Marine Genomics
September-December 2010, Volume 3, Issues 3-4, Pages 133-143
http://dx.doi.org/10.1016/j.margen.2010.08.005

Transcriptomic response of the Pacific oyster *Crassostrea gigas* to hypoxia

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Abstract:

Marine intertidal organisms commonly face hypoxic stress during low tide emersion; moreover, eutrophic conditions and sediment nearness could lead to hypoxic phenomena; it is indeed important to understand the molecular processes involved in the response to hypoxia. In this study the molecular response of the Pacific oyster *Crassostrea gigas* to prolonged hypoxia (2 mg O_2 L^{-1} for 20 d) was investigated under experimental conditions. A transcriptomic approach was employed using a cDNA microarray of 9058 *C. gigas* clones to highlight the genetic expression patterns of the Pacific oyster under hypoxic conditions. Lines of oysters resistant (R) and susceptible (S) to summer mortality were used in this study. ANOVA analysis was used to identify the genes involved in the response to hypoxia in comparison to normoxic conditions. The hypoxic response was maximal at day 20. The principal biological processes up-regulated by hypoxic stress were antioxidant defense and the respiratory chain compartment, suggesting oxidative stress caused by hypoxia or an anticipatory response for normoxic recovery. This is the first study employing microarrays to characterize the genetic markers and metabolic pathways responding to hypoxic stress in *C. gigas*.

Keywords: Mollusca; Hypoxia; cDNA microarray; Gene expression; Oxidative stress; Respiratory chain

1. INTRODUCTION

The hypoxic response in marine mollusks is a challenging subject to study, even if they are adapted to low oxygen concentrations, hypoxia can act as a stress factor too. In particular, benthic intertidal communities can face hypoxic conditions. On one hand, they are exposed twice a day to oxygen deprivation during low tide and they have developed appropriate survival mechanisms [1]. On the other hand, excessive anthropogenic input of nutrients can lead to hypoxic phenomena caused by algal bloom or by anoxic sediment nearness [2]. The first step of the hypoxic response in tolerant organisms is an increase in water pumping-ventilation, in an attempt to maintain oxygen delivery [2, 3]; then, when critical O_2 concentrations occur, the metabolic rate decreases. This mechanism is called “metabolic depression” or “hypometabolism”, and is not a passive shut down of cellular metabolism, but rather a highly organized suppression of energy consuming mechanisms [4]. As the oxygen concentration decreases, anaerobic fermentative pathways replace aerobic ATP-producing mechanisms. Alternative routes of anaerobic carbohydrate catabolism are less efficient in producing ATP and do not provide enough energy to maintain aerobic consumption; hence metabolic depression is a well-regulated response to the lower availability of ATP, facilitating increased survival [4].

In this context, the Pacific oyster *Crassostrea gigas*, a bivalve distributed worldwide owing to aquaculture practices, is an example of an intertidal sessile organism tolerant to oxygen deprivation during low tide emersion. Significant mortality of *C. gigas* is observed during the summer months in several countries since 70’s [5], and it has recently been demonstrated that hypoxic phenomena may have a role in these summer mortality events. Experiments performed within the framework of the multidisciplinary “Morest” project [6] suggested an involvement of stress response under hypoxic conditions. The increased activity of catalase and glutamine

synthetase enzymes and an augmentation of metallothionein concentrations were observed during exposure to hypoxia for 7 days at 30% O_2 saturation [7]. To date, only one study on global gene regulation under hypoxic stress has been conducted on *C. gigas*. A suppression subtractive hybridization (SSH) analysis performed on oysters undergoing prolonged hypoxia highlighted the over-expression of genes implicated in many physiological pathways such as respiration, carbohydrate metabolism, lipid metabolism, oxidative metabolism, and the immune system [8]. Other studies on the hypoxic stress response of *C. gigas* focused on energetic metabolism. Changes in energetic metabolism were observed in oyster muscle under hypoxia, using pyruvate kinase (PK) and phosphoenolpyruvatecarboxykinase (PEPCK) as indicators for the switch between the aerobic and anaerobic pathways of ATP production [9]. The same authors determined that the oxygen critical point threshold (PcO2), at which oysters switch to anaerobic metabolism, is approximately 3 mg O_2 L^{-1} depending on the water temperature [10]. The Morest project also suggested a genetic heritability for resistance to summer mortality [11], which offered the possibility to develop lines of oysters that were 'Resistant' (R) or 'Susceptible' (S) to summer mortality [11]. R and S lines differ in their reproductive effort [11] [12], energetic metabolism [11], immunity [13], and oxidative metabolism [13]; thus, R and S lines could be utilized to identify hypoxic response mechanisms under summer mortality conditions.

The main objective of the present study was to compare normoxic and hypoxic oysters in order to identify the genes and the metabolic pathways involved in the hypoxic response; moreover, R and S oysters were utilized to investigate if there were differences in the gene expression profiles between the two lines, as differences in their hypoxic responses have been observed [7] [13]. We used a microarray that was produced within the Network of Excellence "Marine Genomic Europe" (http://www.marine-genomics-europe.org/) and the European Union-funded project "Aquafirst" (http://www.sigenae.org/aquafirst/), and it contained 9058 *C. gigas*

93 expressed sequence tags (ESTs), isolated from several cDNA libraries and the SSH bank. In
94 this study, R and S oysters were submitted to long-term hypoxia at 2 mg O_2 L^{-1}, the threshold
95 below which bivalves can no longer maintain a sufficient rate of oxygen consumption [14]. The
96 experiment took place in summer during oysters' gametogenesis. This is the first study to
97 investigate the transcriptomic response to hypoxia in oysters using microarray analysis.
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2. MATERIALS and METHODS

2.1 Experimental design and animal sampling

Twelve-months-old oysters from the fourth generation of the R and S *C. gigas* lines were used in the experiment. The lines were produced at Ifremer Hatchery in La Tremblade (Charentes-Maritime, France) in 2004 as described by Fleury *et al*. [15]. At the end of the larval rearing period, each family was settled on cultch (ground oyster shell). The spat was transferred to the Bouin nursery (Vendée, France) for culturing away from mortality risks. Twelve-months-old oysters (March 2005) were transferred in the field at Fort Espagnol (South Brittany, France). The oysters were cultured in triplicate bags fixed on racks. From April to August, the oysters were checked and counted to monitor mortality (April 11, May 9, 16, 25, and 30, and June 6 and 20), and dead oysters were removed.

