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Microarray analysis highlights immune response of Pacific oysters as a determinant of resistance to summer mortality

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Abstract :

Summer mortality of *Crassostrea gigas* is the result of a complex interaction between oysters, their environment, and pathogens. A high heritability was estimated for resistance to summer mortality, which provided an opportunity to develop lines of oysters that were resistant (R) or susceptible (S) to summer mortality. Previous genome-wide expression profiling study of R and S oyster gonads highlighted reproduction and antioxidant defense as constitutive pathways that operate differentially between these two lines. Here, we show that signaling in innate immunity also operates differentially between these lines, and we hypothesize that this is at the main determinant of their difference in survival in the field. A reanalysis of our published microarray data using separate ANOVAs at each sampling date revealed a specific “immune” profile at the date preceding the mortality. In addition, we conducted additional microarray profiling of two other tissues, gills, and muscle, and both showed an overrepresentation of immune genes (46%) among those that are differentially expressed between the two lines. Eleven genes were pinpointed to be simultaneously differentially expressed between R and S lines in the three tissues. Among them, ten are related to “Immune Response.” For these genes, the kinetics of R mRNA levels between sampling dates appeared different just before the morality peak and suggests that under field conditions, R oysters had the capacity to modulate signaling in innate immunity whereas S oysters did not. This study enhances our understanding of the complex summer mortality syndrome and provides candidates of interest for further functional and genetics studies.

Keywords : Mollusca ; *Crassostrea gigas* ; Gene ; Immunity ; cDNA microarray ; Summer mortality

1. Introduction

Significant mortality in Pacific oyster *Crassostrea gigas* has been reported to occur during the summer months in several countries and is a major concern for oyster aquaculture. This phenomenon is multifactorial, resulting from a complex interaction between oysters, their environment and pathogens (Samain and McCombie, 2008). More precisely, summer mortalities of *C. gigas* spat result from opportunistic pathogens, Ostreid herpes virus type 1 (OsHV-1) (Renault et al., 1994; Sauvage et al., 2009; Schikorski et al., 2011) and different species of bacteria belonging to the genus *Vibrio* (Garnier et al., 2007; Labreuche et al., 2010, Le Roux et al., 2002), that infect oysters weakened by a combination of abiotic stress (Lang et al., 2009) and the high energetic demands of reproduction (Fleury et al., 2010).

Variation in resistance to summer mortality is highly heritable (Dégremont et al., 2005; 2007), which provided an opportunity to develop lines of oysters that were "resistant" (R) or "susceptible" (S) to summer mortality (Samain and McCombie, 2008). To further investigate the physiological differences between the R and S lines and identify molecular markers for resistance to summer mortality, we recently conducted a genome-wide expression profiling study of R and S gonads using a 9X cDNA microarray specific to *C. gigas* (Fleury et al., 2010). The ANOVA used for the previous study used a statistical model with three factors : "line" (R or S), "date of sampling" (May, 9th, May, 25th, June 6th and June 20th), and technical replicates (spots deposited in duplicates on the slide). Of the 9058 unigenes analyzed, 34 were appeared systematically differentially expressed between R and S lines during the 3-month period preceding a summer mortality outbreak, (i.e. significant for the factor "line" overall the entire duration of the experiment). Annotation of most of these genes highlights reproduction and antioxidant defense as the main pathways that operate differentially between R and S lines in gonadal tissue during the critical period preceding a summer mortality outbreak. Results also showed that the factor "date of sampling" appeared to play a major influence as 23% of the studied genes were found to vary significantly according to the date (Fleury et al., 2010), but no interaction was found between "line" and "date of sampling".

In the present paper, we performed a different statistical analysis, a separate ANOVA for each of the dates, for the microarray data obtained on the R and S gonad samples already published in (Fleury et al., 2010). Results revealed a specific profile for the date preceding the mortality outbreak (June 20th), with more than 54% of the genes differentially expressed between R and S linked to the "immune response" gene ontology (GO) category. In addition, we conducted an expression profiling of two other tissues, gills and muscle, sampled during the same survey and using the same cDNA microarray. Gills and muscle were analyzed because they are the primary tissues involved in responses to biotic or abiotic environmental variations in the context of pathogens or environmental stress associated with summer mortality (Samain and McCombie, 2008). Statistical analysis of the microarray data was performed to test an over-representation of the GO "immune response" for the genes that appeared differentially expressed between R and S in these two tissues prior to the mortality outbreak (June 20th). The kinetics of gene expression of the immune-relevant genes commonly detected in the three tissues, are then discussed as a potential value in determining resistance to mortality.

2. Material and methods

2.1. Biological material

Following a divergent selection experiment on spat survival (Dégremont et al., 2007; Samain and McCombie, 2008), a fourth generation of *C. gigas* lines resistant (R) and susceptible (S) to summer mortality was produced in March 2004 at the IFREMER hatchery in La Tremblade (France). Intraline crosses of "G3c2" generation gave the "G4R" and "G4S" batches according to the crossing design described in Fleury et al. (2010). Briefly, five hundred oysters per G4R and G4S batch were kept at the Bouin nursery (France) away from mortality risks. After 12 months (March

2005), G4R and G4S pools, with a mean weight of 1.26 and 1.38g respectively, were deployed in the field at Fort Espagnol (South Brittany, France). They were cultured in triplicate plastic bags fixed on racks at 70cm out off the ground as the proximity of sediment have been shown to be a worsening factor in summer mortalities. The density of oysters per bags was 200, and they were cleaned every month during all the *in situ* experiment.. Mortality was monitored as described in (Fleury et al., 2010): respectively 56% and 22% cumulative mortality had been observed for S and R lines during the mortality peak (July, 7th), representing a substantially 2.5 times higher mortality rate in S oysters compared to R oysters. Gonad samples from R and S lines were the same as those previously described in Fleury et al. (2010). Muscle and gills were sampled three times (May 25th, June 6th, June 20th). On each analyzed date, 24 oysters per line were randomly collected (8 per bag) and their tissues were immediately dissected. For each tissue, samples were then pooled (3 pools of 8 oysters), homogenized in Extract-all (Eurobio) at a concentration of 1ml/50mg tissue and stored at –80°C for subsequent total RNA extraction. To ensure that each individual will contribute equally to the pool, a piece of 30 mg of each tissue was sampled per oyster separately. Furthermore, the whole bodies of 10 wild oysters were collected in the Marennes Oleron basin (France), pooled and homogenized in Extract-all (Eurobio) to constitute a single total RNA sample for use as a reference in all slide hybridizations.

2.2. RNA preparation

Samples of gonad, muscle and gills in Extract-all (Eurobio) were taken frozen and solubilized using a Brinkman Polytron tissue disruptor. Total RNA of each tissue was then isolated using Extract-all (Eurobio) procedure. RNA quality was assessed using RNA nano chips and Agilent RNA 6000 nano reagents (Agilent Technologies, Waldbronn, Germany) according to manufacturer's instructions. RNA concentrations were measured using an ND-1000 spectrophotometer (Nanodrop Technologies) at 260 nm using the conversion factor 1 OD = 40 µg/ml RNA.

For microarray hybridizations, 5µg of total RNA were directly labeled by reverse transcription and then purified using the Direct ChipShot Labeling kit (Promega), according to manufacturer's recommendations. This reaction was performed for each of the 18 muscle samples and the 18 gills samples with Cy5 (red) incorporation. The reference sample (wild oysters) was Cy3-labeled (green) in 36 separate tubes following the same protocol. The 36 Cy3-labeled cDNAs were next pooled, and then divided once more into 36 samples to obtain a homogeneous reference. All dye incorporation rates were verified by ND-1000 spectrophotometer (Nanodrop Technologies) prior to any hybridization.

