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Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: Evidence of early infection and down-expression of immune-related genes

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Abstract:

The understanding of reciprocal interactions between *Crassostrea gigas* and *Vibrio* sp., whether these be virulent or avirulent, is vital for the development of methods to improve the health status of cultured oysters. We describe an original non-invasive experimental infection technique using cohabitation, designed to explore these interactions. Using real-time PCR techniques we examined the dynamics of virulent and avirulent *Vibrio* sp. in oyster hemolymph and tank seawater, and made a parallel study of the expression of four genes involved in oyster immune defense: *Cg*-BPI, *Cg*-EcSOD, *Cg*-IκB, *Cg*-TIMP.

No mortality occurred in control animals, but oysters put in cohabitation for 2–48 h with animals previously infected by two *Vibrio* pathogens suffered mortalities from 2 to 16 days post-cohabitation. Our results show that virulent *Vibrio* infect healthy individuals after only 2 h of cohabitation, with values ranging from 4.5×10^{2} to 2×10^{4} cells ml^{-1} hemolymph. Simultaneously, an approximate ten-fold increase of the total *Vibrio* population was observed in control animals, with a 6.6–78.5-fold up-expression of targeted genes. In contrast, oysters exposed to harmful bacteria had mean expression levels strongly down-regulated by a factor of 9.2–29 (depending on the gene) compared with control animals. Although oysters were still found to be infected by virulent *Vibrio* after 6–48 h of cohabitation, no significant differences were noted when comparing levels of each transcript in control and infected oysters at the same sampling times during this period: the important differences were noted before 6 h cohabitation. Taken together, our data support (1) the hypothesis that virulent *Vibrio* disturbs the immune response of this invertebrate host both rapidly and significantly, although this occurs specifically during an early and transient period during the first 6 h of cohabitation challenge, and that (2) expression of targeted genes is not correlated with vibriosis resistance.

Keywords: Oyster-*Vibrio* interactions; Pathogenesis; Immune response; Non-invasive experimental challenge; Real-time PCR

1. Introduction

All organisms are surrounded by harmless and harmful microbes and, as pointed out by Virgin [1], reciprocal interactions during their co-evolution have produced homeostasis between these 'host' organisms and their commensal or pathogenic microbes. Other microbes, in contrast, can be opportunists, which change their status depending on the immune capacity of the host. In *Crassostrea sp.* oysters, several commensal bacterial species have been found not only in the digestive tract but also in the hemolymph and other soft tissues, probably due to the filter-feeding behaviour of oysters and their open circulatory system [2-5]. The dominant bacteria found in *C. gigas* hemolymph to date are Gram negative and belong to the genera *Vibrio*, *Pseudomonas*, *Alteromonas* and *Aeromonas.* Concentrations are normally above 10^2 bacteria per ml, according to estimations made with the classic culture counting method [3]. Such tolerance of commensal bacteria can be explained by a differential response of the oyster immune system to different bacteria. Oyster immune response is mediated via the phagocytic properties of hemocytes and humoral factors and has been seen to vary according to the different bacterial species and strains tested, including some species of concern for human health [5-6]. In the first of these studies, Pruzzo *et al.* showed that fecal pollutants *Escherichia coli* and *V. cholerae* were quickly destroyed by phagocytosis in oyster hemolymph. In contrast, some foodborne pathogens that produce an outer polysaccharide capsule, such as certain strains of *V. vulnificus*, persisted longer in the hemolymph and were seemingly resistant to digestion in hemocyte phagolysosomes, although the mechanism of this resistance is unknown.

Other bacterial species or strains, such as those related to the *V. splendidus* polyphyletic group or to *V. aestuarianus*, have been found to be pathogenic to *C. gigas* oysters in French rearing areas [7-12] and have frequently been identified as part of bacterioplankton communities in the coastal marine environment [13]. These *Vibrio* species or strains have been shown to be closely associated with mortality events, which occur mainly during the hot season. In such periods, these bacteria are found to be dominant and present in abnormal concentrations up to 10^4 colony forming units per ml (CFU ml^{-1}) hemolymph. These strains were also found to be pathogenic by experimental oyster infection [14]. However, the classical distinction between pathogen and opportunist remains problematic, since impairments of the *C. gigas* defense reaction could occur during natural mortality outbreaks or in experimentally-induced infections. Indeed, De Decker *et al*. [15] explored certain host susceptibility components to experimentally-induced vibriosis, such as gametogenesis and ploidy using strains of *V. splendidus* and *V. aestuarianus* revealing that reproductive status can affect host susceptibility.

Because oyster cellular defense mechanisms rely mainly on hemocytes, we decided to focus our study on hemocyte-bacterium interactions at a transcriptional level. Four well-characterized genes known to be involved in *C. gigas* defense mechanisms were chosen: *Cg*-BPI, *Cg*-EcSOD, *Cg*-IκB, *Cg*-TIMP. The expression of the *C. gigas* BPI gene (*Cg-bpi*), encoding a bactericidal-permeability-increasing protein (BPI), is shown to be induced in hemocytes after oyster heat-killed-bacteria challenge, and was detected by *in situ* hybridization [16]. *Cg*-EcSOD has been shown to be the major plasmatic multifunctional protein in *C. gigas*; gene expression and production are restricted to the hemocytes. This protein presumably binds to bacteria and lipopolysaccharide (LPS) and, as it has an unclassical superoxide dismutase (SOD) activity [17-18], could be involved in phagocytic mechanisms [19-21]. A putative *Cg*-IκB protein was chosen that was identified by an expressed-sequence-tag approach [22]. The inhibitors of NF-κB (IκBs) have been identified as major components of the Rel/NF-κB signaling pathway, an important mediator of immune responses in vertebrates and insects. *Cg*-TIMP is an inducible tissue inhibitor of

metalloproteinase in *C. gigas*. Its potential role in wound healing and defense mechanisms in this species was illustrated by the induction of *Cg*-TIMP gene expression in hemocytes following shell damage or non-virulent bacterial challenge [21, 23-24].

To describe the reciprocal interactions between *C. gigas* and virulent or avirulent *Vibrio* sp. we developed a non-invasive experimental infection technnique of *C. gigas* oysters, using a procedure for horizontal transmission of *Vibrio* bacteria through cohabitation. Cohabitation trials consisted of placing healthy oysters in the same tank as oysters that had first been experimentally infected or mock infected by injection with virulent *Vibrio* sp. or sterile artificial seawater, respectively, and sacrificed by severing their adductor muscle at the start (time zero) of cohabitation. The dynamics of both virulent *Vibrio* sp. and the total *Vibrio* population in the hemolymph of individual test animals and tank seawater were followed, in parallel with an expression study on the four defense-related hemocytic genes mentioned above, using specially developed real-time PCR techniques.

