). The values presented on the graph were calculated from the western blot of OYVLG just below. The inhibition of OYVLG protein level ranged from 15% to 100%. In the same samples, protein level of Histone H3 (blot under the graph) was unchanged. (D) OYVLG protein level 1 month post-injection (OD). The values presented on the graph were calculated from the western blot of OYVLG just below. The inhibition ranged from 0 to 83%. In the same samples, protein level of Histone H3 (blot under the graph) was unchanged. The control used for protein measurement is a pool of proteins from all control oysters injected with saline solution. Asterisks (*) indicate oysters showing knock-down phenotype.