2.3. Microarray hybridizations

The 9X cDNA microarray slide, containing a total of 11 088 features spotted in duplicate (9058 cDNA clones plus controls including water, spotting buffer, and calibration control with a standard fluorescence), and the hybridization procedure were the same as in Fleury et al. (2010). The array design is accessible through Gene Expression Omnibus (GSE16448). Briefly, for each tissue, equimolar amounts of cDNA samples and cDNA reference labeled with Cy5 and Cy3, respectively, were SpeedVac evaporated and mixed into a single pool with the hybridization buffer (ChipHybe™ hybridization buffer, Ventana Discovery, Tucson, AZ, USA). They were then co-hybridized on the same microarray slide, in a Ventana hybridization station (Ventana Discovery, Tucson, AZ, USA). Hybridizations were performed at the INRA IFR 140 transcriptomic facility (Rennes, France). Slides were filled with a pre-hybridization buffer for 1 hour at 42°C in a humidified chamber. Following this pre-hybridization step, hybridization was conducted overnight at 42°C in a humidified chamber. The arrays were washed twice at room temperature with Ribowash solution, twice with 0.1×SSC and finally centrifuged for drying.

After the hybridization step, microarray slides were scanned using a Scanner Genepix 4000B (Axon Instruments Inc.) with standard dual laser excitation at 532 nm (17 mW) and 635 nm (10 mW) according to the following parameters: Cy 5 Photo Multiplier Tube (PMT): 550 and Cy 3 PMT:

590. This process was repeated for each of the 36 hybridized slides with the high-resolution mode (5 µm resolution). The images were then analyzed using Genepix pro 5.1 software (Axon Instruments Inc.) according to manufacturer's instructions. Spots were filtered for quality according to the criteria defined in Fleury et al. (2010) and spots not fulfilling these criteria were removed from the data set.

2.4. Microarray data analyses

2.4.1. Correction and normalization

Transformation and normalization of hybridization data were performed by Log and standardisation respectively, in order to minimize variation arising from technical differences in RNA quality, probe labelling, and hybridization conditions between experiments. As normalization without background subtraction resulted in the lowest variability, microarray data background has not been corrected prior to data_correction and normalization, as suggested by Zahurak et al. (2007). First, a logarithmic transformation for each signal intensity was performed in all filtered sets to obtain Log (hybridization values). Considering that expression of the majority of the spotted genes did not change with the experimental conditions, the total quantity of cDNA (sum of Log hybridization values) and the variance of Log (hybridization values) within each set should be the same across the different samples. For this reason, a median normalization has been performed by subtracting the median of Log (hybridization values) in a set (sample) from each Log (hybridization values) for that set (to obtain Log normalized hybridization values), as described in Rezen et al. (2008). Correction for differences in the variance across the range of gene expression levels were next performed by dividing each Log (normalized hybridization values) by the standard deviation of the Log (normalized hybridization values) for each set, as described in Darias et al. (2008). The corrected and normalized data are available in Gene Expression Omnibus (GSE25614). Microarray data obtained on gonad samples were already validated by real-time PCR based on a significant correlation (R^2 = 0.89) of the gene expression assays between the two methods (see Fleury et al., 2010). Furthermore, fold changes between R and S samples appeared extremely similar between the two methods, as the highest difference in fold changes obtained between the two methods was 4.2% attesting the reliability of the present data.

2.4.2. Identification of differentially expressed genes

Genes that were differentially expressed between R and S lines at each sampling date and for each tissue were determined statistically (p-value < 10^{-4}) by variance analysis using GeneANOVA software (Didier et al., 2002). The False Discovery Rate (FDR) associated with the selected genes was thus determined by the formula: [Total number of analyzed genes (9058) x p-value (10^{-4}) / number of differentially expressed genes] x 100. For each analysis (*i.e* each date and each tissue), we constructed a statistical model with 2 factors: "line" (R or S) and "technical replicate" (spots deposited in duplicate on the slide). The common selected genes in gonad, muscle and gills at June 20th were kept for pattern expression analysis, to visually inspect if these genes have similar patterns between each sampling dates. .Student t-tests were realized on the transformed normalized Cy5 log value of each gene between dates, for R and S lines respectively, as detailed in Yuan et al. (2006).

2.4.3. Gene ontology analyses

Gene ontology analysis was carried out for each list of selected genes (by date and by tissue), using the BLAST2GO program (Conesa et al., 2005). This program is a Java application enabling gene ontology (GO) assignment, based on data mining of sequences for which no GO annotation is currently available. FASTA-formatted sequences representing the selected ESTs were uploaded into the program and BLASTX similarity searches were carried out against the Swiss-Prot

database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The program retrieved the GO terms associated with the hits (E-value < 10^{-5}) and queries were annotated based on hit similarity and GO evidence codes. Expression Analysis Systematic Explorer (EASE, Hosack et al., 2003) analysis was performed to test selected list of genes against all genes spotted on the chip to discover enriched biological GO category within gene lists. GO categories that showed EASE scores ≤ 0.05 were considered significantly over-represented.

3. Results

3.1. Identification of Differentially Expressed Genes

Analysis of variance (ANOVA) for the gonad tissue revealed respectively 34, 55 and 60 differentially expressed genes between R and S lines for the 3 studied dates, May 25th, June 6th, June 20th, respectively. For muscle 20, 39, 32 genes and for gills 49, 70 and 72 genes were differentially expressed between R and S oysters for the dates May 25th, June 6th and June 20th, respectively. The level of significance used for all ANOVA analysis to select these ESTs as differentially expressed was *p*-value < 10^{-4}. Consequently, the FDR values of selected ESTs for each date and for each tissue were always under 5% (2.6%, 1.6% and 1.5% for the gonad, 4.5%, 2.3% and 2.8% for the muscle and 1.8%, 1.2% and 1.2% for the gills for the 3 dates, respectively).

3.2. Annotation and Gene Ontology analyses of the selected genes

Compiled BLAST analyses revealed that 84% of the differentially expressed ESTs showed significant hits with Swiss-Prot databank sequences (E-value threshold < 10^{-5}). Gene ontology analyses of the annotated clones for the 3 dates and the 3 tissues are presented in Figure 2. For the 2 first dates (May 25th, June 6th), all the biological processes (reproduction, lipid metabolic process, response to stress, cell death, catabolic process, antioxydant activity, growth, immune response, energy reserve metabolic process, and NA when no GO was associated) are almost equally represented for the 3 tissues, and no significant EASE-score was obtained for any of them, meaning that there was no over-representation of these GO categories for these 2 dates. For the last date (June 20th), 54%, 48% and 45% of the selected genes were associated with the “immune response” biological process for gonad, muscle and gills, respectively. In comparison, the percentage of genes associated with the “immune response” category was 3% in the whole cDNA microarray. EASE scores obtained for “immune response” among the lists of genes selected at the June 20th were always under 0.05, meaning that genes linked with “immune response” biological process appear more often in the given lists than expected under random distribution. The list of the 164 differentially expressed ESTs and their annotation is given for the date June 20th in Table 1. Compiling data from the three tissues, they correspond to 130 single ESTs: 41 specifically selected in gonad, 50 in gills and 16 in muscle (Figure 3). Twenty three were commonly identified in at least two tissues for which 86% fall into the category “immune response”. Eleven genes appeared commonly detected in the 3 tissues, muscle, gills and gonad at the date June 20th. Among these, 10 genes fall in the category “immune response” and were considered as 10 immune-relevant transcripts for further investigation.

3.3. Kinetics of mRNA level of the 10 selected immune genes

Time course expression analyses for these 10 immune-relevant transcripts selected as differentially expressed between R and S oyster lines showed the same pattern for each of the 3 analyzed tissues. Whereas no significant variation of mRNA level was observed in S oysters along the 3 dates, two coordinate patterns of expression were revealed for R oysters with significant variations observed at June 20th in comparison with June 6th and May 25th (Figure 4). First cluster

of co-regulated genes identified the ESTs showing a mean of 3.5x decrease in R mRNA level at the date June 20th for the three tissues in comparison with the 2 previous dates. This cluster grouped ESTs encoding two inhibitors of NF-κB and a suppressor of cytokine signalling. The second cluster corresponded to transcripts over-expressed of around 14x in R at the date June 20th compared to the 2 previous dates. This group identified ESTs encoding Toll-like receptor, B-cell lymphoma/leukemia, peroxinectin, complement C1q-like protein, prostaglandin E2 receptor, and two superoxide dismutases.