2. Material and Methods

2.1. Oysters

Two-year-old diploid oysters (*Crassostrea gigas,* mean total fresh weight = 4.6 ± 1.3 g) from natural recruitment were collected on a commercial farm (Perquis, Bassin de Marennes-Oléron, Charente-Maritime, France) and maintained for six months in an indoor infrastructure (Ifremer, La Tremblade), with daily monitoring of oyster mortality. In order to limit the impact of environmental parameters on immune gene expression levels [25][26] similar environmental conditions (see below) and high quality seawater was used for oyster breeding. These oysters were presumed to be healthy because none died during this six-month observation period. Since herpesvirus OsHV-1 and *V. aestuarianus* have been identified as the main infectious agents associated with *C. gigas* mortality events in France [27], these oysters were tested for the presence of OsHV-1 infection throughout the cohabitation described below. OsHV-1 DNA was not detected by real-time PCR [28] in tissues of the oyster used in our experiment. These oysters were also tested and found to be free of *V. aestuarianus* DNA at time zero, using a Taqman PCR assay [29].

2.2. Bacterial strains and culture conditions

In order to improve virulence towards *C. gigas* for the infection trials and to follow a reproducible experimental procedure [14], the two virulent stains *Vibrio splendidus* LGP32-GFP [30] and *V. aestuarianus* 02/041 [7] were used together in this study. Both strains were cultured in Marine Broth (Difco), prepared as follows: peptone 4 %, yeast extract 1 % diluted in Sterile Artificial Sea Water (SASW 2.3 % NaCl, 20 mM KCl, 5 mM $MgSO_4$, 2 mM $CaCl_2$). Bacteria were grown at 20 °C for 20 h under constant shaking. The bacterial culture concentrations were evaluated spectrometrically to obtain an optical density (OD) at 600 nm wavelength. Cells were centrifuged at 3200 g for 10 min and the resulting pellet resuspended in SASW to an OD of two, corresponding to 2 10^9 CFU ml^{-1}. A mix of both bacterial cells (v/v) was prepared for individual injection.
Both strains, LGP32 [31] and 02/041 (unpublished results) have been completely sequenced.

2.3. Experimental infection by intramuscular injection followed by cohabitation

Oysters were anaesthetised for one to two hours at 20 °C in a solution of magnesium chloride (28 g l^{-1} $MgCl_2$), with added phytoplankton (*Isochrysis galbata* and *Chaetoceros calcitrans*) and aeration to improve anaesthetic efficiency (personal obsevations). Then, 100 µl of a mix of virulent *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041 bacterial suspensions in equal doses of 10^8 CFU ml^{-1}, prepared as described in Saulnier *et al.* [14], were injected into the adductor muscle of oysters. Another group of oysters were injected with SASW (mock infection). After injection, oysters were transferred to tanks filled with 1 µm-filtered seawater at 3.1 % salinity, maintained under static conditions at 20 °C with aeration. Twenty-four hours after these injections, the surviving animals were sacrificed, removing one valve by severing their adductor muscle. The other valve and flesh were used for cohabitation treatments of healthy oysters. Cohabitation was designated as either "control" or "virulent *Vibrio*". Under both conditions, a ratio of five healthy oysters to one injected was used and total initial density was 5 ind. l^{-1}. For the control, 30 healthy oysters were put in cohabitation with sacrificed, mock infected, SASW injected oysters. For the "virulent *Vibrio*" treatment, 200 healthy oysters (referred to as *Vibrio* infected oysters) were put in cohabitation with sacrificed *V. splendidus*- and *V. aestuarianus*-injected oysters. In both cases the injected oysters also harboured a natural commensal *Vibrio* flora [3]. After 48 hours of cohabitation, all injected oysters were removed and the tank seawater was replaced with 1 µm-filtered UV-treated seawater at 3.1 % salinity. All experiments were performed under static water conditions at 20 °C with aeration, but no supplementary food. During the cohabitation and post-cohabitation periods, groups of oysters were sampled from the main cohabitation tanks and used for mortality monitoring, bacterial assessments and gene expression study. These samples were taken at 2 h, 6 h, 8 h, 24 h and 48 h of cohabitation and at days 7 and 19 of the post-cohabitation period. At each sampling, ten oysters from the virulent *Vibrio* treatment were randomly selected for mortality monitoring, rinsed with UV-treated seawater and transferred into individual small aquaria containing UV-treated seawater.

2.4. Hemolymph, oyster soft tissues and water sampling

Oysters are surrounded by waterborne microorganisms. To circumvent or at least minimize contamination during tissue sampling, hemolymph withdrawal from the adductor muscle was performed immediately after removing seawater from the palleal cavity, using a syringe fitted with a 23-gauge needle. Individual samples of hemolymph were collected from live oysters from both the control (n = 3 control oysters) and the virulent *Vibrio* tanks (n = 9 *Vibrio* infected oysters), at the different sampling times during the cohabitation and post cohabitation phases of the experiment. The flesh of each oyster was severed from its shell using a scalpel and stored individually at -20 °C. One ml of seawater was also collected from each tank at each time point (2 h, 6 h, 8 h, 24 h and 48 h for cohabitation; days 7 and 19 post-cohabitation) and stored at -80 °C.

2.5. Bacterial quantification by real-time PCR

2.5.1. *DNA extraction procedures*

Individual samples of hemolymph (200 µl) and tank seawater (500 µl) were centrifuged at 10 000 g for 5 minutes. DNA was extracted from cell pellets using QIAmp DNA Mini Kit (Qiagen), following manufacturer's instructions. The same DNA extraction procedure was followed using 20 mg of oyster tissues collected from different separated organs. Pelleted

DNA was resuspended in 200 µl ultra pure water and DNA yields and their purity determined spectrometrically (Eppendorf). DNA extracts were stored at -20 °C until use.

2.5.2. *Real-time PCR assay analyses*

Three real-time PCR assays were used for the specific detection and quantification of the *V. splendidus* LGP32-GFP strain, for bacteria related to *V. aestuarianus* and the *Vibrio* genus overall, targeting the *GFP, dnaJ* and *16S rRNA* genes, respectively. The GFP-qPCR test was developed for this study using Taqman chemistry and the same PCR conditions as those used for *V. aestuarianus* qPCR detection [29]. The sequences of oligonucleotides and Taqman probes used here are shown in Table 1. They were assessed for species-specificity using a BLAST search to determine homology with known sequences. For the detection and quantification of bacteria related to the *Vibrio* genus, we used the qPCR protocol described by Thompson *et al*. [32], with slight modifications for the quantification of amplicons by SYBR green chemistry. Each PCR assay was run in duplicate in a final volume of 25 µl containing 5 µl DNA sample, 200 nM of each primer (Table 1) and 12.5 µl 2× Brilliant II SYBR® Green QPCR Master Mix (Stratagene). Thermal cycling consisted of an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing and extension at 65 °C for 30 s. Lastly, the amplicon melting temperature curve was analysed over one cycle at 95 °C for 30 s, 55 °C for 30 s and 95 °C for 30 s. A real-time PCR assay was conducted on a MX3000 Thermocycler (Stratagene) and fluorescence intensity was expressed in delta reporters (dR) after background subtraction. The threshold was set using an amplification based algorithm from the MX3000 software (Stratagene) for the initial plate. For the other plates we used an interplate calibrator to set the threshold manually. Threshold Cycle (Ct) value corresponded to the PCR cycle number at which an increase in reporter fluorescence above the baseline signal was first detected.

