



# Expression of candidate genes related to metabolism, immunity and cellular stress during massive mortality in the American oyster *Crassostrea virginica* larvae in relation to biochemical and physiological parameters

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## ABSTRACT

Quantification of mRNA of genes related to metabolism, immunity and cellular stress was examined in relation to a massive mortality event during the culture of American oyster larvae, *Crassostrea virginica* which was probably, in regard to previous microbiological analysis, induced by *Vibrio* infection. To document molecular changes associated with the mortality event, mRNA levels were compared to biochemical and physiological data, previously described in a companion paper. Among the 18 genes studied, comparatively to the antibiotic control, 10 showed a lower relative gene expression when the massive mortality occurred. Six of them are presumed to be related to metabolism, corroborating the metabolic depression associated with the mortality event suggested by biochemical and physiological analyses. Relationships between the regulation of antioxidant enzyme activities, lipid peroxidation, and the mRNA abundance of genes linked to oxidative stress, cytoprotection, and immune response are also discussed. Finally, we observed an increase in the transcript abundance of two genes involved in apoptosis and cell regulation simultaneously with mortality, suggesting that these processes might be linked.

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## 1. Introduction

Massive mortality of bivalve larvae has often been associated with a low quality of water or of microalgae provided as food for aquaculture production, promoting the proliferation of opportunistic pathogenic microorganisms (Olafsen, 2001; Paillard et al., 2004). Many studies have been dedicated to the identification and characterization of pathogenic microorganisms to such mortalities but, to our knowledge, few physiological or molecular studies have yet been carried out to study larvae during such mortality events. This is indeed related to the

technical difficulties to study such rapid phenomenon on very small organisms. In addition to biochemical and physiological approach (Tikunov et al., 2010), recently developed genomic tools can now be used to better document larval responses. In the last decade, cDNA collections and expressed sequence tags (EST) have been developed for several bivalve species, and they account for the large number of RNA sequences available in public databases (Collin et al., 2010; Fleury et al., 2009; Jenny et al., 2007; Quilang et al., 2007; Sussarellu et al., 2010; Tanguy et al., 2008; Zapata et al., 2009). Such an increasing number of EST databases provide the opportunity to characterize bivalve mortality events and infections (Fleury et al., 2009; Huvet et al., 2004; Samain et al., 2007; Travers et al., 2010). In the American oyster, EST collections are being used to identify genes with putative functions in the cellular and biochemical processes implicated in the resistance to *Perkinsus marinus* (Tanguy et al., 2004; Wang et al., 2010).

To date, the majority of genomic approaches have been carried out on adult bivalves and little is known about gene regulation at larval stage. Jenny et al. (2002) produced two EST collections from embryos and hemocytes of the American oyster to identify genes that are potentially related to immune and stress responses. More recently, Tirape et al. (2007) studied the expression of immune-related genes during ontogeny and bacterial challenge in *Crassostrea gigas*.

**Abbreviations:** mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; EST, expressed sequence tags; MDH, malate dehydrogenase; NADH-1, nicotinamide adenine dinucleotide dehydrogenase subunit 1; CC-1, cytochrome C1; AS-6, adenosine triphosphate synthase f0 subunit6; EDL, endothelial lipase; D9-des, delta 9 desaturase; GS, glutamine synthetase; eIF-2B, translation initiator factor; CTB, cathepsin B; ANX, annexin; NK-rec, natural killer receptor; KC-rec, killer cell lectin-like receptor; MYC-h, MYC-homolog; SUP, RAS suppressor; PRDX6, peroxiredoxin 6; HSP70, heat shock protein 70; PRN, permin; CP450, cytochrome P450; RT-PCR, reverse transcriptase polymerase chain reaction; SOD, superoxide dismutase; GPX, glutathione peroxidase; ROS, reactive oxygen species.

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In this context, we selected 18 candidate genes, presumed to be involved in metabolism (energy production, protein synthesis, and lipid remodeling), immunity (pathogen recognition, proteinase, apoptosis, and inflammatory response) and cellular stress (antioxidant defenses, xenobiotic detoxification and cytoprotection). Relative expression of these 18 genes was studied by quantification of mRNA using real time PCR, during a mortality event in larvae of the American oyster *Crassostrea virginica* and compared with an antibiotic-treated control that showed no significant mortality. Results are discussed in the light of biochemical and physiological data reported in a companion paper (Genard et al., 2011).