4. Discussion

In this study, we conducted a genome-wide expression profiling of *Crassostrea gigas* lines selected to be resistant (R) and susceptible (S) to summer mortality to identify specific genes that contribute to resistance to summer mortality, and therefore obtain further insights into the understanding of the complex summer mortality syndrome. We used a cDNA microarray containing 9,085 unigenes from *C. gigas* and to obtain genome-wide expression profiles of muscle and gills samples from R and S lines preceding a summer mortality outbreak. We combined these new data with those previously obtained for the gonad from the same individuals and using the same microarray (Fleury et al., 2010). We originally chose to study gonad based on the negative correlation between reproductive effort and resistance to summer mortality, which suggests that summer mortality could be partly due to a physiological disorder and metabolic disturbance in oysters associated with high reproductive effort (Fleury et al., 2010; Huvet et al., 2010; Samain and McCombie, 2008). Moreover, environmental stress and pathogens are known to interact and lead to summer mortality outbreaks. We added gills in this study because they are bathed by hemocytes (Gonzalez et al., 2007; Seo et al., 2005), which play a key role in the immune defense against pathogens (Bachère et al., 2004; Le Foll et al., 2010; Venier et al., 2011). Gills are also known to be involved in stress response in oysters, such as accumulation and detoxification of heavy metals (Macey et al., 2010). As well, the muscle is of importance in bivalves as one of the primary tissue implicated in the adaptation mechanisms to environmental variations such as hypoxic stress (Le Moullac et al., 2007).

The previously genome-wide expression profiling of R and S oyster gonads during the 3-month period preceding a summer mortality event highlighted reproduction allocation and antioxidant defense as constitutive pathways that operate differentially between R and S lines (Fleury et al., 2010). Here we showed that signalling in innate immunity operates differentially between these two lines and we hypothesized that this is at the main determinant of their difference in survival in the field. Our analysis of the microarray data from gonad, muscle and gills revealed a specific “immune” profile at the date June 20th corresponding to the date preceding the mortality outbreak.. This significant over-representation of differentially expressed genes associated with “immune response” biological process is in contrast with the biological processes equally represented for the genes differentially expressed at the two previous dates and for the cDNA spotted on the microarray.

From the 60, 32, and 72 ESTs selected at the June 20th for gonad, muscle, and gills, respectively, 44% appeared over-expressed and 56% under-expressed in R lines compared to S lines overall the three tissues. This suggests that the difference between the two lines is not only due to a physiological weakness of S oysters causing generalized down-regulation of gene transcription. When focusing on immune selected genes, no statistical differences were observed between R and S lines at May 25th and June 6th. Consequently, the kinetics of immune-relevant gene expression, inferred from the microarray data along the temporal sampling, appeared punctually different between the two lines at June 20th.. For the 10 immune-relevant genes, pinpointed by the Venn diagram to be simultaneously differentially expressed between R and S lines in the three tissues at this date, no significant variation of gene expression was observed in S lines along the three sampling dates whereas significant and strong variations of mRNA level were observed for the R lines. Indeed, two patterns of coordinately regulated immune-relevant genes were identified in R lines, 7 transcripts were over-expressed from June 6th to June 20th and 3 transcripts were

under-expressed from June 6th to June 20th. Interestingly, the sense of mRNA level variation of the selected ESTs observed in R oysters is in agreement with their stimulator / inhibitor role known in some vertebrates. ESTs encoding two inhibitors of NF-κB and a suppressor of cytokine signalling appeared to be down-regulated at the June 20th whereas the other ESTs encoding genes implicated in the detection of pathogens, the activation of signal transduction and of appropriate defenses appeared to be strongly up-regulated. In our field experiment, R oysters displayed capacity to react, in terms of immune gene expression, whereas the S oysters had not or reacted less, and this might be linked to their ability to survive.

Based on the function of these 10 genes, signalling in innate immunity appears to have a key role in resistance to the summer mortality syndrome. Innate immunity consists of three steps: detection of pathogens, activation of signal transduction, and mounting of appropriate defenses (Foley and O'Farrell, 2004). Pathogen signature, such as microbial components, are detected by host recognition receptors including Toll-like receptors (TLRs) responsible for the activation of NF&B pathway and phagocytosis. One differentially expressed transcript encodes a TLR which is different from the already characterized TLR4 in *C. virginica* (Tanguy et al., 2004) or *Cg*-Toll-1 in *C. gigas* (Zhang et al., 2011) and might correspond to a TLR-type 3 known in vertebrates to recognize microbial nucleic acids and to induce antiviral immune response (Kawai and Akira, 2010). Indeed, TLR3 triggers antiviral immune responses through the production of inflammatory cytokines via the NF-κB pathway, which suggests that TLR3 has an essential role in preventing virus infection. Indeed, studies realized in humans showed the association betweenTLR3 deficiency and susceptibility to herpes simplex virus type 1 (Kawai and Akira, 2010). The involvement of B-cell lymphoma/leukemia (BCL)-10, one of the 10 candidate ESTs, in the activation of NF-κB, by both canonical and non-canonical pathways, has been very well documented. B-cell lymphoma protein is a transcriptional repressor which once phosphorylated, is degraded by ubiquitin/proteasome pathway. A TLR–BCL10 pathway and a reactive oxygen species–Heat shock protein27 pathway were demonstrated to activate NF-κB in human in response to LPS (Bhattacharyya et al., 2010).

Two types of IκBs (Inhibitor of κB) were characterized to be differentially expressed between R and S lines and might play a pivotal role in regulating the innate immune response of *C. gigas*. One is 58% identical with the amino acid sequence of a IκB recently identified in the pearl oyster *Pinctada fucata* (Zhang et al., 2009). The second EST displayed the highest identity with an inhibitor κB characterized in *Argopecten irradians* (Mu et al., 2010). The 50 and 36% of amino acid identity obtained, respectively, with the inhibitor of NF-κB already characterized in *C. gigas* and named *Cg*-IκB (Montagnani et al., 2008) is quite low, suggesting newly-characterized inhibitors of NF-κB in *C. gigas*. The conserved lysine and serine residues required for inducible degradation of the IκB proteins are present in the two sequences and full length cDNA should be further characterized to look for ankyrin repeats, a common feature of most IκBs necessary for specific binding to NF-κB. During inflammatory responses, the phosphorylation of inhibitors of NF-κB leads to the dissociation of the inhibitor/ NF-κB complex and ultimately the degradation of inhibitors. The NF-κB protein complex is subsequently free to translocate to the nucleus where it stimulates the transcription to activate target genes that have DNA-binding sites for NF-κB, notably those involved in immune responses.

Cytokines are potent activators of NF-κB. Therefore, the suppressor of cytokine signalling, identified among the 10 immune-relevant genes, is expected to limit NFκB signalling following a classical negative feedback system that regulates cytokine signal transduction (Alexander and Hilton, 2000). The strong decrease of its mRNA level observed in our data suggests the engagement in R oysters of cytokines that trigged the inflammatory response.