Standard curves for the detection of LGP32-GFP and bacteria related to the *Vibrio* genus were established using 1:10 serial dilutions of DNA extracted from *V. splendidus* LGP32-GFP strain and *V. aestuarianus* 02/041 strain. All DNA concentrations used for *Vibrio* genus quantification were artificially spiked in DNA extracted from 10^6 cells ml^{-1} *Photobacterium phosphoreum* reference strain. The quantification of bacterial cells by real-time PCR was compared with the enumeration of the bacterial cells used for DNA extractions, using a *Malassez* counting chamber under a light microscope.

2.6. Analysis of gene expression in hemolymph by real-time PCR

2.6.1. *Hemocyte RNA extraction procedures*

Total RNA was isolated from individual hemolymph samples (600 µl) and centrifuged at 800 g for 5 min using a commercial RNA purification kit (RNeasy Mini Kit, Qiagen) including a DNAse treatment. RNA concentrations in each sample were estimated using the Quant-It™ Ribogreen® reagent and RNA standard curves (Stratagene), reading the plates on a Stratagene MX3000 thermal cycler. The quality of extracted RNA was checked by agarose gel electrophoresis using 20 randomly selected RNA samples and verified to be free of DNA contamination by performing a no reverse transcription assay in real time qPCR expression analysis (see paragraph below).

2.6.2. *Real-time PCR analysis of gene expression*

The expression levels of four different mRNA transcripts, *Cg*-BPI, *Cg*-EcSOD, *Cg*-I B and *Cg*-TIMP, were determined by real-time PCR on a MX3000 Thermocycler (Stratagene). An elongation factor encoded by the *ef I* gene was used to provide a steady-state expression

level of a house-keeping gene, allowing an internal control for gene expression as described in previous work done with the mollusks *C. gigas* and *Mya arenaria* [20-21, 23, 30, 33-34]. Approximately 100 ng of individually-isolated RNA transcripts were reverse-transcribed in a final volume of 20 µl, using an optimized blend of oligo-dT and random primers (Qiagen) and a mix of two reverse transcriptases (Quantiscript Reverse Transcriptase, Qiagen) according to the manufacturer's instructions (Quantitect Reverse Transcription Kit, Qiagen). cDNAs were amplified by real-time PCR using specific primers designed by Primer 3 software, choosing general conditions of 60 +/-1 °C optimal annealing temperature for pairs of oligonucleotides, and an amplicon size under 300 bp (Table 2). The cDNA conserved regions for primer design were deduced from GenBank-available targeted sequence alignment. The primers were checked for specificity using a BLAST search to determine homology with known sequences. Two to three primer pairs per gene were tested for their efficacy and specificity in real-time PCR, leading to the selection of a primer pair for the subsequent gene expression analysis done in this study. The real-time PCR assay was performed in duplicate in a total volume of 25 µL, using the Brilliant SYBR Green QPCR master mix (Stratagene), with 200 nM of each primer. The thermal cycle was performed with a two-step PCR: one cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s, and ending with one cycle at 95 °C for 60 s, 60 °C for 30 s and 95 °C for 30 s, aimed at melting temperature curve analysis. All fluorescence thresholds for the first and consecutive PCR plates were set as indicated above for bacteria quantification by qPCR, using cDNAs prepared from the pool of hemocytes collected at time zero as an interplate calibrator. PCR efficiencies ($E = 10^{(-1/slope)}$) and linear regressions were calculated by drawing standard curves from a serial dilution analysis of these cDNAs, testing each dilution in triplicate.

The calculation of relative mRNA levels was based on the comparative Ct method [35] modified by Pfaffl [36]. The fold change Qr in the target gene, normalized to the *ef I* gene and relative to expression at time zero using a pool of 11 cDNAs obtained from 11 hemolymph samples collected at T0 (just before the beginning of cohabitation trial) was expressed for each sample as $Qr = [(E_{target})^{\Delta Ct_{target}(\text{pool T0} - \text{sample Tx})}]/[(E_{efl})^{\Delta Ct_{efl}(\text{pool T0} - \text{sample Tx})}]$.

This calculation was performed on all individual samples of cDNAs collected from both control oysters (n = 3 at each time point) and *Vibrio*-infected oysters (n = 9 at each time point).

2.7. Statistical analysis

To analyse the degree of linear relationships i) between virulent *Vibrio* quantifications in tank water and in hemolymph of individual oysters and ii) between total *Vibrio* population quantifications in tank water and in hemolymph of individual oysters, at the same time point, Pearson's correlations were calculated (XLSTAT-Pro). Difference in *ef I* Ct individual means between control and virulent *Vibrio* treatments was tested using a Bonferroni test [20]. The ratio changes (mean Qr control/mean Qr *Vibrio*) of defense-associated gene transcripts were tested for significant differences between control and virulent *Vibrio* oysters at the same point in time during the experiment and between two consecutive sampling times for the same batch of oysters, using unpaired Student's t tests (XLSTAT-Pro). A Pearson's correlation test was performed in order to evaluate for each virulent *Vibrio* infected oysters a relationship between virulent *Vibrio* load in hemolymph and the ratio changes (Qr *Vibrio*) of each targeted defense-associated gene transcripts.

3. Results

3.1. Oyster mortality monitoring

No mortality of control oysters occurred during experimentation. In the virulent *Vibrio* cohabitation tank, in contrast, dead oysters were removed daily. Mortality was monitored in the batches of ten randomly-chosen oysters taken from the virulent *Vibrio* tank at the different sampling times during and post cohabitation. These oysters were individualized in order to avoid potential post cohabitation infection by neighboring dying oysters. Similar results were observed at all time points (data not shown): oyster mortality started and peaked at day two, and ended at day 16. After day 16, there was no more mortality until the end of the survey at day 25. Two hours of cohabitation was sufficient to cause significant mortality in test oysters, with 5 dead oysters out of 10 at day 14. Overall, 27 % of the 70 animals transferred in individual aquaria died during the 16 days of mortality that occurred during this trial.

3.2. Detection and quantification of the total Vibrio population and the two virulent Vibrio strains

Specific PCR primers and a Taqman probe were designed in order to provide relevant diagnostic tools for this study. These tools can be used to target and quantify the gene encoding for the Green Fluorescent Protein of a mutated LGP32-GFP strain, which is related to *V. splendidus* and pathogenic for *C. gigas*. Linear values for PCR amplification were obtained for 1:10 dilutions of purified LGP32-GFP DNA corresponding to $1.2\ 10^3$ to $1.2\ 10^9$ cells of LGP32-GFP ml^{-1}, with 0.993 correlation and 99.5 % PCR efficacy (Fig. 1). Size of amplified PCR products was verified by visualizing the expected 194-bp long amplicon (not shown) after agarose gel electrophoresis.