The experiment was performed at the Ifremer laboratory in Argenton (North Brittany, France) in June 2005. The oysters were placed in six 300 L experimental tanks with 20 µm filtered running seawater, renewed with a continuous seawater flow of 50 L h^{-1}. Three tanks were used for each normoxic and hypoxic condition. R and S oysters were placed in each tank in separate small cases and in an equal number. The oysters were first acclimatized for 1 week to the laboratory conditions, and the temperature was progressively increased from the initial temperature of 16°C up to 20°C. This temperature was chosen to mimic the environmental conditions that occur during the summer mortality period. During the acclimatization and experimental periods, the oysters were continuously supplied with the microalga *Isochrysis galbana* at a concentration of approximately 100 cells μL^{-1}. The algae/oyster ratio (dry weight) was maintained at 25%. The medium was continuously homogenized using a submerged pump (1200 W). Normoxic conditions were maintained in 3 tanks continuously supplied with

seawater saturated in oxygen (8.5 mg L^{-1}, 100% O_2 saturation at 20°C). Hypoxia was permanently maintained in the 3 remaining tanks for 20 d, and the oxygen levels were reduced by bubbling nitrogen to obtain 2 mg O_2 L^{-1} (30% oxygen saturation at 20°C). To avoid the exchange of oxygen between the seawater and the air, floating PVC plates were placed on the surface of the water. Each tank was connected to a system that collected measurements every 2 h 30 min to control for dissolved oxygen and chlorophyll levels [16].

Oysters were sampled at days 0, 2, 10, and 20 after the beginning of hypoxia. On each date, the digestive gland from 6 oysters was dissected, pooled, and stored at -80°C in Extract-All Reagent (Eurobio) at a concentration of 1 mL/50 mg tissue until total RNA was extracted. For each condition (hypoxia and normoxia) and oyster line (R and S), 4 pools were sampled (biological replicates) for a total of 56 pools during the 4 days of sampling. To ensure that each individual oyster contributed equally to the pool, 30 mg of tissue was sampled from each oyster. Furthermore, the entire tissue from 10 wild oysters was collected, pooled, and homogenized in Extract-all Reagent (Eurobio) to constitute a single total RNA sample to use as a reference in all slide hybridizations and Real-Time PCR analyses.

2.2 RNA preparation

Samples were homogenized using a Pro Polytron tissue disruptor. Total RNA was then isolated using Extract-all Reagent (Eurobio). All of the extractions were performed by the same experimenter. RNA quality was assessed using an Agilent Bioanalyzer 2100 and RNA 6000 Nano Kit (Agilent Technologies) according to the manufacturer's instructions. RNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific), and the samples were stored at −80°C until use. Five µg of total RNA were directly labeled by reverse transcription using the Direct ChipShot Direct Labeling and Clean-Up Kit (Promega),

according to the manufacturer's recommendations. The samples were labeled with Cyanine-5 (Cy5). The reference samples were labeled with Cyanine-3 (Cy3) in separate tubes following the same protocol. The Cy3-labeled cDNAs were pooled and re-divided to obtain a homogeneous reference sample. All dye incorporation rates were verified using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

For Real-Time PCR analysis, RNA samples were first treated with 0.5 U RQ1 RNase-Free DNAse (Promega) per µg of total RNA, precipitated in 3 M sodium acetate and 95% ethanol, washed twice in 70% ethanol, and finally diluted in 10 µL of RNAse/DNase-free water. Total RNA quality was assessed using an Agilent Bioanalyzer 2100 and RNA 6000 Nano Kit (Agilent Technologies), and RNA quantity was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Reverse transcription was carried out with the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas), according to the manufacturer's instructions, on 1 µg of total RNA using random hexamer primers to start the reaction.

2.3 Microarray hybridization and scanning

The cDNA microarray slides were prepared and printed as described by Fleury *et al.* [15]. Briefly, the slides contained 11088 features spotted in duplicate and were printed at the Max Planck Institute (Berlin, Germany) in May 2007. cDNA samples and references were evaporated in a SpeedVac and mixed into a single pool in equimolar amounts with the Chip Hybe hybridization buffer (Ventana Discovery). Hybridization was performed at the INRA IFR 140 Transcriptomic Facility (Rennes, France) using a Ventana automatic hybridization station (Ventana Discovery). The slides were filled with a pre-hybridization buffer (Chip Spread buffer containing 4× SSC and 0.2% SDS) for 1 h at 42°C. Hybridization was conducted

overnight at 42°C in the automatic station according to the manufacturer's instructions. After hybridization, the arrays were washed twice with Ribowash solution (0.1 M Tris, 0.05 M EDTA, and 0.4 M NaCl) and once with 0.1× SSC, and finally centrifuged (6,000 rpm, 15 s, room temperature) for drying. Microarray slides were scanned using a Genepix 4000B microarray scanner (Axon Instruments Inc.) with standard dual laser excitation at 532 nm (17 mW) and 635 nm (10 mW) according to the following parameters: Cy5 Photo Multiplier Tube (PMT) 550 and Cy3 PMT 590. Images (16-bit TIF) were analyzed using GenePix pro 5.1 software (Axon Instruments Inc.) according to the manufacturer's instructions. Spots were filtered for quality according to the parameters described by Fleury *et al*. [15]; spots not fulfilling these criteria were eliminated.

2.4 Microarray data analysis

Microarray data were initially processed using the language R/BioConductor [17] (R Development Core Team 2008). LOESS normalization and background correction were performed with the limma package [18]. All corrected and normalized hybridization values are deposited in the gene expression omnibus (GEO) repository with the accession number GSE23883.

Statistical analyses to identify the differentially expressed genes ($p < 1.0 \times 10e^{-4}$) were carried out by variance analysis using GeneANOVA software [19]. The false discovery rate (FDR) associated with the selected genes was determined by the following formula: [total number of analyzed genes (9058) × *p* value / number of differentially expressed genes] ×100; the FDR cut-off value was < 5%. Three ANOVA were performed using GeneANOVA software: an initial global ANOVA with all hybridized samples, a "line" ANOVA accounting for the hypoxic R and S oysters, and an ANOVA on day 20 samples only. Factors for the global

199 ANOVA were: “duplicate” (technical replicate within the array), “day” (days 0, 2, 10, and 20),
200 “stress” (hypoxia or normoxia), and “line” (R or S). The factors for the ANOVA on the R and
201 S lines were “duplicate” (technical replicate within the array) and “line” (R or S oysters). The
202 factors for the ANOVA on day 20 samples were “duplicate” (technical replicate within the
203 array) and “stress” (hypoxia or normoxia).
204 Annotations according the Swiss Prot Database (E-value < $1.0 \times 10e^{-5}$) were identified using
205 the GigasDatabase [20]. Hierarchical clustering analysis using the average linkage method was
206 performed with TMev software (Multi Experimental Viewer, TM4 Microarray Software Suite
207 [21]). Gene ontology (GO) analysis was performed using Blast2GO software [22] to identify
208 the biological processes in which the selected genes were involved.
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210 ***2.5 Real-Time PCR analysis***
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212 Eight identified-microarray genes were selected for their relevant putative annotation and
213 screened using real-time RT-PCR to validate the microarray results. Five different
214 housekeeping genes were screened: four classic housekeeping genes (ribosomal 28s, actin,
215 glyceraldehyde-3-phosphate dehydrogenase GAPDH, elongation factor 1 alpha EF1) and a
216 non-annotated EST (AM854995) from the microarray chosen on the basis of its low variability.
217 The geNorm algorithm [23] and the variation coefficient were used to determine the most
218 stable gene directly from its cycle threshold (Ct) value.
219 All primers, except for EF1 [24], were designed using Primer Express software V 2.0 (Applied
220 Biosystems). PCR efficiency (*E*) was estimated for each primer pair by serial dilutions (from
221 1/20 to 1/640) of the reference cDNA sample (the same sample used for the common Cy3
222 reference in the microarrays). The primers efficiency was determined by the slope of the

standard curves by the following formula: $E = 10^{[-1 / slope}$ [25]. Primer sequences, GenBank accession numbers of the sequences, and PCR efficiencies are listed in Table 1.