Pathogens induce phagocytosis and elicit a large amount of reactive oxygen species (ROS) in the host, which had stimulatory effects on NF-κB signalling. ROS, together with lysosomal cytotoxic components, kill pathogens but also produce oxidative damage to biological macromolecules such as DNA, RNA, protein and lipids (Cutler, 1991; Feuers et al., 1993). A greater capacity to rapidly detoxify the ROS contributes to cellular host protection by preventing host cellular oxidative damage and may therefore improve oyster survival. This is the function of SOD, an enzyme up-regulated after hypoxic stress in oysters (Sussarellu et al., 2010), that converts O_2^- into the less

damaging H_2O_2. Two SOD sequences were already characterized to be differentially expressed between R and S lines during the critical period (Fleury et al., 2010; Huvet et al., 2004). The first one was identified as a plasma extracellular Cu/Zn SOD (*Cg*-EcSOD) (Gonzalez et al., 2005) and the second as a manganese-SOD (Genbank accession ABZ90958). Here, two ESTs differentially expressed before the mortality outbreak, displayed significant hits with another Cu/Zn SOD already characterized in *C. gigas* (Boutet et al., 2004). These findings suggest that the capacity of antioxidant enzymes to detoxify the ROS could be critical in determining susceptibility or resistance to summer mortality, as already hypothesized by Lambert et al. (2007). Activity of antioxidant enzymes was already reported for the mollusk *Biomphalaria glabrata* to be positively correlated with resistance to the parasite *Schistosoma mansoni* (Goodall et al., 2004). In *C. virginica*, challenge with *Perkinsus marinus* caused the up-regulation of a large set of genes likely involved in the regulation of oxidative stress emphasizing the antioxidant defense system as a main pathway for protection against pathogens and diseases (Wang et al., 2010).

Other immune candidates are clearly of interest based on their previous identification in low-surviving oyster families produced in west coast of USA (Lang et al., 2009; Taris et al., 2009). For example, peroxinectin is a cell-adhesion protein with peroxidase activity and is synthesized and stored in hemocytes. When released, peroxinectin mediates hemocyte adhesion to foreign particles and stimulates phagocytosis (Liu et al., 2007). A prostaglandin receptor and C1q domain-containing gene were reported to be constitutively higher in low-surviving oyster families as it is the case in our S oyster lines for ESTs annotated as a Complement C1q-like protein and a Prostaglandin E2 receptor. Prostaglandins are oxygenated compounds derived from polyunsaturated fatty acids that play various physiological roles when linked to their receptors including mounting immune and inflammatory responses (Taris et al., 2009). In *C. gigas*, the EP4 subtype of prostaglandin E2 receptor appeared very strongly up-regulated in gills following bacterial challenge allowing the authors to conclude that this EST is very interesting for further understanding of inflammation in oysters (Roberts et al., 2009). The C1q domain-containing proteins include a wide range of signalling molecules and are known to participate in the control of inflammation, innate immunity and energy homeostasis (Gerdol et al., 2011; Taris et al., 2009).

Lastly, another immune relevant-candidate is annotated as Defensin, , a recently characterized antimicrobial peptide in oyster, which is an important component of the innate immune system and constitutes a first line of defense against pathogen colonization (Gueguen et al., 2006). Defensin was mainly expressed in mantle edge in oysters (Gueguen et al., 2006) whereas other immune-relevant genes characterized here were expected to have hemocyte-specific expression (for example *Cg*-EcSOD) (Gonzalez et al., 2005). The common identification of immune-relevant genes in gonad, gills and muscle of R and S lines, suggest they were expressed by tissue-bathing hemocytes. Immune genes were already reported in marine bivalves to be highly expressed in gills (He et al., 2011; Zhang et al., 2011). For some of them, more precise investigation revealed their mRNA expression in tissue-infiltrating hemocytes (Gonzalez et al., 2005) at possibly higher levels than in circulating hemocytes (Itoh et al., 2011). Gills were characterized as one organ target for the viral replication of the *Ostreid herpes virus 1* implicated in some summer mortality outbreaks (*e.g.* Schikorski et al., 2011) and thus might be recommended for investigation in challenges employed with the aim of mimicking experimentally summer mortality outbreak.

5. Conclusion

Where most of the immune questions were addressed experimentally in marine bivalves, the originality of the present work was addressing the complex summer mortality syndrome in the field where it occurs and where the R and S lines were selected. Our data emphasize immune response of oysters in playing a pivotal role in determining susceptibility or resistance to summer mortality, and especially for molecules regulating the NF-κB signalling pathway. The most significant outcome of this study is the identification of transcripts which enhances our understanding of the process that contribute to the differential survival between R and S lines in the context of a summer mortality outbreak. Genes linked to immune response provide basic insights into oyster biology and

candidates genes for further study, with the aim of enabling marker-aided selection to improve *C. gigas* oyster survival. More specific and precise individual investigation of these candidate genes is now required in response to bacterial and viral infections, as well as unravelling their role using functional studies such as RNA interference, now available in oyster (Fabioux et al., 2009).

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Tables

Table 1 List of the differentially expressed ESTs in gonad, muscle, and gills between resistant and susceptible oyster lines at the date Jun

Tissue	Genbank accession	Sigenae name	Swiss-Prot hit description	*E* value	*p* Value ANOVA	Fold c R/S
Gonad	AM853409	cdn19p0001i24	Complement regulatory protein	8.00E-06	1.40E-05	0,19
	AM853211	cdn19p0001a09	Calcitonin receptor	3.00E-29	1.10E-05	0,24
	CU686145	**oypm10b03f16r1**	**Suppressor of cytokine signaling 5**	**3.00E-12**	**2.73E-04**	**0,26**
	FP091130	oyio10b01k20r1	S-crystallin	3.00E-13	2.86E-04	0,30
	CU682837	oygd10b09c10r1	NA		1.99E-04	0,34
	AM856743	**cdn21p0002p18**	**NF-kappa-B inhibitor cactus**	**6.00E-07**	**7.60E-05**	**0,35**
	AM854660	cdn20p0001d03	Kinesin-related protein	5.00E-06	4.00E-05	0,39
	AM856987	cdn21p0003k18	NA		9.40E-05	0,42
	AM853504	**cdn19p0001n04**	**NF-kappa-B inhibitor cactus**	**4.00E-33**	**2.20E-05**	**0,44**
	AM858687	cdn37p0004o20	DNA-dependent protein kinase catalytic subunit	7.00E-38	1.24E-04	0,45
	AM859022	cdn37p0005n23	Mitogen-activated protein kinase	2.00E-28	1.28E-04	0,47
	CU683455	oyge09b12j11r1	NA		2.25E-04	0,50
	CU685152	oypg10b07j13r1	Kinase D-interacting substrate	7.00E-04	2.57E-04	0,54
	AM853699	cdn19p0002f20	Ankyrin repeat and KH domain-containing protein	1.00E-12	2.50E-05	0,57
	CU682447	oygd10b10n08r1	NA		1.88E-04	0,58
	CU685072	oypg10b07d22r1	Putative ankyrin repeat protein	3.00E-04	2.47E-04	0,59
	CU685188	oypg10b07f04r1	Fibulin-5	7.00E-10	2.63E-04	0,60
	CU683708	oyge09b12m14r1	Neurogenic locus notch homolog protein 2	3.00E-13	2.42E-04	0,63
	AM857213	cdn21p0004f05	Mitogen-activated protein	2.00E-54	1.01E-04	0,64
	AM855685	cdn20p0004m19	NA		5.60E-05	0,65
	CU681533	oygd09b07p15r1	NA		1.48E-04	0,66
	CU684209	oyge10b13a10r1	Tubulin alpha-1 chain	9.00E-07	2.43E-04	0,66
	AM854778	cdn20p0001i06	Putative ankyrin repeat protein	8.00E-10	4.70E-05	0,67
	CU681818	oygd09b07l12r1	NA		1.86E-04	0,67
	AM857078	cdn21p0003o22	Complement C1q tumor necrosis factor-related protein	7.00E-07	9.80E-05	0,68
	CU681588	oygd09b07o14r1	NA		1.51E-04	0,70
	AM856921	cdn21p0003h18	Set1/Ash2 histone methyltransferase complex subunit	3.00E-48	8.30E-05	0,70
	BQ426809	BQ426809	NA		1.40E-04	0,70
	CU683013	oygd10b09g16r1	NA		2.12E-04	0,71
	CU683326	oyge09b11p23r1	NA		2.19E-04	0,73
	FP090952	oyio10b01c01r1	NA		2.73E-04	0,74
	AM857298	cdn21p0004j22	Prostaglandin E2 receptor	2.00E-20	1.05E-04	0,75
	CU681629	oygd09b07m17r1	NA		1.52E-04	0,76
	AM857028	cdn21p0003m17	Tripartite motif-containing protein 2	9.00E-18	9.60E-05	0,76
	AM855543	cdn20p0004d22	Inhibitor of nuclear factor kappa-B kinase subunit	2.00E-21	5.20E-05	0,78
	CU683323	oyge09b11i11r1	Chitotriosidase-1	4.00E-26	2.14E-04	0,78
	AM856093	cdn21p0001c15	Superoxide dismutase [Cu-Zn]	7.00E-23	6.20E-05	0,79
	AM853576	cdn19p0002a07	Neurogenic locus notch protein homolog	3.00E-14	2.40E-05	0,80
	CU681673	oygd09b07j05r1	NA		1.53E-04	1,13
	AM856775	**cdn21p0003b03**	**Prostaglandin E2 receptor**	**7.00E-22**	**7.90E-05**	**1,18**
	AM855291	cdn20p0003f01	NA		5.20E-05	1,22
	AM853924	cdn19p0002p24	Ras-like GTP-binding protein Rho1	5.00E-25	3.20E-05	1,25
	AM866067	cdn37p0026k10	Zinc metalloproteinase	9.00E-15	1.88E-04	1,27
	AM854726	**cdn20p0001f21**	**Toll-like receptor 3**	**2.00E-09**	**4.10E-05**	**1,29**
	AM856242	cdn21p0001j01	Fucolectin-7	8.00E-07	6.30E-05	1,33
	AM854377	cdn19p0004f01	Mitoferrin-1	5.00E-80	3.50E-05	1,38
	AM853235	**cdn19p0001b12**	**Complement C1q-like protein 4**	**2.00E-09**	**2.11E-04**	**1,39**
	FP089924	oyio09b02n18r1	Soma ferritin	2.00E-21	2.73E-04	1,43