Similarly, the PCR primers described by Thompson *et al*. [32] to quantify the total *Vibrio* population were evaluated using real-time PCR based on SYBR Green chemistry. The same dilution ranges of LGP32-GFP DNA and purified *V. aestuarianus* 02/041 DNA as above were performed in DNA extracted from 10^6 cells ml^{-1} cultures of a *P. phosphoreum* reference strain. Dissociation curve analysis revealed that a single amplicon was obtained for all dilutions, with melting temperatures of 81.9 °C and 82.5 °C for LGP32-GFP and 02/041 strains respectively. Both quantification curves were similar, exhibiting correlations of r^2 = 0.997 and 0.976 and PCR reaction efficacies of 99 % and 96 %, respectively, when dilutions of LGP32-GFP and 02/041 strains ranged from $1.1\ 10^8$ to $1.1\ 10^2$ cells ml^{-1} (data not shown). Adding exogenous DNA from a phylogenetically-close bacterial strain unrelated to the *Vibrio* genus had no influence on PCR-cycle threshold values, regardless of the LGP32-GFP and 02/041 DNA concentrations used. Treatment of Ct values collectively yielded a single quantification curve with 0.992 correlation and 95 % PCR efficacy (Fig. 2).

The quantification of the total *Vibrio* population in the tank water used in cohabitation trials revealed high concentrations of $0.9\ 10^4$ and $1.1\ 10^4$ cells ml^{-1} in control and virulent *Vibrio* treatments respectively at time zero (Fig. 3A). This population increased by a factor of two in the control tank as early as two hours from the start of cohabitation, and later stabilized at approximately $3\ 10^4$ cells ml^{-1}, strongly suggesting that the total *Vibrio* population issued from the injected sacrificed oysters was released into the tank seawater. Bacterial increase reached a factor of 20 in the virulent *Vibrio* tank seawater and later stabilized at approximately $3\ 10^5$ cells ml^{-1}. Individual assessments of the bacterial strains used showed both LGP32-GFP and 02/041 at high concentrations in the tank seawater two hours following the start of the cohabitation challenge, at $3\ 10^3$ and $2\ 10^4$ cells ml^{-1}, respectively. A slight decrease or increase depending on strain was then observed in the water between 8 and 24

hours post cohabitation. After having replaced the tank seawater at the end of cohabitation time (48 h), a significant decrease in the total *Vibrio* population was noticed at day seven in both tanks. At this time, LGP32-GFP was nearly undetectable, whereas 02/041 could still be found in the tank water.

Mean total *Vibrio* population quantification in the hemolymph of oysters from the control tank increased from $2.6 \pm 1\ 10^4$ to $1.7 \pm 2.9\ 10^5$ cells ml^{-1} in the first two hours of cohabitation and at six hours (Fig. 3B), already returned to lower value of 10^4 cells ml^{-1}. In contrast, virulent *Vibrio* were found to infect almost all of the tested oysters very quickly, with concentrations in the hemolymph increasing from a undetectable level at time zero, to $5 \pm 8.9\ 10^2$ or $7 \pm 6.8\ 10^3$ cells ml^{-1} for *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041, respectively, after only two hours of cohabitation. Furthermore, virulent *Vibrio* were detected at concentrations ranging from $3.6\ 10^2$ to $1.04\ 10^6$ cells g^{-1} in the different oyster organs that were tested separately, including gills, mantle, labial palps and digestive gland. After a slight increase of *V. aestuarianus* 02/041 and decrease of *V. splendidus* LGP32-GFP strains in oyster hemolymph during the 48 hours of cohabitation, all tested animals were found to be negative for *V. splendidus* LGP32-GFP at day seven and for both strains by day 19.
A positive correlation (Pearson's $r = 0.745$, $p = 0.005$) was found between the mean logarithmic load of total *Vibrio* in the hemolymph (n=13) and in the tank seawater (n=13) sampled from T0 to day seven. A similar positive correlation (Pearson's $r = 0.445$, $p = 0.004$) was found between 02/041 and LGP32-GFP, analyzing hemolymph taken from 40 individual oysters sampled during the first 48 h of cohabitation.

3.3. Monitoring the expression of genes involved in defense reactions

The expression of four selected genes, known from the literature to be involved in defense reactions mediated by hemocytes, was analyzed by real-time PCR to examine their pattern of expression during the course of the trial in control or *Vibrio* infected oysters. In a preliminary step, the efficacy of qPCR for all targeted genes, including the elongation factor gene and its ability to generate a single qPCR amplicon, were evaluated using a 1:5 serial dilution of a pool of cDNA transcripts obtained from the 11 samples of hemolymph collected at time zero and dissociation curve analysis. Efficiencies for *Cg*-BPI, *Cg*-EcSOD, *Cg*-IκB, *Cg*-TIMP and *ef I* genes were 104.7 %, 102.3 %, 105.6 %, 105.6 % and 95.9 %, respectively. For all genes studied, the square regression correlation coefficient of detection ranged between 0.95 and 0.99. Dissociation curve analysis revealed that a single amplicon was obtained for all genes studied, with melting temperatures of 81 °C, 80 °C, 82 °C, 82 °C and 76 °C respectively. The specificity of qPCR was further verified by agarose gel electrophoresis of qPCR products, visualising as expected one amplicon of the attempted size calculated upon the gene sequence data available on GenBank (not shown).
The detected amount of the housekeeping gene elongation factor mRNA did not show significant difference between control and *Vibrio*-infected animals (p-value > 0.05), with mean threshold values and standard errors of 18.8 ± 2.2 and 19.9 ± 1.8, respectively (n=48 individual RNA samples). The relative expression ratio of immune genes could thus be estimated by normalizing against the *ef I* gene. A significant up-regulation of the four targeted genes was observed after normalization, especially for *Cg*-EcSOD, in each of the three oysters from the control tank two hours after cohabitation (Fig. 4). Transcript levels for *Cg*-TIMP, *Cg*-BPI, *Cg*-IκB and *Cg*-EcSOD genes were 6.6, 9.7, 15.9 and 78.5 fold greater, respectively, than their expression at time zero. In contrast, the mean relative transcript levels of genes from the nine *Vibrio*-infected oysters were approximately ten fold lower compared to expression in control animals, with values ranging from 0.11 (corresponding to

a 9.2-fold down-regulation) for *Cg*-TIMP gene to 0.03 (corresponding to a 29-fold down-regulation) for the *Cg*-BPI gene. Thereafter, no significant differences were observed between control and virulent *Vibrio*-infected oysters (Fig. 4). Noticeably, low levels of *Cg*-TIMP transcripts were reported in both treatments at 24 h and 48 h post-cohabitation, with a decrease in values ranging from 0.25 to 0.13 (corresponding to a down-regulation from a factor of 4 to 7.7). Whatever the considered defense-associated gene tested, no correlation (Pearson's $p > 0.05$) was found between the logarithm load of each virulent *Vibrio* and the ratio changes (Qr *Vibrio*) of transcripts, analyzing hemolymph taken from 40 individual oysters sampled during the first 48 h of cohabitation.