Real-Time PCR was carried out in triplicate in a final volume of 10 µL, using 4.86 µL of cDNA (1/80 dilution) with 5 µL of Absolute QPCR SYBR Green ROX Mix (Thermo Scientific) and 0.07 µL of each primer (70 nM) in a 7300 Real-Time PCR System (Applied Biosystems). Runs started with a 15 min activation of the Thermo-Start DNA Polymerase at 95°C, followed by 45 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, and a melting curve program from 95–70°C by decreasing the temperature 0.5°C every 10 s in order to assess the specificity of the amplification reaction. Each run included the cDNA common control, a no template control, and a water control. Real-Time PCR Ct values were subtracted from the respective housekeeping gene (hkg) values (for each sample: ΔCt = Ct target gene – Ct hkg), the obtained data were then compared to the microarray values.

3. RESULTS

3.1 Identification of differentially expressed genes

Good hybridization quality resulted in 2% of eliminated spots for each array after the filtering step, and the mean correlation coefficient between technical duplicates on each slide was 0.8. The first global ANOVA with all factors identified 1694 genes that were differentially expressed for the factor "day" ($p < 1.0 \times 10e\text{-}^{4}$, FDR = 0.05%), 26 for the factor "stress" ($p < 1.0 \times 10e^{-4}$, FDR = 3.31%), and 73 for the factor "line" ($p < 1.0 \times 10e^{-4}$, FDR = 1.21%). No differentially expressed genes were found for the "duplicate" factor. No significant interactions were found between the factors "day", "stress", and "line". The differentially expressed genes for the factors "day" and "line" are listed in Appendices A and B, respectively, including the GenBank accession numbers, ANOVA *p*-values, and putative annotations according to the Swiss-Prot database (E-value < $1.0 \times 10e^{-5}$). Among the 26 differentially expressed genes for the factor "stress", 10 had a putative annotation according to the Swiss-Prot database. Clusters, GenBank accession numbers, ANOVA *p*-values, putative annotations, and E-value for the genes differentially expressed for the factor "stress" are presented in Table 2. The hierarchical clustering of genes differentially expressed in the first global ANOVA for the factor "stress" is presented in Figure 1. The 26 differentially expressed genes did not show a clear trend of expression depending on the different conditions, but they were clearly divided into two groups: the first one contained genes that were slightly under- or over-expressed, and the second one contained genes that were strongly over-expressed. The first cluster contained the following 5 annotated genes: a protein similar to the human fibrinogen alpha chain protein involved in blood coagulation (AM854350, UniProtKB P02671), an epidermal grow factor-like domain 10 involved in the phagocytosis of apoptotic cells by macrophages (BQ427312,

UniProtKB Q96KG7), S-adenosylmethionine synthetase involved in one-carbon metabolism (AM854702, UniProtKB Q91X83), a dehydrogenase whose ligand is NADP (AM855507, UniProtKB Q6DF30) and a peroxisomal oxidoreductase (CU685657, UniProtKB A4FUZ6). Conversely, the second gene-cluster contained 10 genes, 5 of which were annotated as: ferritin involved in iron storage and potentially involved in antioxidant response via Fenton's reaction (AM854714, UniProtKB P42577), superoxide dismutase converting the superoxide anion into oxygen peroxide (CU681762), a phosphotransferase (AM854767, UniProtKB A2RU49), a ganglioside activator involved in lipid metabolism (CU681763, UniProtKB Q8HXX6), and a transcription inhibitor (CU685227, UniProtKB A5LFW4). Two main sample-clusters were identified from visual inspection. All samples from the hypoxic conditions (days 2, 10, and 20) were clustered together, opposite to the normoxic conditions (days 0, 2, and 10). The only exception was that the normoxic samples from day 20 were regrouped with the hypoxic samples, but their expression profile differed considerably.

In order to assess if there were differences in gene expression under the hypoxic conditions between the R and S oysters, ANOVA was performed only on the hypoxic R and S samples. No gene was differentially expressed with an acceptable FDR according to the factor "line"; therefore, no differences between the R and S oysters were considered for the following analysis.

The results from the global approach showed a greater impact of the factor "day" and a clear gene expression signature at day 20, in which the differences between the hypoxic and normoxic oysters appeared stronger; thus, for the following analysis, we focused only on data from day 20 of sampling. ANOVA on day 20 revealed 647 differentially expressed genes for the factor "stress" ($p < 1.0 \times 10e^{-4}$, FDR = 0.14%), and no genes for the "duplicate" factor. From these 647 differentially expressed genes from the factor "stress", 319 accounted for 50% of the variance. Among these 319 genes, 152 showed a homology with genes of known

function (Swiss-Prot database E-value < $1.0 \times 10e^{-5}$), and were retained for further analysis. Hierarchical clustering by average linkage on these genes (Figure 2) was performed to identify similar patterns of gene expression. Three main clusters were identified from a visual inspection. The first cluster contained 19 genes that were slightly under-expressed in normoxic conditions and strongly under-expressed in hypoxic conditions. The second cluster contained 103 genes that were under-expressed in normoxic conditions, but slightly over-expressed in hypoxic conditions. The third cluster contained 30 ESTs that were slightly over or under-expressed in normoxic conditions and strongly over-expressed in hypoxic conditions. The clusters, GenBank accession numbers, ANOVA *p*-values, putative annotations, and E-values are presented in Table 3 for the genes retained from the day 20 ANOVA for the factor "stress." GO analysis was performed using Blast2GO on the genes retained from the ANOVA analysis on day 20. The following 10 main biological processes (GO level 2) were represented among these selected genes: cellular processes (51.1%), multicellular organismal processes (20.6%), developmental processes (16.6%), response to stimulus (16.6%), metabolic processes (15%), biological regulation (12.5%), localization (9%), growth (6.6%), reproduction (4.7%), and immune system processes (1.2%). The biological processes (GO levels 2 and 3), the number of sequences, and the percentages from GO analysis are presented in Table 4 and in Appendix C (ordered by score).