## 2. Materials and methods

### 2.1. Experimental design and physiological characterization of a bacterial mortality event

Details about the rearing procedure and experimental design are presented in Genard et al. (2011). Briefly, this work was done at the hatchery of the Coastal Zone Research Institute (CRZI, Shippagan, New Brunswick, Canada) during winter of 2006. After fertilization, larvae were reared in a 415 L Xactic tank in filtered seawater. Larvae were fed with a mixture of three microalga species *Isochrysis sp.* (T-ISO), *Pavlova lutheri* (MONO), and *Chaetoceros muelleri* (CHGRA). Two days after fertilization, D-larvae were collected, enumerated and split equally into two experimental treatments. Larvae were cultivated in triplicate with or without antibiotic (chloramphenicol at 4 mg L<sup>-1</sup>; Fluka, Mississauga, ON). Larval samples (100,000 larvae per sample) were collected at 6, 13, and 20 dpf (days post fertilization).

Mortality event occurred 20 days after fertilization in untreated tanks (see Genard et al., 2011 for further details). Mortality was associated with (1) strong changes in the bacterial community structure, (2) a progressive decrease in feeding activity, (3) higher levels of some lipid classes (free fatty acids, diglycerides, and acetone mobile phospholipids), (4) lower levels of phospholipids and protein, (5) higher contents of non-methylene interrupted dienoic fatty acids (22:2 NMI), (6) a decrease in energy metabolism activity (citrate synthase and cytochrome oxidase activities), (7) a higher oxidative stress (lipid peroxidation level), and (8) an activation of antioxidant defenses before mortality (glutathione peroxidase and superoxide dismutase).

### 2.2. Real-time PCR

Total RNA was extracted for each replicate (between 2 and 3 depending on larval availability) using TRIzol™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was resuspended in RNase-free water and concentrations were determined by OD260 measurements. Ten micrograms of total RNA extracted from each developmental stage was reverse transcribed using the oligo(dT) anchor primer and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). Real-time PCR was performed with 5 µL cDNA (1/20 dilution) in a total volume of 25 µL, using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The concentrations of the reaction components were 1 × Absolute QPCR SYBR Green ROX Mix (ABgene, Epsom, UK) and 70 nM of each primer. Reactions began with the activation of Thermo-Start DNA polymerase at 95 °C for 15 min followed by amplification of the target cDNA (50 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 1 min), and melting curve analysis was performed with continuous fluorescence acquisition (95 °C to 70 °C at a temperature transition rate of 0.5 °C every 10 s) to determine the amplification specificity. Each run included a negative control (non reverse-transcribed total RNA) and blank controls (water) for each primer pair.

The relative level of target gene expression was based on a comparative method (Livak and Schmittgen, 2001; Pfaffl, 2001). The threshold value (Ct) was determined for each target as the number of cycles at which the fluorescence curve entered exponential phase. The relative quantification value of a sample is expressed as  $2^{-\Delta\Delta Ct}$ , where  $\Delta\Delta Ct = \Delta Ct$  (target sample) –  $\Delta Ct$  (reference sample) and  $\Delta Ct = Ct$  (target gene) –  $Ct$  (housekeeping gene). Three genes coding for a ribosomal protein subunit were selected as putative housekeeping genes. All of these reference genes were amplified at all stages and in the two treatments. Ribosomal 18s was found to have the lowest variation during the experiment and was then chosen as the housekeeping gene for the rest of the analyses. Larvae treated with antibiotics from each sampling day were used as reference samples to determine treatment differences.

### 2.3. Studied genes

Oligonucleotide primer sequences used to amplify specific gene products are shown in Table 1. Selected genes could be classified into four groups: metabolism, immunity, cell regulation, and cellular stress. To monitor the putative metabolic changes induced by mortality emergence, we characterized expression of four genes involved in ATP synthesis (malate dehydrogenase (*MDH*), NADH dehydrogenase subunit 1 (*NDH-1*), cytochrome C1 (*CC-1*) and ATP synthase f0 subunit 6 (*AS-6*)), two genes of lipids metabolism (endothelial lipase (*EDL*) and delta9 desaturase (*D9-des*)) and two of protein synthesis (glutamine synthetase (*GS*) and translation initiator factor eIF-2B delta subunit (*eIF-2b*)).