Table 1 (continued)

Tissue	Genbank accession	Sigenae name	Swiss-Prot hit description	*E* value	*p* Value ANOVA	Fold chang R/S
	AM853394	**cdn19p0001i09**	**Superoxide dismutase [Cu-Zn]**	**2.00E-04**	**1.20E-05**	**1,51**
	AM857760	**cdn37p0002c03**	**Superoxide dismutase [Cu-Zn]**	**1,00E-50**	**5,49E-05**	**1,52**
	AM856953	cdn21p0003j05	Leukocyte common antigen	1.00E-08	9.10E-05	1,54
	CU683194	oyge09b11m02r1	Ras-like GTP-binding protein Rho1	6.00E-94	2.13E-04	1,59
	CU683707	oyge09b12m06r1	Toll-like receptor 1	3.00E-06	2.36E-04	1,61
	AM858229	**cdn37p0003j12**	**T-complex-associated testis-expressed protein**	**1.00E-50**	**1.15E-04**	**1,64**
	AM854090	cdn19p0003h13	C-type lectin domain family	3.00E-05	1.49E-04	1,72
	FP091069	oyio10b01h21r1	Dopamine receptor 1	7.00E-23	2.84E-04	1,75
	CU681715	oygd09b07k07r1	Integrin beta-1	1.00E-17	1.63E-04	1,79
	CK172373	CK172373	NA		1.43E-04	1,81
	CU681745	**oygd09b07i13r1**	**B-cell lymphoma/leukemia**	**1.00E-11**	**1.72E-04**	**1,85**
	CU682412	**oygd10b10j02r1**	**Thyroid peroxidase**	**7.00E-27**	**2.24E-04**	**1,87**
Muscle	FP091019	oyio10b01f10	NA		1,10E-04	0,21
	AM853269	cdn19p0001c24	NA		5,10E-05	0,25
	AM858613	cdn37p0004l12	NA		1,74E-04	0,28
	AM856743	**cdn21p0002p18**	**NF-kappa-B inhibitor cactus**	**6,00E-07**	**1,79E-04**	**0,30**
	AM853504	**cdn19p0001n04**	NF-kappa-B inhibitor cactus	**4,00E-33**	**4,90E-05**	**0,30**
	CU686145	**oypm10b03f16**	**Suppressor of cytokine signaling 5**	**3,00E-12**	**6,70E-05**	**0,34**
	AM855031	cdn20p0002e17	NA		1,10E-04	0,36
	CU683232	oyge09b11m21	Apoptosis-inducing factor 3	2,00E-68	1,22E-04	0,40
	CU681645	oygd09b07p14	Collagen alpha-2(IV) chain	1,00E-25	1,77E-04	0,43
	AM854151	cdn19p0003k14	Programmed cell death protein 10	6,00E-38	1,91E-04	0,44
	CU685677	oypg10b08f14	Pyruvate kinase muscle isozyme	5,00E-36	1,83E-04	0,48
	AM857924	cdn37p0002k10	Glucokinase	2,00E-60	1,87E-04	0,49
	AM854961	cdn20p0002a23	NA		8,40E-05	0,53
	CU686063	oypm10b03a15	Paramyosin	2,00E-14	2,03E-04	0,58
	AM859122	cdn37p0006c18	Programmed cell death protein 6	6,00E-71	1,34E-04	0,70
	CU681507	oygd09b07n23	Dihydropyrimidine dehydrogenase [NADP+]	9,00E-36	1,47E-04	0,79
	AM853394	**cdn19p0001i09**	**Superoxide dismutase [Cu-Zn]**	**2,00E-04**	**4,40E-05**	**1,10**
	AM866876	cdn37p0029d10	Phosphatidylinositol-4,5-bisphosphate 3-	5,00E-68	7,60E-05	1,32
	AM856775	**cdn21p0003b03**	**Prostaglandin E2 receptor**	**7,00E-22**	**1,11E-04**	**1,32**
	AM853235	**cdn19p0001b12**	**Complement C1q-like protein 4**	**2,00E-09**	**1,98E-04**	**1,34**
	AM867953	cdn37p0032f24	Heat shock 70 kDa protein	3,00E-89	1,16E-04	1,39
	AM853618	cdn19p0002c05	Catalase	1,00E-101	1,61E-04	1,41
	CU682412	**oygd10b10j02**	**Thyroid peroxidase**	**7,00E-27**	**1,52E-04**	**1,45**
	AM860311	cdn37p0009m15	Sperm-associated antigen 6	2,00E-99	2,80E-05	1,48
	AM857078	cdn21p0003o22	Complement C1q tumor necrosis factor-related protein	7,00E-07	1,65E-04	1,49
	AM858229	**cdn37p0003j12**	**T-complex-associated testis-expressed protein**	**1,00E-50**	**1,28E-04**	**1,56**
	CU685371	oypg09b06j05	Metallothionein	1,00E-06	1,44E-04	1,63
	AM854726	**cdn20p0001f21**	**Toll-like receptor 3**	**2,00E-09**	**2,70E-05**	**1,72**
	AJ582629	AJ582629	Defensin	9,00E-07	2,09E-04	1,74
	AM856765	cdn21p0003a17	Bone morphogenetic protein 15	4,00E-06	5,50E-05	1,77
	AM857760	**cdn37p0002c03**	**Superoxide dismutase [Cu-Zn]**	**1,00E-50**	**5,49E-05**	**1,82**
	CU681745	**oygd09b07i13**	**B-cell lymphoma/leukemia**	**1,00E-11**	**8,50E-05**	**1,84**
Gills	AM858409	cdn37p0004c02	NA		5,61E-05	0,19
	AM865171	cdn37p0024a03	Glycogen debranching enzyme	1,00E-104	6,50E-05	0,22
	AM866254	cdn37p0027c21	NFKB inhibitor interacting Ras-like 1	6,00E-41	6,69E-05	0,27
	AM856242	cdn21p0001j01	Fucolectin-7	8,00E-07	4,83E-05	0,30