3.2 Real-Time PCR analysis

The analysis of the stability of the five housekeeping genes (28s, GAPDH, actin, EF1, cdn20p0002c12) using the geNorm algorithm indicated that actin had the lowest expression stability measure (M = 0.085) and variation coefficient (0.043) (Table 1). However, given that the actin gene resulted differentially expressed between oysters exposed to hypoxic conditions

311 in a previous study [8], we consider that the actin gene was not suitable for this study.
312 Therefore, the GAPDH gene was chosen as housekeeping gene for internal standardization,
313 because it was the most stable gene after actin (M = 0.088) and previously used as reference
314 gene in several oysters gene expression studies [26, 27, 28]. Gene expression levels for Real-
315 Time PCR and microarray analysis are presented in Figures 3a and 3b, respectively. Results
316 showed no significant differences were found between normoxia and hypoxia samples using
317 Real-Time PCR. To determine which technique was more reliable we hypothesized that the
318 more repeatable observations were the more reliable. Firstly, the standard deviation of the
319 expressed value was calculated for each gene on technical replicate (duplicate of spot for
320 microarray, and Real-Time PCR triplicates). Standard deviation was then plotted against the
321 gene expression value (log ratio for microarray and Ct for Real-Time PCR). For the Real-Time
322 PCR results, we observed a statistically significant augmentation of the standard deviation with
323 lower levels of gene expression (Ct > 25) ($p << 0.01\%$, correlation coefficient = 0.305). We did
324 not observe the same result for microarrays ($p = 1.2\%$, correlation coefficient = -0.113), the
325 standard deviations remained stable for all ranges of gene expression. Furthermore, we
326 performed a comparison of the standard deviations of the two techniques. The Real-Time PCR
327 data were firstly normalized (ΔCt – average / standard deviation), and the distribution of the
328 two series of standard deviations was compared (Figure 3c for Real-Time PCR data and Figure
329 3d for the microarrays). The median was two times more elevated for Real-Time PCR data than
330 for the microarrays (0.160 and 0.088, respectively), and was statistically significant ($p <<$
331 0.01%, Mann-Whitney bilateral test). This result showed that Real-Time PCR is two times less
332 accurate than microarray analysis.
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4. DISCUSSION

In this study we investigated the transcriptomic response of *C. gigas* to hypoxia using cDNA microarray analysis. Oysters kept in normoxic conditions were compared to oysters exposed to long-term experimental hypoxic conditioning (2 mg O_2 L^{-1}, approximately 30% oxygen saturation, at 20°C for 20 days). First global ANOVA identified 1694 genes that varied significantly according to the four dates of sampling (factor “day”) independently from the effects of hypoxia or the oyster line. This high number of genes could be explained by the fact that the oysters underwent strong gene regulation during the active gametogenesis period, as shown by Fleury *et al*. [15] who used the same microarray slides on R and S oyster lines in an *in situ* experiment. We hypothesized that hypoxic stress may play a role as an environmental stressor during summer mortality events. The Morest project indicated that R and S oysters appear to be different in terms of oxidative management when subjected to hypoxia [13]. S oysters produced more reactive oxygen species than R oysters, and R oysters activated antioxidant enzymes such as catalase and glutathione transferase under hypoxic conditions [13]; however, no genes were differentially expressed between the R and S lines exposed to hypoxia in this study. This may confirm, as already suggested [11, 12], that the resistance mechanisms would rather take place at the reproduction level, initially *via* differential energy investment between the two lines.

The hypoxic response was more important after 20 days of low oxygen exposure; therefore, we can infer that the hypoxic effects appeared late in such a low oxygen-tolerant species. Gray *et al*. [29] classified marine organisms according to their tolerance to hypoxia, and bivalves demonstrated the least sensitivity. Their growth was affected by oxygen concentrations lower than 1.5 mg O_2 L^{-1}, while the growth and metabolism of fish and crustaceans were affected by concentrations of 6 and 4 mg O_2 L^{-1}, respectively. Furthermore, Le Moullac *et al.* [10]

demonstrated that 3 mg O_2 L^{-1} is the oxygen critical point threshold (PcO2) at which oysters switch to anaerobic metabolism.

We observed that the 152 annotated differentially expressed genes accounted for 50% of the variance were generally over-expressed in comparison to the normoxic conditions, except for a limited set of genes (cluster 1) that were under-expressed in the hypoxic conditions; indeed, we could not infer a global metabolic depression at the transcriptional level. This result is not surprising because, as shown in other organisms, metabolic depression induced by hypoxia mainly concerns protein synthesis, while transcription remains at the basal level, is slightly reduced, or can even be activated, e.g., some genes required for the re-oxygenation process [1]. Heterologous array analysis on *Littorina littorea* after exposure to anoxia demonstrated that only 0.6% of the sequences were down-regulated [1]. Some studies support the idea that transcripts remain untranslated in sequestrating complexes and hidden from the translation apparatus [30, 31]. Transcripts are then kept available as an anticipatory response for a quick restart of metabolism when normoxic conditions return [32]. Nonetheless, GO analysis of the selected genes differentially expressed in the hypoxic conditions at day 20 indicated that 6 sequences showed GO codes for negative regulation of transcription (4.6%). Moreover, the differential expression of a transcriptional inhibitor of cell differentiation (GB CU685227) identified in the first global ANOVA suggests that some transcriptional processes were repressed. These results indicate that even if gene expression appears to be maintained or even activated during hypoxia from the genes present on our microarray, some transcriptional processes are negatively regulated. Furthermore, 8 sequences (1.8%) were identified using Blast2GO analysis as negative regulators of cellular metabolic processes; indeed, this result indicates that a slowing down of metabolism occurs after 20 days of exposure to hypoxia. In comparison to previous studies of energetic metabolism in oysters undergoing hypoxic stress, we observed no change in the regulation of the PK and PEPCK genes using the microarray

approach. Le Moullac *et al.* [9] observed that PEPCK transcription and enzymatic activity were induced after 10 days of exposure to hypoxia (2 mg O_2 L^{-1}), and PK was initially down-regulated at the enzyme activity level, at day 10, and later at the transcriptomic, level at day 20. The difference of results in this study is probably due to the tissue specific regulation of the energetic metabolism; the switch between aerobic and anaerobic pathways observed by Le Moullac *et al*. [10] occurred in the muscle, the first storage organ in oysters. Effects of air exposure on energetic metabolism are even more rapid, as previously observed in a study on acid-base changes in oysters *C. gigas* exposed to air [33]. Muscle resulted affected within the first 12 h of air exposure by a significant acidification of the tissue pH, but no results are presented on digestive gland. In another recent study on hemolymph biochemical changes on oysters *C. gigas* exposed to air, an acidification of the hemolymph pH after 3 days of exposure was observed [34]; anaerobic metabolism, in fact generally causes acidification because the final acid end products of fermentative pathways are accumulated in tissues.