To investigate immune response and cell regulation, six genes (cathepsin B (*CTB*), annexin (*ANX*), natural killer receptor (*NK-rec*), killer cell lectin-like receptor (*KC-rec*), MYC-homolog (*MYC-h*) and RAS suppressor (*SUP*)) were selected from previous EST collections. Finally, cellular stress was investigated through expression of genes involved in antioxidant defense (peroxiredoxin 6 (*PRDX6*)), cytoprotection (heat shock protein 70 (*HSP70*) and pernin (*PRN*)) and xenobiotic detoxification (cytochrome P450 (*CP450*)).

### 2.4. Statistical analysis

Analyses were carried out using Software SAS® system (8.2). The significance value for all analyses was set at  $p < 0.05$ . PROC GLM (one-way ANOVA) was used to compare relative gene expression between treatments at each sampling days and to show ontogenetic effects on each treatment. Where differences were detected, LSMEANS (*t*-test) multiple comparisons tests were used to determine which means were significantly different. Residuals were graphically assessed for normality using the PROC PLOT function coupled with univariate procedure (PROC UNIVARIATE). Homogeneity was tested using the O'Brien test. When normality precept was not realized, LOG transformation was used. Linear regressions were carried out using Sigma plot 8.0 (SYSTAT Software Inc, USA). Data were presented with mean ± standard deviation.

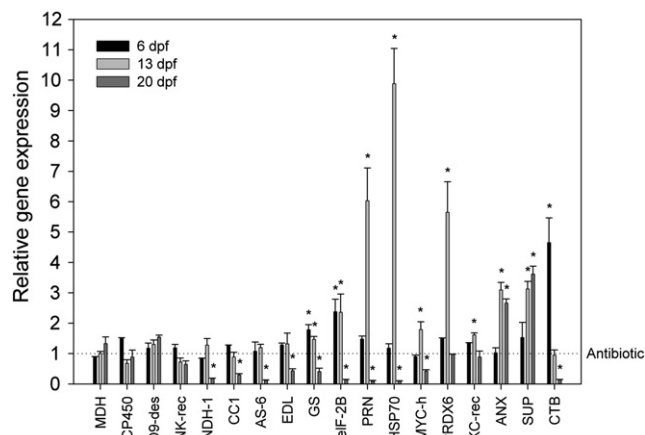
## 3. Results

RT-PCR was performed with RNA isolated from larvae of 6 to 20 dpf; transcripts were detected in all larval stages. Transcript levels were compared between treatments at each sampling day (using the antibiotic treatment as a reference sample), which led us to classify the 18 genes into six groups of response (Fig. 1). In the first group, which included *MDH*, *CP450*, *D9-des*, and *NK-rec*, no significant differences were found between treatments. *NDH-1*, *CC1*, *AS-6*, and *EDL* constituted the second group and were characterized by a significantly lower gene expression in untreated larvae at 20 dpf, when massive mortality occurred. The third group included the two genes involved in protein synthesis (*GS* and *eIF-2B*); it showed over-expression at 6