Table 1 (continued)

Tissue	Genbank accession	Sigenae name	Swiss-Prot hit description	*E* value	*p* Value ANOVA	Fold ch R/S
	AM856743	**cdn21p0002p18**	**NF-kappa-B inhibitor cactus**	**6,00E-07**	**5,21E-05**	**0,35**
	AM858487	cdn37p0004f18	Cell division control protein 42	4,00E-47	5,64E-05	0,39
	CU686145	**oypm10b03f16**	**Suppressor of cytokine signaling**	**3,00E-12**	**2,82E-04**	**0,44**
	AM861125	cdn37p0012e07	Peroxisomal leader peptide-processing protease	4,00E-07	5,86E-05	0,48
	AM862314	cdn37p0015j15	Pancreatic lipase-related protein	6,00E-25	6,14E-05	0,48
	BQ426737	BQ426737	Integrin beta-1	2,00E-04	1,46E-04	0,50
	CU685188	oypg10b07f04	Fibulin-5	7,00E-10	2,79E-04	0,51
	AM858676	cdn37p0004o08	NA		5,78E-05	0,51
	AM855451	cdn20p0003o08	Ubiquitin carboxyl-terminal hydrolase	5,00E-80	4,16E-05	0,52
	AM853504	**cdn19p0001n04**	**NF-kappa-B inhibitor cactus**	**4,00E-33**	**3,37E-05**	**0,52**
	FP091048	oyio10b01g19	NA		2,95E-04	0,56
	CU684835	oypg09b05h15	Soma ferritin	5,00E-29	2,74E-04	0,57
	CU682043	oygd09b08f20	Tissue inhibitor of metalloproteases	2,00E-04	2,25E-04	0,59
	CU682416	oygd10b10h10	NA		2,35E-04	0,59
	AM853924	cdn19p0002p24	Ras-like GTP-binding protein Rho1	5,00E-25	3,58E-05	0,59
	CU682923	oygd10b09j18	NA		2,40E-04	0,60
	AM853819	cdn19p0002l04	Cathepsin L	1,00E-102	3,40E-05	0,61
	AM862518	cdn37p0016c22	Stress response protein nhaX	3,00E-07	6,16E-05	0,61
	CU681765	oygd09b07h14	Glutathione-requiring prostaglandin D synthase	1,00E-05	2,24E-04	0,61
	AM855176	cdn20p0002n15	Cell death-related nuclease	5,00E-24	3,82E-05	0,61
	CU681565	oygd09b07a19	Soma ferritin	1,00E-25	1,71E-04	0,62
	AM857924	cdn37p0002k10	Glucokinase	2,00E-60	5,57E-05	0,66
	AM856068	cdn21p0001b14	Testicular haploid expressed gene protein	7,00E-11	4,40E-05	0,67
	AM854685	cdn20p0001e04	Inositol hexakisphosphate kinase	4,00E-22	3,71E-05	0,68
	AM854877	cdn20p0001m18	Phospholipid hydroperoxide glutathione peroxidase	3,00E-51	3,80E-05	0,68
	CU683232	oyge09b11m21	Apoptosis-inducing factor 3	2,00E-68	2,41E-04	0,80
	AM855818	cdn20p0005d23	Glutathione peroxidase 3	1,00E-35	4,20E-05	0,82
	CU681645	oygd09b07p14	Collagen alpha-2(IV) chain	1,00E-25	1,82E-04	0,84
	CU684230	oyge10b13d08	Big defensin	3,00E-20	2,49E-04	0,86
	AM857294	cdn21p0004j16	Carnitine O-palmitoyltransferase	2,00E-17	5,29E-05	0,87
	AM863247	cdn37p0018f04	Chorion peroxidase	3,00E-22	6,30E-05	0,87
	CU682536	oygd10b10o09	NA		2,39E-04	0,88
	AM867148	cdn37p0030b11	NA		1,07E-04	0,88
	AM857637	cdn37p0001l15	Peroxiredoxin-5, mitochondrial	1,00E-29	5,47E-05	0,89
	AM857760	**cdn37p0002c03**	**Superoxide dismutase [Cu-Zn]**	**1,00E-50**	**5,49E-05**	**1,14**
	AM854726	**cdn20p0001f21**	**Toll-like receptor 3**	**2,00E-09**	**3,72E-05**	**1,14**
	AM853394	**cdn19p0001i09**	**Superoxide dismutase [Cu-Zn]**	**2,00E-04**	**3,37E-05**	**1,16**
	CU682412	**oygd10b10j02**	**Thyroid peroxidase**	**7,00E-27**	**2,32E-04**	**1,21**
	AM869445	cdn37p0036k04	Testis-specific serine/threonine-protein kinase	4,00E-13	1,45E-04	1,23
	AM856093	cdn21p0001c15	Superoxide dismutase [Cu-Zn]	7,00E-23	4,70E-05	1,25
	AM858229	**cdn37p0003j12**	**T-complex-associated testis-expressed protein**	**1,00E-50**	**5,61E-05**	**1,27**
	CU685371	oypg09b06j05	Metallothionein	1,00E-06	2,81E-04	1,28
	AM856775	**cdn21p0003b03**	**Prostaglandin E2 receptor**	**7,00E-22**	**5,26E-05**	**1,28**
	AM856052	cdn21p0001a22	Heat shock protein 105 kDa	2,00E-27	4,40E-05	1,32
	AM855723	cdn20p0004p01	Gametogenetin-binding protein	2,00E-66	4,17E-05	1,32
	AM855417	cdn20p0003m12	Peroxisomal carnitine O-octanoyltransferase	1,00E-64	3,87E-05	1,34
	FP089924	oyio09b02n18	Soma ferritin	2,00E-21	2,91E-04	1,36
	CU681715	oygd09b07k07	Integrin beta-1	1,00E-17	2,00E-04	1,40

Table 1 (continued)

Tissue	Genbank accession	Sigenae name	Swiss-Prot hit description	*E* value	*p* Value ANOVA	Fold change R/S
	AM856731	cdn21p0002p05	Glutathione peroxidase	4,00E-55	5,11E-05	1,42
	AM855992	cdn20p0005n21	NA		4,38E-05	1,44
	AM853235	**cdn19p0001b12**	**Complement C1q-like protein 4**	**2,00E-09**	**2,90E-05**	**1,46**
	CU682055	oygd09b08b20	Heat shock protein 75 kDa, mitochondrial	2,00E-48	2,25E-04	1,47
	AM855989	cdn20p0005n15	Metallothionein	1,00E-06	4,26E-05	1,48
	AM854299	cdn19p0004b14	Spermatogenesis-associated protein	2,00E-21	3,69E-05	1,49
	AM866067	cdn37p0026k10	Zinc metalloproteinase	9,00E-15	6,62E-05	1,50
	AM854090	cdn19p0003h13	C-type lectin domain family	3,00E-05	3,60E-05	1,53
	AM854760	cdn20p0001h11	Glutathione peroxidase	8,00E-25	3,76E-05	1,53
	CU684330	oyge10b13e24	Thyroid peroxidase	1,00E-16	2,52E-04	1,53
	BQ427137	BQ427137	Metallothionein	4,00E-10	1,68E-04	1,56
	AM857298	cdn21p0004j22	Prostaglandin E2 receptor	2,00E-20	5,38E-05	1,59
	AM859290	cdn37p0006k17	Peroxiredoxin	1,00E-81	5,81E-05	1,59
	CU681745	**oygd09b07i13**	**B-cell lymphoma/leukemia**	**1,00E-11**	**2,10E-04**	**1,62**
	AJ582629	AJ582629	Defensin	9,00E-07	1,20E-01	1,63
	AM853310	cdn19p0001e18	Extracellular superoxide dismutase [Cu-Zn]	8,00E-10	3,21E-05	1,68
	AM866641	cdn37p0028g21	Catalase	1,00E-101	9,30E-05	1,71
	AM856573	cdn21p0002h14	C-type lectin domain family 4 member	5,00E-15	4,96E-05	1,77
	AM867444	cdn37p0030o16	Bone morphogenetic protein 15	4,00E-06	1,35E-04	1,80
	AM853865	cdn19p0002n04	Heat shock protein 10 kDa, mitochondrial	5,00E-35	3,47E-05	1,82