Between the genes differentially expressed for the factor “stress” in the global ANOVA, 4 of the 10 annotated ESTs are involved in redox balance or antioxidant defense: 2 oxidoreductase genes (a dehydrogenase whose ligand is NADP, AM855507, and a peroxisomal oxidoreductase, CU685657), ferritin (AM854714), and superoxide dismutase (CU681762). This may indicate a role for the cellular redox balance in the hypoxic response and an involvement of antioxidant defense mechanisms. Furthermore, it is of interest the over-expression of many genes coding for enzymes involved in antioxidant defense and reactive oxygen species (ROS) detoxification within the genes differentially expressed at day 20: glutathione-S-transferase (AM858066), extracellular superoxide dismutase (AM853310), superoxide dismutase (AM856093), peroxiredoxin 5 (CX068955), ferritin (AM854714), and NADP transhydrogenase (AM857387), another enzyme involved in the maintenance of the cellular redox state. Indeed, this study highlights the importance of the antioxidant

409 compartment in the hypoxic response. Moreover, the Blas2GO analysis demonstrated that
410 “response to stimulus” was the fourth most prevalent biological process (16.62% of the
411 selected sequences, including 29 sequences). Within this process “response to stress” and
412 “response to abiotic stimulus” contained 16 (10.7%) and 11 sequences (2.9%), respectively
413 (Table 4).
414 The relationship between low oxygen concentration and oxidative stress are still unclear.
415 Logically, a lower oxygen concentration would result in the reduced production of ROS, as
416 shown by a study on oyster hemocytes (*C. virginica*) exposed to hypoxia, in which ROS
417 production was 33% of that under normoxic conditions [35]. However, many studies on
418 hypoxic regulation in marine organisms indicated that antioxidant responses were induced
419 during hypoxia. Ferritin was up-regulated at 6 h and 48 h of hypoxic exposure in the grass
420 shrimp *Palaemonetes pugio* [36]. Studies on fish are quite discordant, in a transcriptomic study
421 of the Japanese medaka *Oryzias latipes* (Actinopterygii), 4 genes linked with oxygen and ROS
422 metabolism were down-regulated in the liver after exposure to strong hypoxia (0.17–0.21 mg
423 O_2 L^{-1}) for 6 d [37]; however, in many cases, the genes and enzymes involved in the
424 antioxidant response are up-regulated, e.g. the ferritin gene in the hypoxia-tolerant goby fish
425 *Gillichthys mirabilis* after a short exposure (90 min) to hypoxia (0.8 mg O_2 L^{-1}) [38]. The
426 enzymatic activity of superoxide dismutase (SOD) and catalase in the estuarine fish *Leistomus*
427 *xanthurus* was increased during exposure to hypoxia (0.8 mg O_2 L^{-1}) for 12 h [39]. SOD
428 enzymatic activity was also increased in the scallop *Chlamys farreri* at 4.5 and 2.5 mg O_2 L^{-1}
429 after 12 h [40]. Finally, a previous study on the transcriptomic response to hypoxia using SSH
430 on *C. gigas* under the same hypoxic conditions indicated that some genes of the oxidative
431 metabolism (glutathione peroxidase and glutathione transferase) were up-regulated after 7–10 d
432 of exposure to hypoxia [8]. Recently, authors have agreed that low oxygen concentrations can
433 cause the augmentation of ROS [41], at least at the beginning of hypoxia. The underlying

mechanisms are still unknown, but it was suggested that a slowing down of the respiratory chain, caused by a lower oxygen concentration, may increase the lifetime of intermediate ubisemiquinone forms (a link between complexes II and III) in the electron transport chain, leading to ROS production [41]. Hence, in this study, the activation of the oxidative stress response could be a direct effect of hypoxia or an anticipatory technique for the reoxygenation process. In fact, an oxidative “burst” can occur when returning to normoxic conditions. This mechanism of cellular hypoxia-reoxygenation injury is well known and described in studies on ischemia [42].

In our study, many genes of the respiratory chain were also over-expressed: NADH-ubiquinone oxidoreductase chains 1, 2, 4, and 5 (complex I of the respiratory chain), succinate dehydrogenase (complex II), cytochromes b and c (complex III), and cytochrome c oxidase subunits 1, 2, 3, and 5A (complex IV), indicating that all of the complexes of the electron transport chain were involved. This up-regulation has already been observed in others animals under low oxygen conditions such as *Littorina littorea*, *Chrysemys picta*, and *Rana sylvatica* [4], as well as in the *C. gigas* hypoxia SSH library [8]. The reason for this up-regulation is not known, but one explanation could be that the majority of these genes, being encoded in the mitochondria, are all transcribed from a unique mitochondrial promoter, as proposed by Storey [4]. Another hypothesis is that the activation of the respiratory chain is necessary to maintain the energy requirement to survive prolonged hypoxia, as suggested by Le Moullac *et al.* [10]. Finally, a stock of untranslated mRNA of respiratory chain subunits may be a mechanism that anticipates the return to normoxic conditions, as suggested by Larade and Storey [1], in order to rapidly re-activate electron transfer in the respiratory chain.

Finally, the comparison of 8 selected genes between Real-Time PCR and microarray analysis demonstrated that the trend of gene expression detected by these two techniques goes in the same direction; however, the Real-Time technique was less precise and accurate, while

459 microarray analysis was more repeatable and reliable. Real-Time PCR is the most commonly
460 used tool to validate the result from microarray analyses; however, the results from these
461 techniques could be in disagreement. It is well documented that both techniques have pitfalls
462 that may influence the data interpretation [43]. It has also been demonstrated that the
463 correlation between these techniques increases with increased gene expression, commonly a 2-
464 fold change is considered as the limit below which microarray analysis and Real-Time PCR
465 begin to lose correlation [43]. Actually, within the genes studied using Real-Time PCR, only
466 NADP transhydrogenase and peroxiredoxin had a fold change > 2. Furthermore, it has recently
467 been demonstrated that elevated genetic polymorphisms in the Pacific oyster can generate
468 artifacts in Real-Time PCR [44] and call into question the use of this technique as a tool to
469 validate transcriptome analysis.
470
471 **5. CONCLUSIONS**
472
473 The present study is the first microarray-based approach to assess the response to hypoxic
474 stress in the pacific oyster *Crassostrea gigas*. We observed the involvement of the oxidative
475 stress response compartment and the up-regulation of respiratory chain genes. This study
476 constitutes an initial high yield screen of the hypoxic response of oysters. More precise
477 investigations of individual genes should be performed using transcriptomic and physiologic
478 approaches. In particular the link between oxidative stress, hypoxia, and respiratory chain
479 regulation should be investigated by coupling gene expression analysis to enzymatic activity.
480 Furthermore, measures of ROS production should be performed and coupled to measures of
481 mitochondrial respiration under hypoxic conditions.
482
483

Acknowledgements This study was conducted under the network “REX Marine Genomics Europe” and the European program “Aquafirst”. We thank P. Boudry, A. Huvet and J. Moal from IFREMER Brest for involvement on these European projects. We tank IFREMER La Tremblade staff for the oyster production, and IFREMER Brest and Argenton staff for the animal conditioning and sampling. The authors are also grateful to A. Hénaut from UMR CNRS 8116, Tour Evry (France), for the precious help on microarray/Real-Time PCR comparison. We thank A. Le Cam and J. Montfort from INRA-SCRIBE (Rennes, France) for the technical assistance on the transcriptomic facility. We acknowledge the Max Plank Institute of Berlin, Germany, for the preparations on cDNA slides. R. Sussarellu was founded by the French Research Ministry doctoral grant.