**Table 1**  
Details of genes and primers used in the quantitative real-time PCR relative expression analysis.

Genes	Label	Genbank ref.	Function	Forward primer (5'–3')	Reverse primer (5'–3')
Ribosomal protein 18s	R18s	CX069129	Putative housekeeping gene	GTCGTGTAATCCGATAACGAACGGAACCTCA	TGCTCAATCTCGTGTGGCTAAACGCAACTG
Malate dehydrogenase precursor	MDH	BG624325	Energy metabolism (acid citric cycle)	ACTGAAGTTGTGGAGGCTAAGGCT	ATTCTAGATTTTTCTCCACACC
NADH dehydrogenase subunit 1	NDH-1	CD648997	Energy metabolism (electron chain transport complex I)	TCGCGCGCTGCTTTCCACGGTT	TTACCGCACCAAGAGCTACGCAGC
Cytochrome C1	CC-1	CD526711	Energy metabolism (electron chain transport complex III)	CTTGCTCTTTTTTCAGACACATAGA	GCATTATCCAGACAGGTAGTCTTATGAG
ATP synthase f0 subunit 6	AS-6	EE677700	Energy metabolism (electron chain transport complex V)	ATGCCAAGCATGTTCTACAGAGT	GCAAAGGATCGCTCTACCAAAGC
Delta9 desaturase	D9-des	CX069227	Lipid metabolism (fatty acid desaturation)	TACTGTCTTCTGCTAAACGCCAC	GTCGTGATATTGAGGTGCCAGCC
Endothelial lipase precursor	EDL	CD647348	Lipid metabolism (phospholipase)	GCCACACCATGGGATACGCCGG	TGGCACCCCGTTGTCTCGTCCC
Glutamine synthetase	GS	CG1753	Protein metabolism (amino acid synthesis)	ACGGAGTTGACGGGACTT	GCTGGACCACGATTGG
Translation initiation factor eIF-2B delta subunit	eIF-2B	EE677861	Protein synthesis regulation (DNA translation)	GGCTGGTATCCCTTGCTCTA	CACITTAGTAGCCTCTTGCAATTGC
Cathepsin B	CTB	BG624471	Proteolysis, pathogen recognition	CCTACTCCATCAAGTCATGTGACCA	ATACCCAGTGAAGCGCCCTCT
NKR-P1B (natural killer receptor)	NK-rec	BG624594	Non-self recognition (lectin receptor family)	GCCTGATTAAGTGTCCCATGATGGTTT	GAGTGTCCAGTCATGGGCCACACCCA
Killer cell lectin-like receptor	KC-rec	BG624932	Non-self recognition (lectin receptor family)	GGTCTTGGCAGTTTCGGGTTTATAAC	TGGTCTCGGGAGACACCAATATGGCCT
Annexin	ANX	CD648336	Apoptose, inflammation response	GATAAGAATCATCGTCACTCGGGCCGA	AATTTTCACTTAAACTCCACATAC
Peroxiredoxin 6	PRDX6	CX069146	Oxidative stress (antioxidant enzyme)	GATGACGTCCCACTATGAGGGGTGGTC	TGGGGATGGAGGTAAGACCATACACT
Cytochrome P450	CP450	CD526847	Xenobiotic detoxification	GTGCATCAAAGAATTTGGATAC	TGCAATAATTTTTGAAGCCCGG
Heat shock protein 70	HSP70	CX069205	Chaperone protein (cytoprotection)	ATGAGTAAACACCAACAGGCCATCGG	AAGATAGTGTTCGATGGGTTTCATGGC
Pernin precursor	PRN	CD526735	Iron chelator (cytoprotection)	CTCTGATCATGCTGAACCT	GATCATGTTTGTTCGGTCATC
RAS suppressor	SUP	BG624647.1	Cell division inhibitor	ACTGAAGTTGTGGAGGCTAAGGCT	ATTCTAGATTTTTCTCCACACC
MYC-homologue	MYC-h	CX069136	Cell division, proto-oncogene	TTCTATAACGGAACATTATACCAACAAGG	CAACATTTACCTGGGGCAGGTGGGTTTCAG

and 13 dpf and under-expression when mortality occurred in the untreated larvae. The fourth group, *PRN*, *HSP70*, and *MYC-h*, had significantly higher expression levels at 13 dpf and significantly lower levels at 20 dpf in untreated larvae. *PRDX6* and *KC-rec* showed a similar pattern except at 20 dpf, when no significant difference was found. *ANX* and *SUP* composed the last group, in which we measured significantly higher expression levels between 13 and 20 dpf in untreated larvae. Finally, the unclassified *CTB* was characterized by a higher transcript level at 6 dpf, a similar level at 13 dpf, and a lower level at 20 dpf in untreated larvae.