Figures

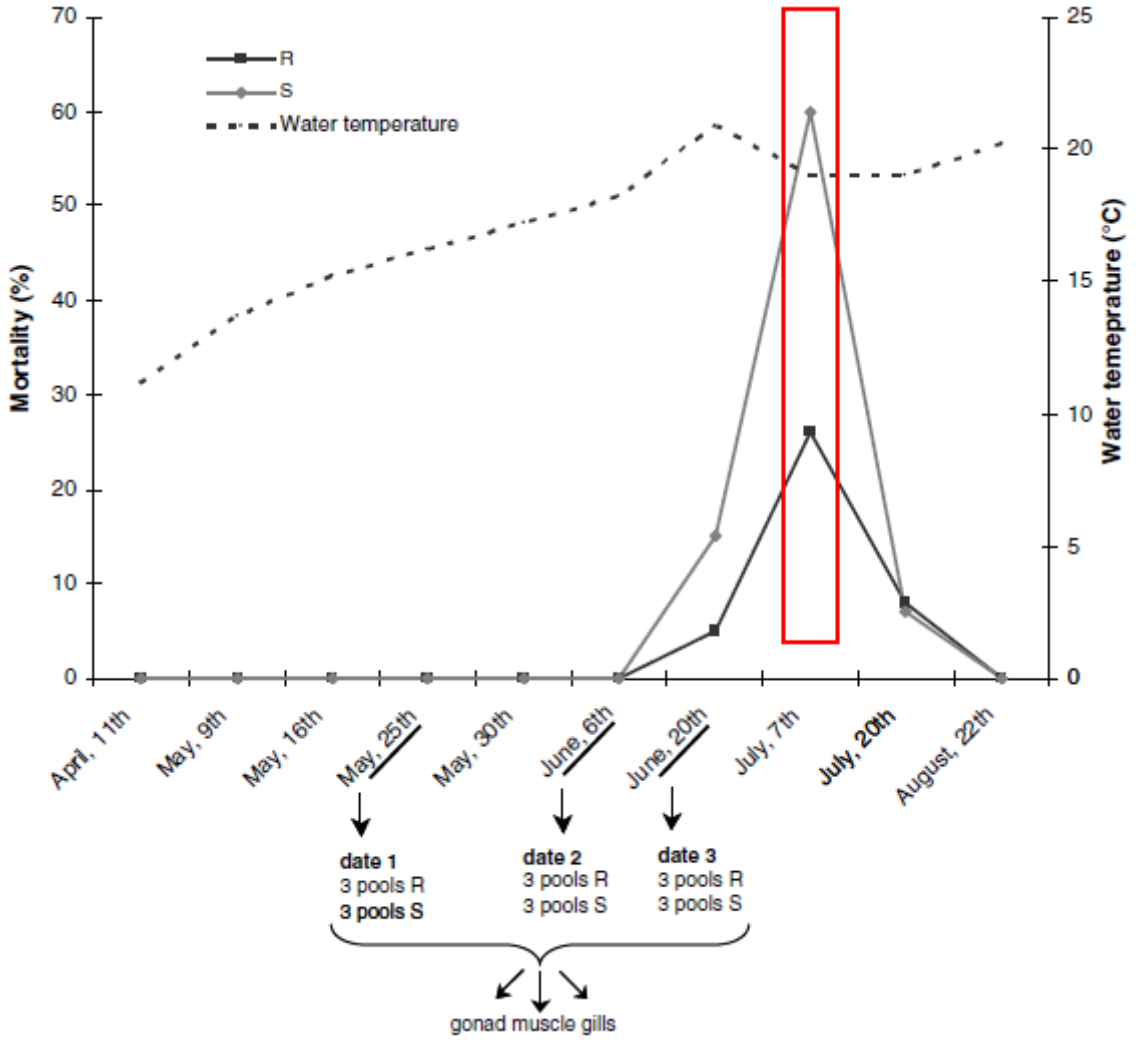

Figure 1: Mortality rates of Resistant (black) and Sensitive (gray) oysters lines during a field experiment (Fort Espagnol, Britanny, France 2005). The sampling dates were used to assess the mortality rates of the studied oysters. The three sampling dates used for microarray analyses are underlined; date 1: May, 25th, date 2: June, 6th, and date 3: June, 20th. For each date, three tissues were sampled in 3 pools containing 8 oysters: gonad, muscle and gills. The box indicates the mortality peak.

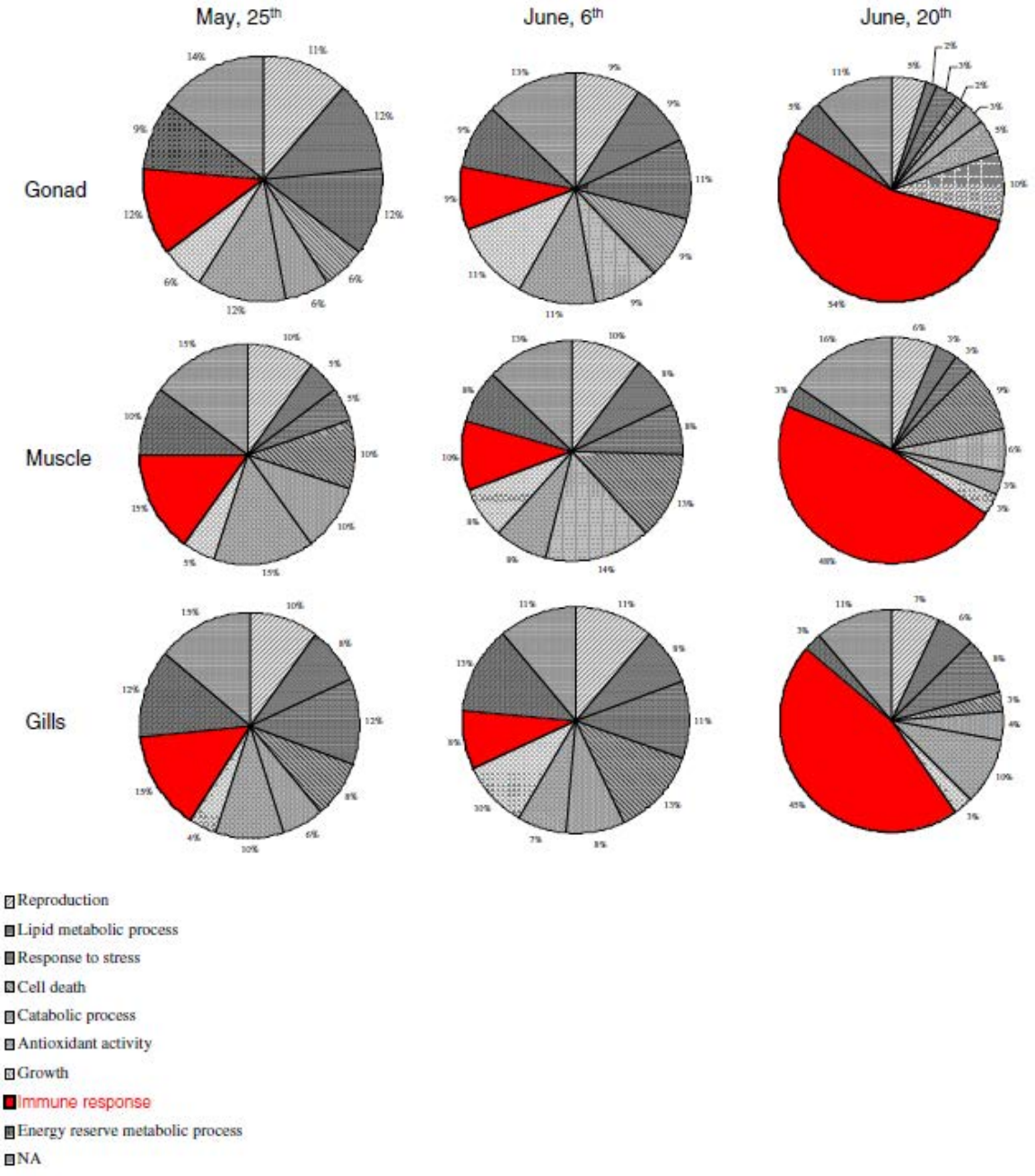

Figure 2: Gene ontology analysis of the differentially expressed genes between resistant and susceptible oyster lines for three sampling dates and three tissues (muscle, gills and gonad). The three dates, May 25th, June 6th and June 20th, are in columns. Each part of the graph represents the percentage of a specific biological process among the selected genes. More specifically, the percentage of genes associated with "immune response" biological process for each date and each sample are shown in red.