REFERENCES

[1] K. Larade, K.B. Storey, Chapter 3 A profile of the metabolic responses to anoxia in marine invertebrates, in: K.B. Storey and J.M. Storey (Ed.), Sensing, Signaling and Cell Adaptation, Elsevier, 2002: pp. 27-46.

[2] R.S.S. Wu, Hypoxia: from molecular responses to ecosystem responses, Mar. Pollut. Bull. 45 (2002) 35-45.

[3] D. Tran, A. Boudou, J. Massabuau, Mechanism for maintaining oxygen consumption under varying oxygenation levels in the freshwater clam Corbicula fluminea, Can. J. Zool. 78 (2000) 2027-2036.

[4] K.B. Storey, Molecular mechanisms of anoxia tolerance, Int. Congr. Ser. 1275 (2004) 47-54.

[5] A. Parache, Growth performance of oyster Crassostrea angulata and Crassostrea gigas reared in Arcachon Bay between 1950 and 1986: First results, Haliotis. 19 (1989) 227-236.

[6] J.F. Samain, H. McCombie, Summer mortality of Pacific oyster Crassostrea gigas. The Morest Project, Quae Eds, 2008.

[7] T. Burgeot, B. Gagnaire, T. Renault, D. Moraga, E. David, I. Boutet, et al., Oyster summer morality risks associated with environmental stress, in: Summer Mortality of Pacific Oyster Crassostrea Gigas. The Morest Project, Éd. Ifremer/Quæ, J.F. and McCombie H. (eds), 2008: pp. 107-151.

[8] E. David, A. Tanguy, K. Pichavant, D. Moraga, Response of the Pacific oyster Crassostrea gigas to hypoxia exposure under experimental conditions, FEBS Journal. 272 (2005) 5635-5652.

[9] G. Le Moullac, H. Bacca, A. Huvet, J. Moal, S. Pouvreau, A. Van Wormhoudt, Transcriptional regulation of pyruvate kinase and phosphoenolpyruvate carboxykinase in the adductor muscle of the oyster Crassostrea gigas during prolonged hypoxia, J. Exp. Zool. A.Ecol. Genet. Physiol. 307A (2007) 371-382.

[10] G. Le Moullac, I. Quéau, P.L. Souchu, S. Pouvreau, J. Moal, J.R.L. Coz, et al., Metabolic adjustments in the oyster Crassostrea gigas according to oxygen level and temperature, Mar. Biol. Res. 3 (2007) 357-366.

[11] J.F. Samain, L. Degremont, P. Soletchnik, J. Haure, E. Bedier, M. Ropert, et al., Genetically based resistance to summer mortality in the Pacific oyster (Crassostrea gigas) and its relationship with physiological, immunological characteristics and infection process, Aquaculture. 268 (2007) 227 - 243.

[12] A. Huvet, J. Normand, E. Fleury, V. Quillien, C. Fabioux, P. Boudry, Reproductive effort of Pacific oysters: A trait associated with susceptibility to summer mortality, Aquaculture. 304 (2010) 95-99.

[13] A. Huvet, J. Royer, J. Moal, T. Burgeot, S. Lapègue, V. Buolo, et al., Phenotypic characteristics of "R" and "S" oysters lines, selected for resistance or susceptibility to summer mortality, in: Summer Mortality of Pacific Oyster Crassostrea Gigas. The Morest Project, Éd. Ifremer/Quæ, J.F. and McCombie H. (eds), 2008: pp. 197-241.

[14] S. Bricker, C. Clement, D. Pirhalla, S. Orlando, D. Farrow, National Estuarine Eutrophication Assessment: Effects of Nutrient Enrichment in the Nation's Estuaries, National Oceanic and Atmospheric Administration (NOAA), Science Silver Spring, 1999.

[15] E. Fleury, J. Moal, V. Boulo, J. Daniel, D. Mazurais, A. Hénaut, et al., Microarray-Based Identification of Gonad Transcripts Differentially Expressed Between Lines of Pacific Oyster Selected to Be Resistant or Susceptible to Summer Mortality, Mar. Biotechnol. 12 (2010) 326-339.

[16] S. Blain, J. Guillou, P. Treguer, P. Woerther, L. Delauney, E. Follenfant, et al., High frequency monitoring of the coastal marine environment using the MAREL buoy, J. Environ. Monitor. 6 (2004) 569-575.

[17] R. Gentleman, V. Carey, D. Bates, B. Bolstad, M. Dettling, S. Dudoit, et al., Bioconductor: open software development for computational biology and bioinformatics, Genome. Biol. 5 (2004) R80.

[18] G.K. Smyth, T. Speed, Normalization of cDNA microarray data, Methods. 31 (2003) 265-273.

[19] G. Didier, P. Brezellec, E. Remy, A. Henaut, GeneANOVA-gene expression analysis of variance, Bioinformatics. 18 (2002) 490-491.

[20] E. Fleury, A. Huvet, C. Lelong, J. de Lorgeril, V. Boulo, Y. Gueguen, et al., Generation and