**Fig. 1.** Relative expression of selected genes in *Crassostrea virginica* larvae untreated with antibiotic. A massive mortality was observed at 20 dpf. Relative gene expression data were estimated by comparison with the corresponding level from larvae that had the antibiotic treatment. Data are means  $\pm$  SD, \* significant difference ( $p < 0.05$ ), – reference level (antibiotic); see Table 1 for gene abbreviations.

## 4. Discussion

### 4.1. Mortality emergence and variation of gene expression

In the present study, we report, for the first time, molecular and physiological responses of oyster larvae during massive mortality process, particularly at the metabolism, immunity, cellular stress and cell regulation levels. Due to the microscopic size of pelagic larvae, the study of immunity at cellular level is very difficult, but we showed that the use of relative gene expression regulation is a powerful tool. As the transcript level is in equilibrium between production and degradation, it is indeed not possible to translate directly to cellular activity, but observed changes of gene expression can be related to their putative functions. The emergence of massive mortality was characterized by a decrease of relative gene expression of 15 out of 18 genes in moribund larvae (untreated larvae that showed subsequent mortality at 20 dpf). Beside this down-regulation, we measured that, comparative to antibiotic control, 9 genes were up-regulated at 13 dpf, suggesting that gene regulation began 1 week before mortality emergence and depended to the infection intensity. Recently, the impact of infection intensity on gene regulation was demonstrated in adults of *C. virginica* challenged by *P. marinus* (Wang et al., 2010). Similarly, Chaney and Gracey (2011) found up-regulated genes associated to massive mortality emergence from hemolymph of *C. gigas*.

### 4.2. Genes related to metabolism

For genes related to energy metabolism, moribund larvae at 20 dpf had lower transcript abundances of *NDH-1*, *CC-1*, and *AS-6*. The NADH dehydrogenase subunit 1 (*NDH-1*), cytochrome C1 (*CC-1*), and ATP synthase f0 subunit 6 (*AS-6*) genes code for protein subunits of electron chain transport complexes I, III, and V, respectively. Interestingly, as described in Genard et al. (2011), we observed a decline in the activities of two enzymes involved in energy production (citrate

synthase and cytochrome oxidase) along with these other molecular changes in moribund larvae, illustrating the correspondence between the molecular and physiological energy responses to mortality emergence. As discussed previously in the companion paper (Genard et al., 2011), the decreased metabolic activity could be associated with a metabolic depression explained by a lack of energy substrate (principally lipids and proteins) due to limited feeding and a higher energy demand resulting from the immune process activation. To our knowledge, little information is yet available on the regulation of genes related to energy metabolism as a function of feeding activity in bivalves (Bacca et al., 2005). Salem et al. (2007) demonstrated that genes from complex III (Ubiquinol–cytochrome C oxidoreductases), complex IV (cytochrome C oxidase), and complex V (ATP synthase) were down-regulated in starved rainbow trout.

The metabolic depression associated with mortality emergence could also be suggested by the down regulation of two genes related to protein metabolism (*GS* and *eIF-2B*). Glutamine synthetase (*GS*) is a ubiquitous enzyme involved in complex cellular functions, including nitrogen metabolism, ammonia detoxification, and the conversion of glutamate to glutamine. In *C. gigas*, Tanguy et al. (2005) suggested that glutamine produced by glutamine synthetase is required in the formation of amino acids, purines, and pyrimidines, all of which are essential for protein synthesis during oogenesis and embryogenesis. Previous studies on *Drosophila melanogaster* showed that the loss of glutamine could depress both protein and DNA synthesis and lead to a variety of mitotic defects in this embryonic system (Frenz and Glover, 1996). Translational initiation factor 2 (*eIF2*) is a multifunctional heterotrimeric G protein which plays a crucial role in the regulation of the initiation step of protein synthesis (Proud, 2005; Suragani et al., 2006). Thus, the low *GS* and *eIF-2B* transcript abundances measured in moribund larvae could reflect a decrease in protein synthesis; this has been observed in marine snails when temperatures approach lethal levels (Tomanek and Somero, 1999). This hypothesis is supported by our biochemical results: we observed that moribund larvae stopped protein accumulation (Genard et al., 2011).