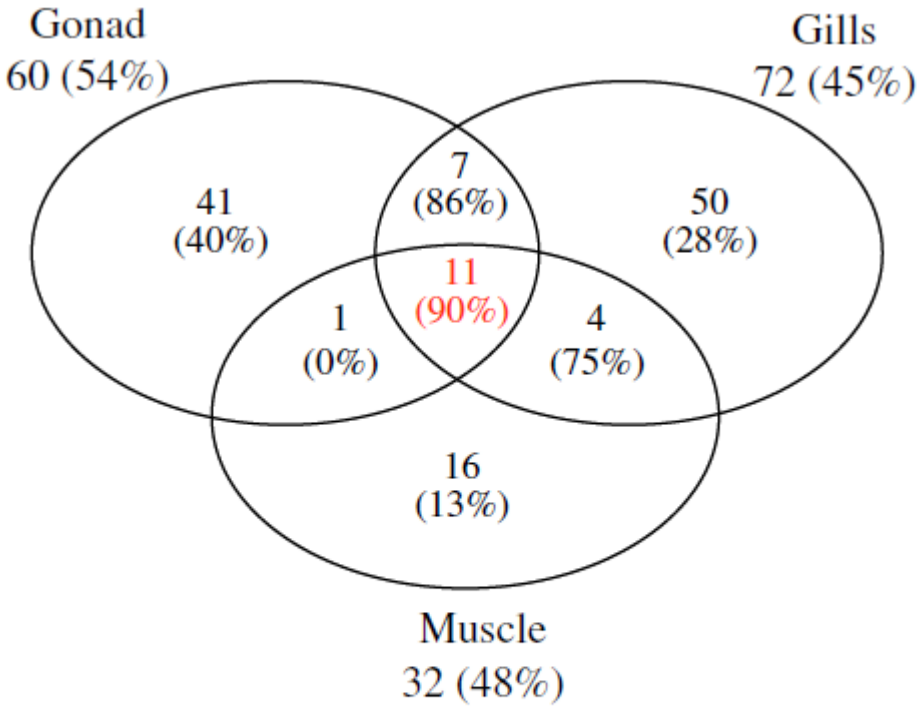

Fig. 3 Venn diagram representing the common selected ESTs between resistant and susceptible oyster lines sampled at the date June 20th for three tissues (muscle, gills, and gonad). The number of selected ESTs is given followed into brackets by the percentage of ESTs linked to "immune response" gene ontology category

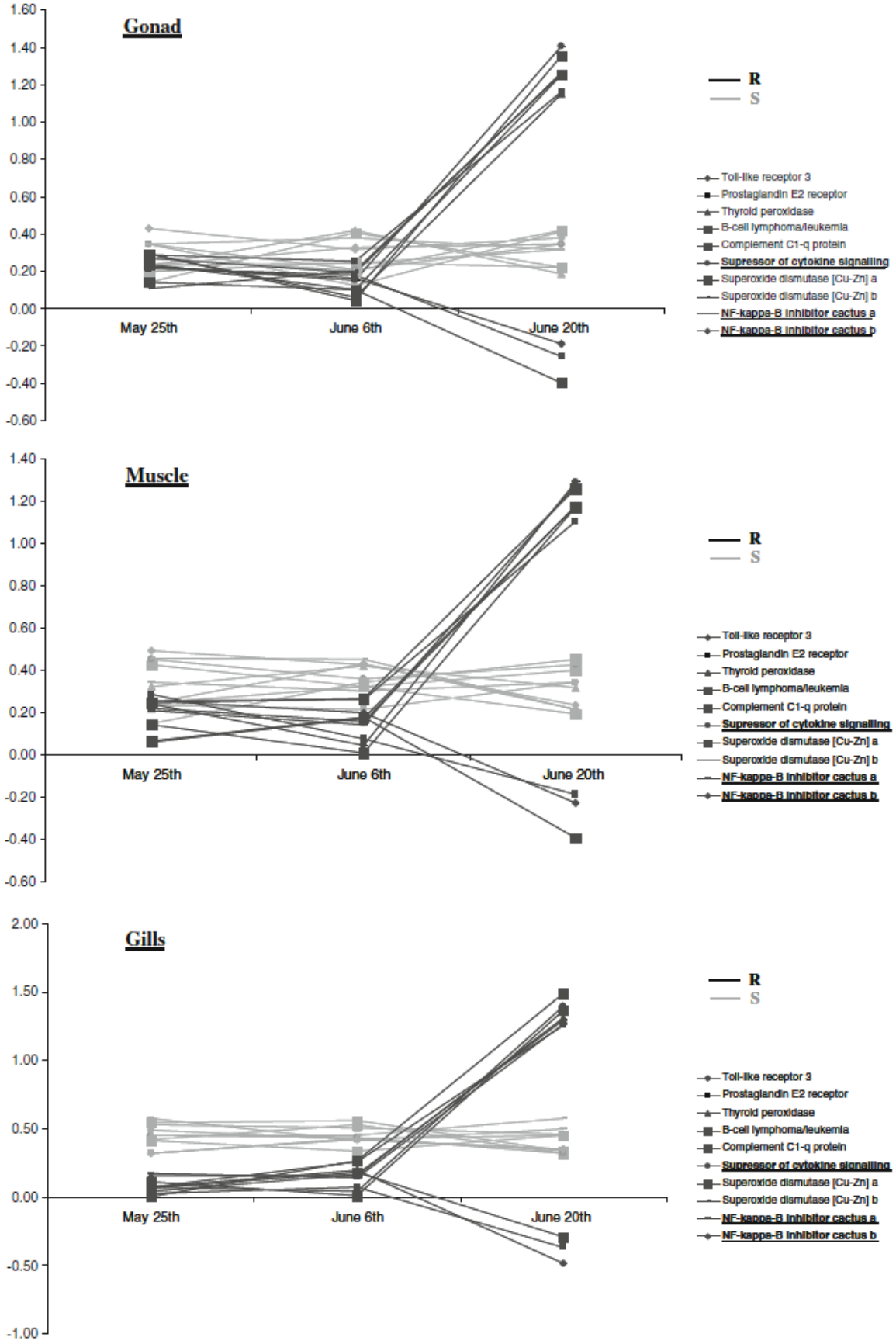

Figure 4: Time-course patterns for the 10 immune-relevant ESTs selected as differentially expressed between resistant and susceptible oyster lines inferred from the microarray data. X axis represents the three dates of sampling (May 25th, June 6th and June 20th) for susceptible (S, grey) and resistant (R, black) oysters. Y axis represents the transformed normalized Cy5 log value obtained for R and S samples for each dates. Genes which are significantly under-expressed in R compared with S samples at June 20th are underlined. The results of each tissue, gonad, muscle and gills are presented separately.