549 analysis of a 29,745 unique Expressed Sequence Tags from the Pacific oyster (Crassostrea gigas)
550 assembled into a publicly accessible database: the GigasDatabase, BMC Genomics. 10 (2009)
551 341.
552 [21] A.I. Saeed, N.K. Bhagabati, J.C. Braisted, W. Liang, V. Sharov, E.A. Howe, et al., TM4
553 Microarray Software Suite, in: DNA Microarrays, Part B: Databases and Statistics, Academic
554 Press, 2006: pp. 134-193.
555 [22] A. Conesa, S. Gotz, J.M. Garcia-Gomez, J. Terol, M. Talon, M. Robles, Blast2GO: a universal
556 tool for annotation, visualization and analysis in functional genomics research, Bioinformatics. 21
557 (2005) 3674-3676.
558 [23] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, et al., Accurate
559 normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal
560 control genes, Genome Biology. 3 (2002) research0034.1 - 0034.11.
561 [24] C. Fabioux, A. Huvet, C. Lelong, R. Robert, S. Pouvreau, J.Y. Daniel, et al., Oyster vasa-like gene
562 as a marker of the germline cell development in Crassostrea gigas, Biochem. Biophys. Res.
563 Commun. 320 (2004) 592 - 598.
564 [25] A. Stahlberg, P. Aman, B. Ridell, P. Mostad, M. Kubista, Quantitative Real-Time PCR Method
565 for Detection of B-Lymphocyte Monoclonality by Comparison of κ and λ Immunoglobulin Light
566 Chain Expression, Clin. Chem. 49 (2003) 51-59.
567 [26] E. Farcy, C. Fleury, C. Lelong, M. Dubos, C. Voiseux, B. Fiévet, et al., Molecular cloning of a
568 new member of the p53 family from the Pacific oyster Crassostrea gigas and seasonal pattern of
569 its transcriptional expression level, Marine Environmental Research. 66 (2008) 300-308.
570 [27] C. Lelong, F. Badariotti, H. Le Quéré, F. Rodet, M. Dubos, P. Favrel, Cg-TGF-[beta], a TGF-
571 [beta]/activin homologue in the Pacific Oyster Crassostrea gigas, is involved in immunity against
572 Gram-negative microbial infection, Developmental & Comparative Immunology. 31 (2007) 30-
573 38.
574 [28] I.D. Medeiros, M.N. Siebert, G. Toledo-Silva, T.B. Rodrigues, M.R. Marques, A.C. Bainy,
575 Induced gene expression in oyster Crassostrea gigas exposed to sewage, Environmental
576 Toxicology and Pharmacology. 26 (2008) 362-365.
577 [29] J.S. Gray, R.S. Wu, Y.Y. Or, Effects of hypoxia and organic enrichment on the coastal marine
578 environment, Mar. Ecol. Prog. Ser. 238 (2002) 249-279.
579 [30] A. Spirin, Masked and translatable messenger ribonucleoproteins in higher eukaryotes, in:
580 Translational Control, JWB Hershey, MB Mathews, N Sonenberg, eds, Cold Spring Harbor, NY,
581 Cold Spring Harbor Laboratory Press, 1996: pp. 319-334.
582 [31] H. Ruan, H. Heider, I. Hohfeld, R. Lyck, E. Schmidt, L. Nover, Analysis of ribosome loading onto
583 mRNA species: implications for translational control, in: mRNA Formation and Function, Richter,
584 J., Ed., Academic Press New York, 1997: pp. 305-321.
585 [32] R.O. Laine, N.F. Shay, M.S. Kilberg, Nuclear retention of the induced mRNA following amino
586 acid-dependent transcriptional regulation of mammalian ribosomal proteins L17 and S25., J. Biol.
587 Chem. 269 (1994) 9693-9697.
588 [33] B. Michaelidis, D. Haas, M.K. Grieshaber, Extracellular and Intracellular Acid‐Base Status with
589 Regard to the Energy Metabolism in the Oyster Crassostrea gigas during Exposure to Air, Physiol.
590 Biochem. Zool. 78 (2005) 373-383.
591 [34] S. Kawabe, M. Takada, R. Shibuya, Y. Yokoyama, Biochemical changes in oyster tissues and
592 hemolymph during long-term air exposure, Fish. Sci. (2010) 10.1007/s12562-010-0263-1.
593 [35] J. Boyd, L. Burnett, Reactive oxygen intermediate production by oyster hemocytes exposed to
594 hypoxia, J. Exp. Biol. 202 (1999) 3135-3143.
595 [36] T. Li, M. Brouwer, Gene expression profile of grass shrimp Palaemonetes pugio exposed to
596 chronic hypoxia, Comp. Biochem. Physiol. Part D Genomics Proteomics. 4 (2009) 196-208.
597 [37] Z. Ju, M.C. Wells, S.J. Heater, R.B. Walter, Multiple tissue gene expression analyses in Japanese
598 medaka (Oryzias latipes) exposed to hypoxia, Comp. Biochem. Physiol. C Pharmacol. Toxicol.
599 Endocrinol. 145 (2007) 134-144.
600 [38] A.Y. Gracey, J.V. Troll, G.N. Somero, Hypoxia-induced gene expression profiling in the euryoxic
601 fish Gillichthys mirabilis, Proc. Natl. Acad. Sci. USA. 98 (2001) 1993-1998.
602 [39] R.U. Cooper, L.M. Clough, M.A. Farwell, T.L. West, Hypoxia-induced metabolic and antioxidant
603 enzymatic activities in the estuarine fish Leiostomus xanthurus, J. Exp. Mar. Biol. Ecol. 279

(2002) 1-20.
[40] J. Chen, K. Mai, H. Ma, X. Wang, D. Deng, X. Liu, et al., Effects of dissolved oxygen on survival and immune responses of scallop (Chlamys farreri Jones et Preston), Fish. Shellfish. Immunol. 22 (2007) 272-281.
[41] R.D. Guzy, P.T. Schumacker, Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia, Exp. Physiol. 91 (2006) 807-819.
[42] C. Li, R.M. Jackson, Reactive species mechanisms of cellular hypoxia-reoxygenation injury, Am. J. Physiol., Cell Physiol. 282 (2002) C227-241.
[43] J. Morey, J. Ryan, F. Van Dolah, Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR, Biol. Proced. Online. 8 (2006) 175-193.
[44] N. Taris, R. Lang, M. Camara, Sequence polymorphism can produce serious artefacts in real-time PCR assays: hard lessons from Pacific oysters, BMC Genomics. 9 (2008) 234.

Table 1. Forward, reverse primers sequences and efficiency (*E*) for Real-Time PCR analyzed genes. The results for the housekeeping genes tested are showed, gene expression stability measure (M) with geNorm algorithm [23] and variation coefficient (Var Coef).

Table 2. Clusters, GenBank access, ANOVA *p*-value (GeneANOVA software, [19]) and putative annotation (Swiss Prot Database, E value < $1.0 \times 10e^{-5}$) for the 26 differentially expressed genes in the global ANOVA for the factor "stress".

Table 3. Clusters, GenBank access, ANOVA *p*-value (GeneANOVA software, [19]) and putative annotation (Swiss Prot Database, E value < $1.0 \times 10e^{-5}$) for the 152 differentially expressed annotated genes for the factor "stress" that explain 50% of variance in the ANOVA on day 20.

Table 4. Gene ontology analysis (Blast2Go analysis [22]) on the 152 annotated genes differentially expressed for factor "stress" that explain 50% of variance in the ANOVA on day 20.

Figure 1. Expression profile of 26 genes resulting differentially expressed with a Global ANOVA (GeneANOVA software, [19]) for the factor "stress". Each column represents one condition of day (Day 0, 2, 10, 20), stress (normoxia=N, hypoxia=H) and lines (resistant=R, sensible=S), and is the average of 4 replicates. Hierarchical gene clustering by average linkage was obtained with TMev software [21]. Red and green represents increase and decrease in transcript abundance, respectively, compared to the reference sample. Sample-cluster, are evidenced with blue, cluster 1, and yellow, cluster 2. Gene-cluster, are evidenced with green, cluster 1, and red, cluster 2. On the right there are the Genbank accession numbers.