4. Discussion

Oyster production represents the majority of the aquaculture industry in France, with the dominant species *C. gigas* grown in a near monoculture. This situation has arisen from the evolution of the French oyster industry from traditional harvesting of wild beds to very intensive cultures. The high rearing densities of French *C. gigas* cultures are favored by the settlement behavior of oysters occurring when swimming larvae reach metamorphosis. In addition, the presence of large populations of wild oysters and other filter-feeding mollusks leads to high trophic competition, which may pose difficulties for the management of rearing sites and impair oyster health status [27]. Indeed, exceptional mortality rates of 40 % have been regularly reported in young cultured *C. gigas* oysters over the past decade or more, occurring mainly during spring and summer seasons in France. Classically, the professional oyster breeders cope with these mortalities by increasing the numbers of these, less costly, young oysters (*i.e.* spat) in their rearing systems. Amongst pathogens found in close association with these mortality events reported in France, two *Vibrio* species, *V. splendidus* and *V. aestuarianus*, were found (saulnier et al, 2010). We therefore focused our study on cohabitation experiments using these *Vibrio* species, purposely choosing a non-invasive method of infection to explore the possibility of horizontal transfer of these *Vibrio* from diseased to healthy oysters. A pathogenesis study was undertaken to describe the pathway of virulent and avirulent *Vibrio*, quantifying them in oyster hemolymph and tank water, while quantifying transcripts of well-characterized hemocytic genes involved in host response, ranging from tissue damage repair to recognition and degradation of pathogenic bacteria.

Litle data is available on non-invasive experimental infection methods for mollusks, although experimental vibriosis was conducted by Travers et al [37] and Gay et al. [8] using balneation and cohabitation procedures in abalone *Haliotis tuberculata* and *C. gigas* respectively. Indeed, experimental vibriosis was recently induced in *C. gigas* infected by bath challenge using only mature and spawning animals (De Decker et al, 2010). Nevertheless this procedure exhibit poor repeatability in mortality rates. The same authors highlighted the importance of sexual maturity for vibriosis infection processes. In the same way we cannot exclude also that *in vitro* cultivation of virulent bacterial strains using synthetic medium may change their virulence, an inconvenient that cohabitation procedure should avoid. Regarding oysters, other non-invasive experimental infection assays have been described using a virus (OsHV-1) in larvae [38], and parasites such as *Bonamia ostreae* and *Perkinsus* spp. [39-40]. The present article relates the development of the first experimental infection technique targeted at better understanding the early steps of pathogenesis events in adult *C. gigas*. We obtained the first evidence of successful experimental horizontal transfer of vibriosis in four independent preliminary cohabitation assays. Significant mortality rates were seen in oysters in the virulent *Vibrio* treatment, whereas no mortality was observed by contact of healthy oysters with oysters that had been injected with sterile artificial seawater injected (mock infected). All moribund animals collected and tested during these preliminary assays harbored a high concentration of the challenge *Vibrio* in their hemolymph, as shown by the

classic plate-counting method. This protocol aimed at mimicking oyster death caused in the wild by disease, predator attack, natural death or resulting from bad zootechnical practices.

The use of real-time PCR in microbial diagnostics is a valuable alternative to time-consuming traditional methods such as classical bacteriological analysis based on the cultivability of organisms. Although based on the detection of DNA and not on the culture of live organisms, this molecular biology-based technique offers multiple advantages during surveys. Notably, its specificity and sensitivity allow the detection and quantification of low concentrations of epidemiologically significant microbial agents in aquatic animals and their environment [29, 41-44]. In the present study, development and evaluation were made of a Taqman real-time PCR to detect a mutated *V. splendidus* LGP32 strain tagged with GFP, known to be pathogenic for *C. gigas* oyster by experimental injection. This particular strain was chosen to overcome the risk of detection and quantification of other strains of avirulent *V. splendidus* present in the background flora of oysters [8, 14].

Our technique successfully produced a rapid and heavy infection ($> 5\ 10^3$ cells ml^{-1}) in all test oysters in the virulent *Vibrio* treatment. *Vibrio* from the injected oysters were detected in the hemolymph and organs of the healthy test oysters after just two hours of cohabitation. At this time an approximate ten-fold increase of the total *Vibrio* population was also observed in the hemolymph of healthy oysters in the control treatment in cohabitation with mock-infected specimens coinciding with its slight increase in control tank water. Because no mortality was observed in these control oysters suffering an increase of *Vibrio* population in their hemolymph, we considered that oysters experienced a challenge with avirulent *Vibrio*. Strong individual variations in bacterial load were also reported during the first 48 h of the post cohabitation challenge, suggesting that individual oysters have different susceptibility levels to vibriosis under this mode of infection. Such variability could be related, in part, to the high genetic variability of this mollusk species [45]. Nevertheless the level of *V. aestuarianus* infection was highly correlated ($p < 0.01$) with that of *V. splendidus* LGP32-GFP, strengthening the hypothesis of a potential inter-strain collaboration previously suggested after experimental injection challenges [14]. Our study of the dynamics of detection of *Vibrio* DNA in tank water revealed that avirulent and virulent *Vibrio* were both transmitted horizontally *via* a waterborne mode of action, probably facilitated by the open circulatory system of this mollusk species as well as its filter feeding. Importantly, *Vibrio* bacterial loads were found to be correlated between the hemolymph of control oysters and the tank water ($p < 0.01$). It is also notable that 7 and 19 days after the 48 h cohabitation trial, no detectable levels of *V. splendidus* LGP32-GFP or *V. aestuarianus* DNA, respectively, were found in the hemolymph of survivors of the virulent *Vibrio* cohabitation, supporting the hypothesis that some oysters succeeded in recovering from infection due to efficient defense mechanisms, even if potential presence of *Vibrio* in other tissues or organs cannot be excluded. The contrasting level of natural *Vibrio* flora supports the hypothesis that the oyster immune system might distinguish between virulent and avirulent, potentially beneficial, *Vibrio* species. Natural background *Vibrio* persisted at a level of $1\ 10^4$ cells ml^{-1} during this 19-day survey, despite the use of UV-treated seawater. A qualitative characterization of this flora by classical bacteriological methods, coupled with metagenomics or by other molecular typing approaches will open new perspectives for a better comprehension of the mechanisms involved in defense reactions, leading to either tolerance or destruction of invading bacteria [46-47].