The impact of massive mortality on lipid metabolism was illustrated by the lower transcript abundance of endothelial lipase (*EDL*) in moribund larvae. *EDL* is a phospholipase which has a high affinity for high-density lipoprotein (HDL) phospholipids (Hirata et al., 2000; Ishida et al., 2003). In addition, it has been demonstrated that *EDL* efficiently cleaves saturated as well as unsaturated fatty acids from HDL phospholipids, making them available for incorporation into endogenous lipids (Gauster et al., 2005; Kratky et al., 2005; Strauss et al., 2003). To our knowledge, no studies have been done on the function of *EDL* in bivalves. Nevertheless, the presence and importance of HDL have been demonstrated in molluscs, bivalves, and crustaceans (Dreon et al., 2002; Heras and Pollero, 2002; Walker et al., 2003), suggesting that *EDL* could play a significant function in fatty acid remodeling and lipid synthesis. Interestingly, we observed a good correspondence between *EDL* expression and phospholipid content, which was reported in the companion paper (Genard et al., 2011), suggesting a putative role of *EDL* in phospholipid accumulation in bivalves.

#### 4.3. Cellular stress and immunity

As discussed in the companion paper (Genard et al., 2011), the higher activity levels of superoxide dismutase (*SOD*) and glutathione peroxidase (*GPX*) in untreated larvae at 13 dpf led us to suggest an activation of antioxidant defenses before the mass mortality event. We had hypothesized that the increased antioxidant activity was induced to manage reactive oxygen species (*ROS*) that can be accumulated during an immune response, thus avoiding oxidative damage to the cell. In untreated larvae, the antioxidant defense activation at 13 dpf was confirmed by the higher transcript abundance of peroxiredoxin 6 (*PRDX6*), a key antioxidant enzyme that protects the

cells from the damaging action of  $H_2O_2$  (Shuvaeva et al., 2009). Recently, the activation of genes associated to *PRDX6* in particular and to antioxidant processes in general was observed in *C. virginica* after *P. marinus* infection (Wang et al., 2010).

Simultaneous with the higher *PRDX6* transcript level, we found an over-expression at 13 dpf of two other genes involved in cellular stress: heat shock protein 70 (*HSP70*) and pernin (*PRN*). *HSP70* is a stress-response protein implicated in cytoprotection that acts as a molecular chaperone, preventing premature folding of proteins and aiding in the translocation to organelles. Increased levels of *HSP70* in various tissues were observed in fish species exposed to bacterial pathogens (Ackerman and Iwama, 2001; Deane et al., 2004). In bivalves, studies showed that the expression of *HSP70* increased in *C. virginica* hemocytes with increasing intensities of *Perkinsus* infection (Brown et al., 1993); similar observations were made for *HSP70* RNA transcripts in the hemocytes of scallops infected by *Vibrio anguillarum* (Song et al., 2006). PERNIN, also called cavortin and first discovered in the mussel *Perna viridis* (Scotti et al., 2001), is a glycosylated protein possessing sequences clearly homologous to the active-site domain of Cu–Zn SODs (superoxide dismutases) which acts both as an iron chelator and as a serine protease inhibitor (Scotti et al., 2001). PERNIN has also the capacity to inhibit in vitro *P. marinus* growth (Gauthier and Vasta, 1994). Tanguy et al. (2004) identified a gene coding for pernin among *C. virginica* hemocytes and observed an increase of mRNA expression after 45 days of *P. marinus* exposure, suggesting the possible involvement of pernin in the response to parasite infection. Moreover, Huvet et al. (2004) suggested that cavortin (or pernin) can act in cellular host protection against the reactive oxygen intermediate (ROI) in *C. gigas* infected by *Vibrio splendidus*. The properties of *HSP70* and pernin indicate that these proteins could play similar functions in cytoprotection and immunity. As for *PRDX6*, we can hypothesize that the over-expression at 13 dpf in untreated larvae enhances cells protection against reactive oxygen species or intermediates (*ROS* or ROI) produced during the immune response. Inversely, the down-expression at 20 dpf could indicate a decline of cytoprotective abilities as supported by the higher lipid peroxidation level observed in moribund larvae.