In an attempt to characterize host-*Vibrio* interactions, the second aim of our study was to evaluate the kinetics of immune-related gene expression in the oyster *C. gigas* in response to virulent or avirulent *Vibrio* sp.. Our non-invasive experimental infection technique aimed at reproducing horizontal transfer of vibriosis to healthy oysters. For mollusk bivalves and *C. gigas* in particular, most studies reported in the literature have used a form of immune

stimulation based on a treatment by inactivated microorganisms, mainly *Micrococcus luteus*, *V. splendidus*, *V. anguillarum*, *V. alginolyticus*, *V. tubiashii*, *V. metschnikovii* [16, 23, 48-49], or microbial components such as lipopolysaccharides poly I:C, poly G:C and dsDNA [48, 50-51], with the main purpose of confirming the involvement of newly identified putative immune genes in defense reactions and studying them at a transcriptional level. These studies and some others used live bacteria, which were mostly harmless strains of *Micrococcus luteus*, *V. alginolyticus*, *V. tasmaniensis*, *V. anguillarum*, *V. splendidus, V. tubiashii* and *V. metschnikovii* [20, 23-24, 33, 52-53]. To our knowledge, *V. aestuarianus* is the only virulent organism tested in juvenile *C. gigas* that has been seen to induce a significant down-expression of *Cg*-EcSOD transcripts by 3.6 and 8.3 fold, on days one and three post injection challenge, respectively, compared with controls [21]. In this last experiment, relative *Cg-TIMP* expression remained stable for the duration of the trial in both *Vibrio*-injected and control animals [21]. Nevertheless, the experimental infection procedure used remains problematic because the intramuscular injection of pathogen at high dose ($5\ 10^7$ CFU oyster^{-1}) could have both short-circuited some potentially important first line immune defenses or activation systems and disturbed normal host immune response against undesirable invaders. In this study, live virulent bacteria were used for the first time in a non-invasive challenge method for *C. gigas* immune gene expression monitoring. The same individual hemolymph samples used for the *Vibrio* kinetics investigation were also used for RNA extractions and real-time PCR quantification of four immune-related target genes and a housekeeping control gene extensively used in previous studies [20-21, 23, 33, 52, 54-55]. Our results on control animals are in agreement with those reported in the above studies: the use of non-pathogenic bacterial strains induced an up-expression of most of the studied immune genes. In this study, an increase of the total *Vibrio* population found in control animals after two hours of cohabitation coincided with a significant up-regulation of the four targeted genes *Cg*-BPI, *Cg*-EcSOD, *Cg*-IκB and *Cg*-TIMP ($p < 0.001$), with expression value increases ranging from 6.6 to 78.5 fold depending on the targeted gene. This significant up-expression was remarkable for its intensity in comparison with data reported in the literature [16, 18, 19, 21] but only transient, since it was not observed after six hours post cohabitation. Conversely, despite the variability recorded in individual response, all targeted transcripts tended to be down-regulated at a later stage, after being normalized. The use of virulent *Vibrio* species *i.e. V. splendidus* and *V. aestuarianus* caused a significant ($p < 0.001$) decrease of expression levels in all studied genes, ranging from a 9.2 to a 29-fold decline, depending on the gene, compared to expression levels in control animals, two hours after the start of cohabitation. These results suggest that virulent *Vibrio* highly disturbed the immune response, which might be understood as a strategy of pathogenic *Vibrio*, aimed at enhancing their colonization skills into the *C. gigas* host. Nevertheless, this apparent immunodepression, occurring less than six hours after the start of the cohabitation challenge, was short-term and no significant differences ($p > 0.05$) were later observed when comparing levels of each transcript in control and infected oysters at the same sampling time, with the noticeable exception of *Cg*-IκB gene at 48 h. None of the expression levels of the targeted genes were correlated with *V. splendidus* and *V. aetuarianus* bacteremia in this study, suggesting that expression of these genes is not correlated withvibriosis resistance.

5. Conclusion

We present a preliminary study on *Vibrio* infection levels and expression patterns of immune-related genes following cohabitation of *C. gigas* with oysters harboring commensal or virulent *Vibrio*. Our results show that in contrast to avirulent *Vibrio*, which induces significant up-expression of four targeted immune related genes, virulent *Vibrio* depress the immune response of this invertebrate host both rapidly and significantly. This disturbance is an early and transient state occurring during the first six hours challenge with pathogenic *Vibrio.* The original horizontal method mimicks natural vibriosis and the development of molecular tools

designed to characterize pathogenesis open up possibilities for identifying new genes involved in disease resistance and virulence of *Vibrio*. Research could be developed in this direction combining our new non-invasive infection method with other high-throughput methods such as microarrays. Such approaches would permit the simultaneous analysis of expression profiles of multiple *C. gigas* and virulent bacterial genes made available by expressed sequenced tag approaches [56] and whole genome sequencing of *Vibrio* species pathogenic for oysters.

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Tables

Table 1

Nucleotide sequences, melting temperature (Tm) and qPCR efficiency of primers and probes used in Taqman and SYBR Green real-time PCR assays.

Table 1

Name	Sequence (5′–3′) and flurochromes	qPCR efficiency (%)	Tm (°C)	Ref.
GFP-F	TCAGTGGAGAGGGTGAAGGT	99.5	60	This study
GFP-R	TACATAACCTTCGGGCATGG		61	This study
GFP probe	Cy5- TGTTCCATGGCCAACACTTGTCA -BHQ1		68	This study
DNAj aes F1	GTATGAAATTTTAACTGACCCACAA	98	58	(Saulnier et al, 2009)
DNAj aes R1	CAATTTCTTTCGAACAACCAC		59	(Saulnier et al, 2009)
DNAj probe	Texas Red-TGGTAGCGCAGACTTCGGCGAC-BHQ2		71	(Saulnier et al, 2009)
567F	GGCGTAAAGCGCATGCAGGT	95	64	(Thompson et al., 2004)
680R	GAAATTCTACCCCCCTCTACAG		66	(Thompson et al., 2004)

Table 2

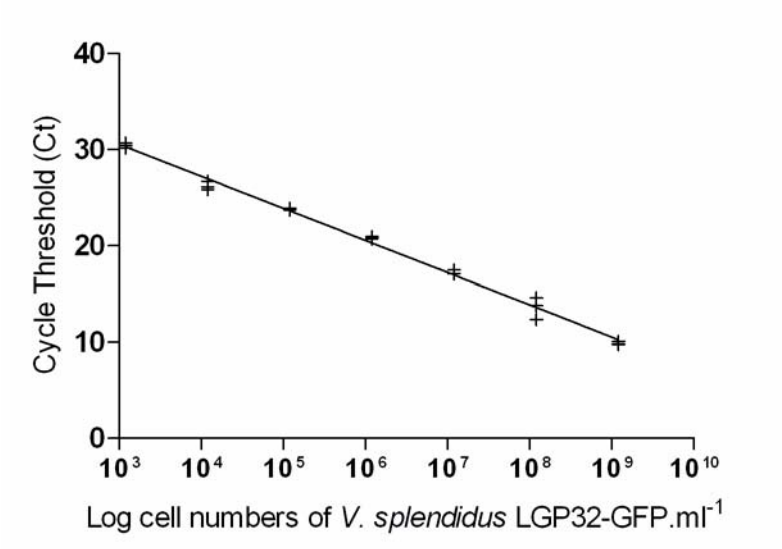
Primers paired used and amplification efficiency of primer sets in real time qPCR expression analysis.