In untreated larvae, the evolution of *MYC* homologue (*MYC-h*) transcript abundance was significantly correlated with the expression profiles of *HSP70* and *PRN*, suggesting that *MYC-h* expression was similarly regulated when mortality event occurred. *MYC-h* belongs to the proto-oncogene family and is involved in the control of metabolism, protein biosynthesis, cell cycle regulation, cell adhesion, and the cytoskeleton (Dang et al., 2006); it is also able to elicit the adverse process of programmed cell death (Evan et al., 1992; Nilsson and Cleveland, 2003). In *C. gigas*, the *MYC* homologue appeared to be up-regulated after 21 days of hydrocarbon exposure (Boutet et al., 2004) and under hypoxic conditions (David et al., 2005). According to these authors, *MYC-h* over-expression could enhance cell protection. In this context, like *HSP70* and *PRN*, the over-expression at 13 dpf could be linked to increased cell protection, while the down-expression at 20 dpf reflected the decrease of cytoprotective abilities in moribund larvae.

Activation of the immune response can be seen in the over-expression at 13 dpf of one gene linked to pathogen recognition and killer cell regulation: killer cell lectin-like receptor (*KC-rec*). This gene was similar to various C-type lectin receptors (Jenny et al., 2002). Lectins are specialized proteins that have the ability to recognize specific pathogen-associated carbohydrate structures and cause agglutination of cells, promote cellular adhesion, and mediate the innate immune response (Allam et al., 2006; Geijtenbeek et al., 2004; McGreal et al., 2004; Yamaura et al., 2008). Recently, the activation of C-type lectin related genes was found after *P. marinus* infection in adults of *C. virginica* (Wang et al., 2010).

The presence of an immune response could also be suggested by the level of cathepsin B (*CTB*) transcript in untreated larvae at 6 dpf.

Cathepsins are lysosomal cysteine proteinases involved in the intracellular protein degradation and in the regulation of melanization (Iwanaga and Lee, 2005; Kanost, 1999; Soderhall and Cerenius, 1998). EST of cathepsins were identified in *C. virginica* (embryo and hemocytes) by Jenny et al. (2002), in the hemocytes of bacterial-challenged *C. gigas* (Gueguen et al., 2003) or before mass mortality occurrence in the same specie (Chaney and Gracey, 2011). In regard to these cathepsin properties, the high transcript abundance measured at 6 dpf in untreated larvae could be linked to high protein degradation induced in the first moments of the immune response while the decline at 20 dpf could be related to the degradation of immune abilities.

Annexin (*ANX*) and RAS suppressor (*SUP*) also showed an over-expression at 13 and 20 dpf that could be associated with the activation of immune response. The annexins have diverse functions in cellular activities that include vesicle trafficking, cell division, apoptosis, inflammatory response, calcium signaling, and growth regulation. *ANX* acts as a secondary mediator of anti-inflammatory glucocorticoids (Perretti and Flower, 2008) and has been identified as one of the “eat-me” signals that allows apoptotic cells to be recognized and ingested by phagocytes (Parente and Solito, 2004; Perretti and Solito, 2004). RAS suppressor (*SUP*) is a member of RAS effectors thought to regulate cell proliferation and apoptosis involved in tumor regulation (Agathangelou et al., 2005; Kumari and Mahalingam, 2009). RAS associated genes were known to be involved in neoplasia in *Mytilus trossulus* (Ciocan et al., 2006), suggesting a putative function during pathology. While little is known about *ANX* and *SUP* functions in oyster larvae, the over-expression of these two genes at 13 and 20 dpf (the only ones) in moribund larvae indicate that both played an important function during pathogenic infection, probably to manage the inflammatory response and the apoptotic process as suggested in adult oysters during *P. marinus* infection in *C. virginica* (Wang et al., 2010) or before mass mortality in *C. gigas* (Chaney and Gracey, 2011).

## 5. Conclusion

To our knowledge, this study is the first to report the molecular and physiological processes of bivalve larvae reared in contrasting microbial environments where massive mortality occurred. Comparisons of biochemical and physiological results provided additional evidence supporting previous suppositions, such as the metabolic depression in moribund larvae and the activation of immune response and antioxidant defenses before the appearance of massive mortality. Moreover, our results illustrated the importance of cytoprotection processes, inflammatory response management, and cell regulation during a massive mortality event.

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