Table 2

Targeted Genes	Primers name	Oligonucleotide sequences (5' – 3')	qPCR efficiency (%)	Amplicon length (bp)
Cg-ef1	EFF	AGTCACCAAGGCTGCACAGAAAG	95.9	199
	EFR	TCCGACGTATTTCTTTGCGATGT		
Cg-BPI	S-BPI1F	TGACGTCAGCCCAATGTAAG	104.7	200
	S-BPI1R	ACTGCTGGGCTTTGTGAACT		
Cg-EcSOD	S-SOD2F	CGAGATGGAACCAAACCACT	102.3	124
	S-SOD2R	GACGTTGAAACCGGTCAGAT		
Cg-IκB	S-IKB3F	TCCAGACCCCTCTTCATTTG	105.6	152
	S-IKB3R	CAGGGCGATATCGTCATACC		
Cg-TIMP	S-TIMP1F	TGGTGTTGACCGTTCTCTGT	105.6	227
	S-TIMP1R	AGAACCCAGCAGAGATGACC		

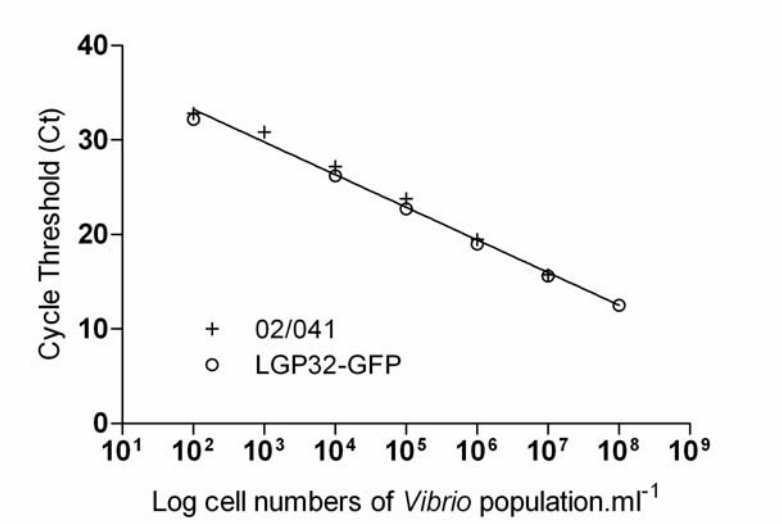
Figures

Fig. 1



Standard curve for *V. splendidus* LGP32-GFP real-time PCR in 1:10 serial dilutions of purified LGP32-GFP DNA, corresponding to $1.2\ 10^3$ to $1.2\ 10^9$ cells of LGP32-GFP ml^{-1}. Standard curves were generated by plotting the log cell number on the mean threshold cycle (Ct) value obtained from triplicate assays. The linear regression, represented here was obtained by collectively treating all the data; $r^2 = 0.993$; PCR reaction efficacy was 99.5%.

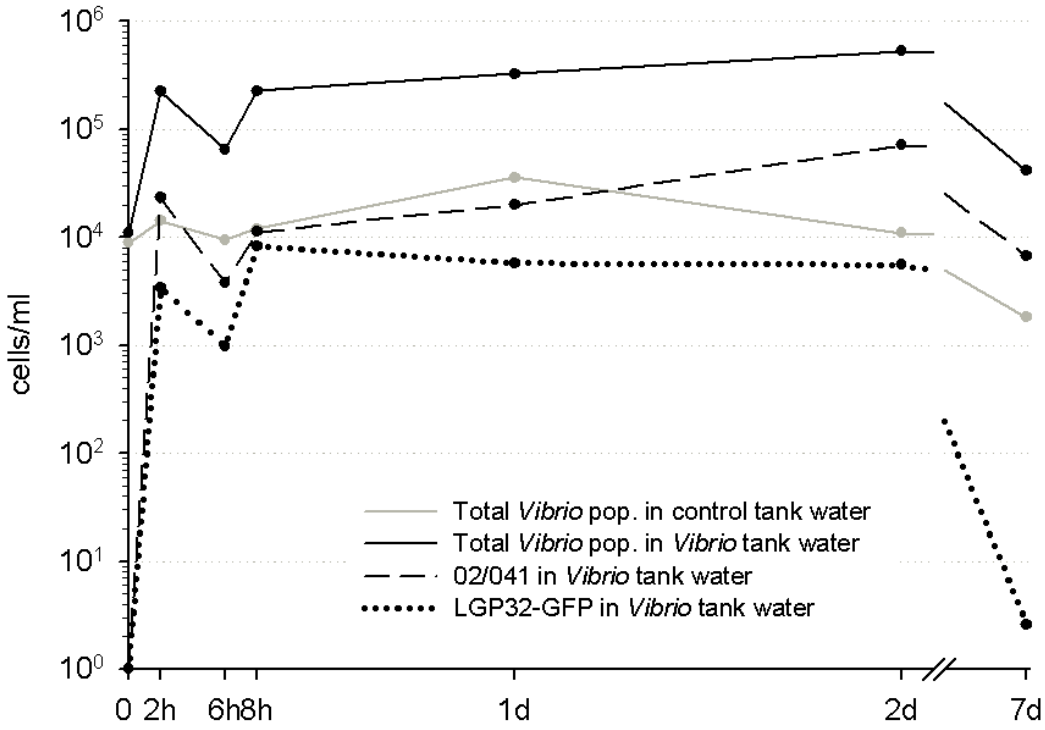
Fig. 2



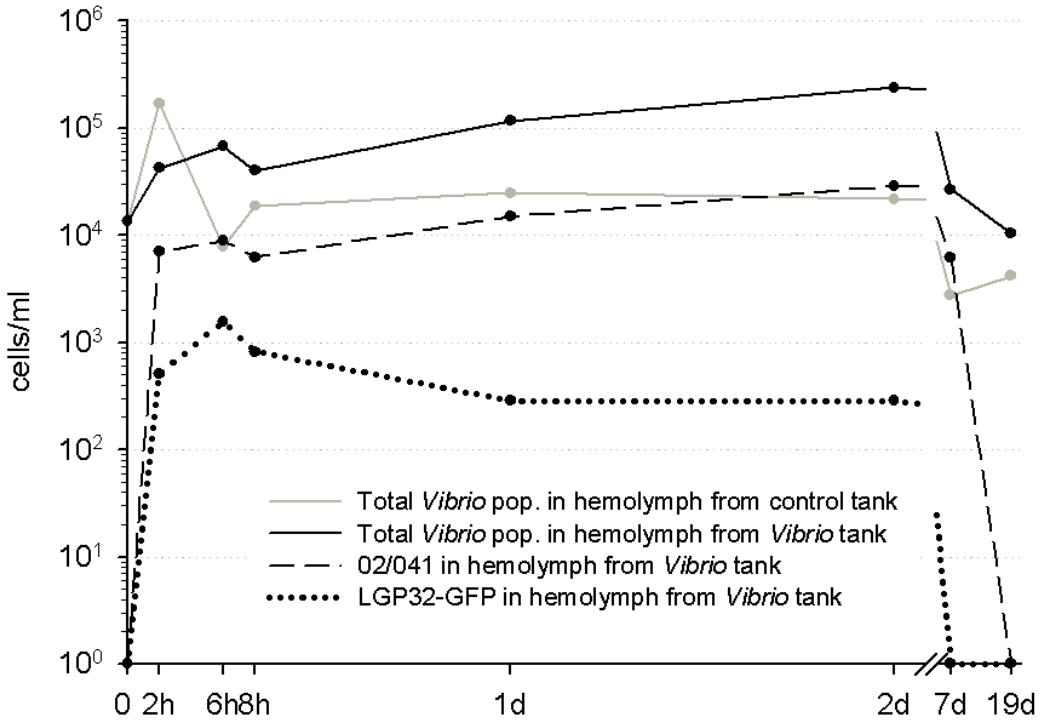
Standard curve for total *Vibrio* population real-time PCR in 1:10 dilutions of purified LGP32-GFP (+) and *V. aestuarianus* 02/041 DNA (○) done in DNA extracted from 10^6 cells ml^{-1} of *P. phosphoreum* reference strains. Standard curves were generated by plotting the log cell number versus the mean threshold cycle (Ct) value obtained from triplicate assays. A single linear regression, represented here, was obtained by collectively treating all the data; $r^2 = 0.992$; PCR reaction efficacy was 95 %.

Fig. 3

A

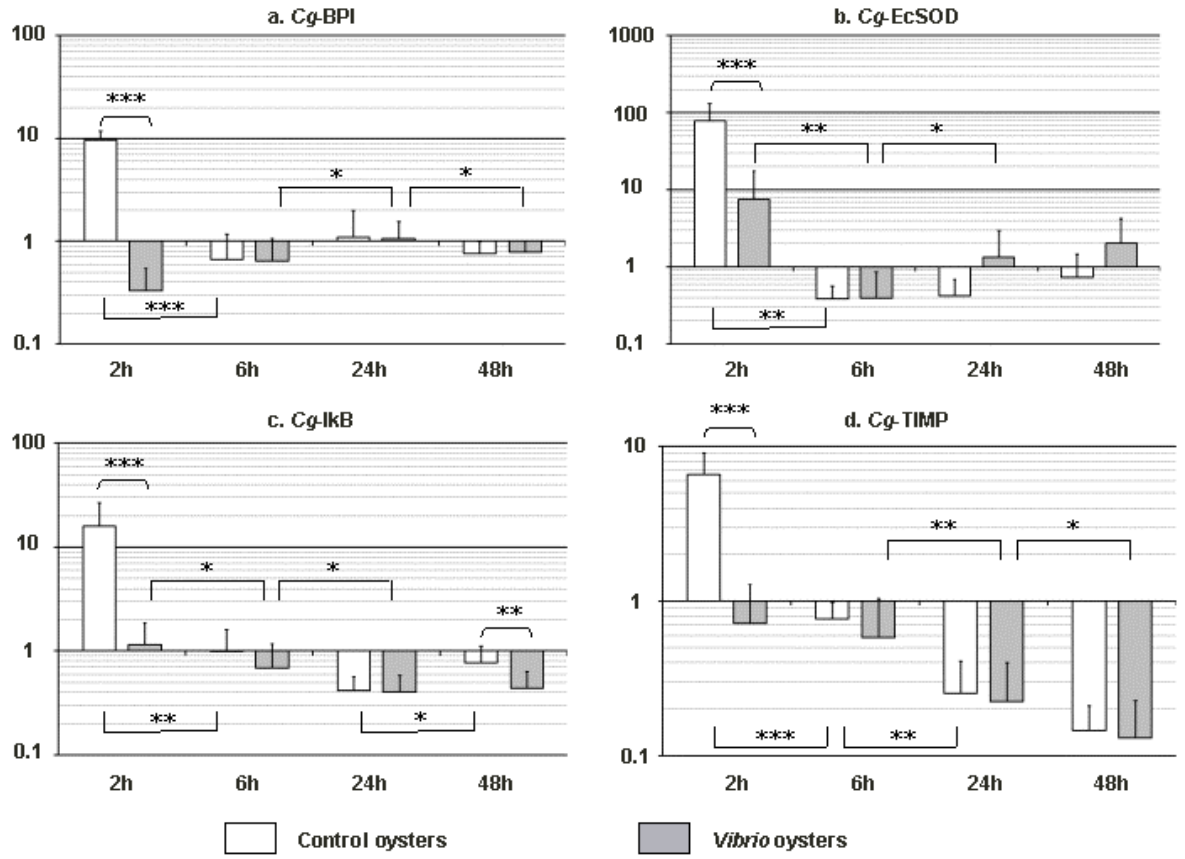

cells/ml
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0 2h 6h8h 1d 2d 7d
Total *Vibrio* pop. in control tank water
Total *Vibrio* pop. in *Vibrio* tank water
02/041 in *Vibrio* tank water
LGP32-GFP in *Vibrio* tank water

B


cells/ml
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0 2h 6h8h 1d 2d 7d 19d
Total *Vibrio* pop. in hemolymph from control tank
Total *Vibrio* pop. in hemolymph from *Vibrio* tank
02/041 in hemolymph from *Vibrio* tank
LGP32-GFP in hemolymph from *Vibrio* tank

Kinetics of the total *Vibrio* population and the two virulent LGP32-GFP & 02/041 *Vibrio* strains quantified by qPCR in seawater (A) or hemolymph (B) of both control and virulent *Vibrio* tanks from T0 to day 7. Limit of detection for total *Vibrio*, LGP32-GFP and 02/041 was 10^2 cells ml^{-1} for all the quantification assays corresponding to 2-3 cells/well. Mean values obtained from individual hemolymph samples collected from control oysters (n = 3 for each time point) and *Vibrio* infected oysters (n = 9 for each time point) were plotted. No LGP32-GFP or *V. aestuarianus* DNA was detected in any samples collected from the control tank.

Fig. 4



Kinetics of the mean (± SD) relative expression of a. *Cg*-BPI, b. *Cg*-EcSOD, c. *Cg*-IκB, d. *Cg*-TIMP in hemolymph sampled in control (n = 3) and *Vibrio* infected oysters (n = 9). Standard bars correspond to integrated technical and individual variability. Gene expression is normalized to the ef I gene and relative to expression at time zero. Asterisks indicate a significant difference in expression levels between control and *Vibrio* infected oysters or between two consecutive sampling times (*: $p<0.05$; **: $p<0.01$; ***: $p<0.0001$).