Figure 2. Expression profile of 152 annotated genes resulted differentially expressed in the ANOVA on day 20 (GeneANOVA software, [19]) for the factor "stress" that explain 50% of variance. Each column represents the samples averaged gene expression of normoxia (N) and hypoxia (H) conditions. The expression profiles were obtained by hierarchical gene clustering with average linkage with TMev software [21], 3 main clusters were found (cluster 1: yellow, cluster 2: orange, cluster 3: blue). Red and green represents increase and decrease in transcript abundance, respectively, compared to the reference sample.

Figure 3. 3a. Log ratio for samples at day 20 of genes tested by Real-Time PCR; 3b relative expression at day 20 of genes tested by microarray (GST= glutathione transferase, NADoxi2= NAD oxidoreductase chain 2, SOD= superoxide dismutase, CCOI= cytochrome c oxidase I, NADPTr= NADP transhydrogenase, ferritin, NADPmal= NADP malic enzyme, PRX= peroxiredoxin; bars represent standard deviation); 3c Real-Time PCR standard deviation distribution; 3d Microarray standard deviation distribution.

Appendix A. GenBank access, ANOVA *p*-value and putative annotations in accord to the Swiss-Prot database (E-value < $1.0 \times 10e^{-5}$) for the 1694 genes resulted as differentially expressed for the factor "day" of the global ANOVA.

Appendix B. GenBank access, ANOVA *p*-value and putative annotations in accord to the Swiss-Prot database (E-value < $1.0 \times 10e^{-5}$) for the 73 genes resulted as differentially expressed for the factor "line" of the global ANOVA.

Appendix C. Gene Ontology Biological processes ordered by score (Blast2Go analysis [22]), from the analysis on annotated sequences resulted differentially expressed for the factor "stress" on the day 20 ANOVA that explain 50% of the variance.

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+
Day 0 S
Day 0 R
Day 2 PN
Day 2 SN
Day 10 PN
Day 10 SN
Day 20 PN
Day 20 SN
Day 2 PH
Day 2 SH
Day 10 PH
Day 10 SH
Day 20 PH
Day 20 SH
AM857844
AM853400
CU684059
AJ565581
CU683150
AM853641
AM854350
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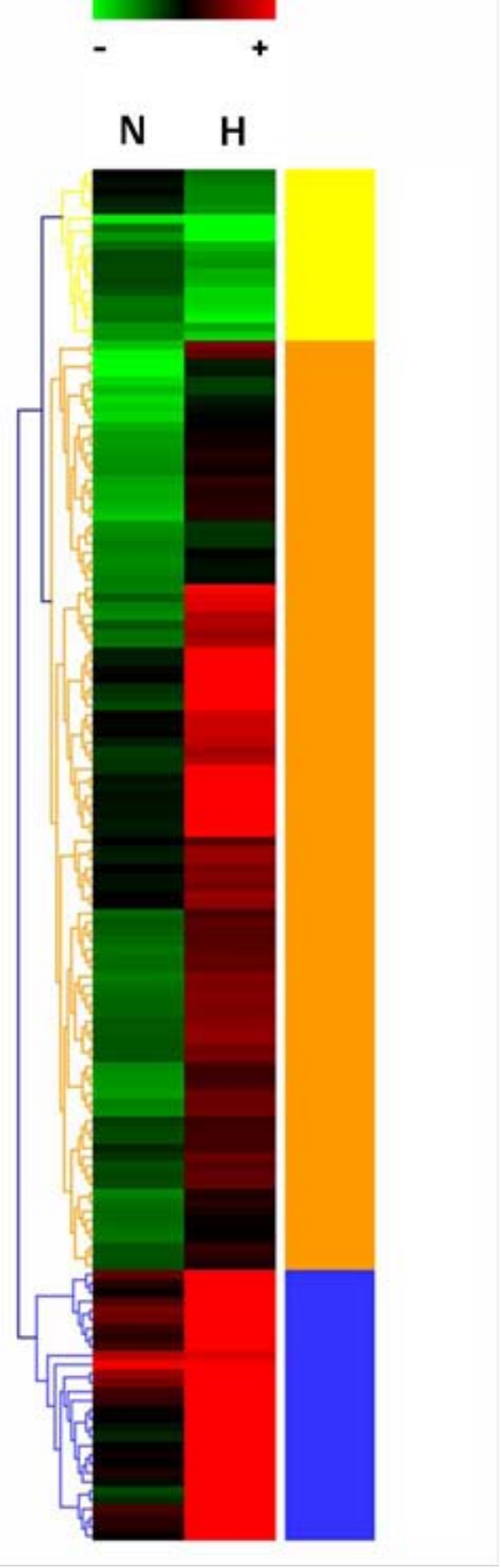
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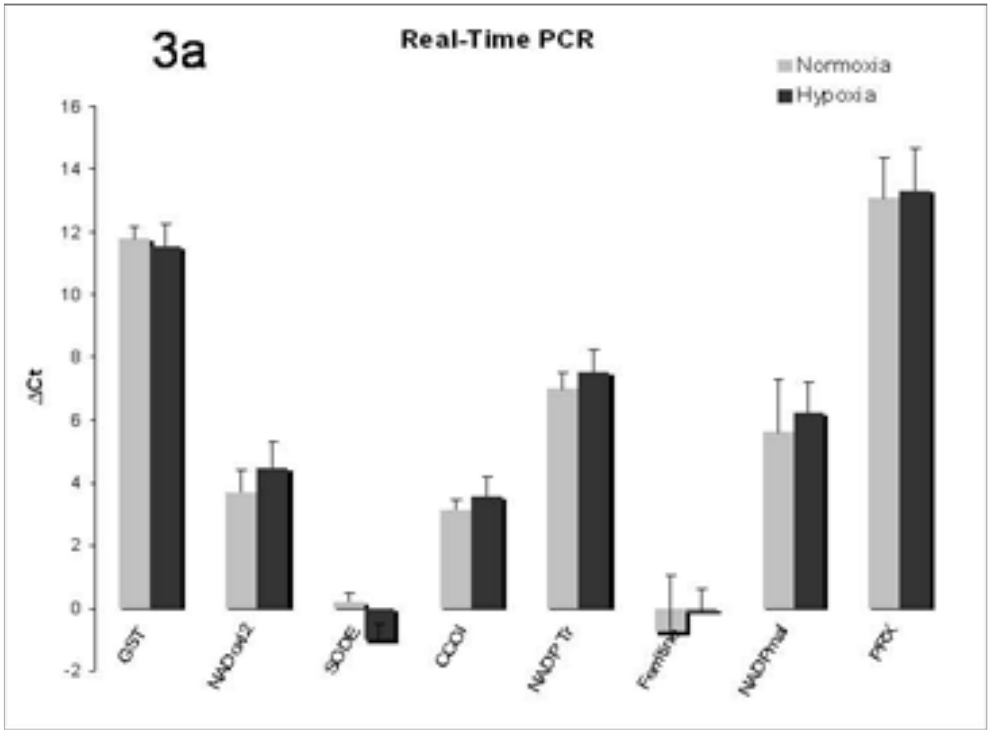
3a
Real-Time PCR
Normoxia
Hypoxia
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14
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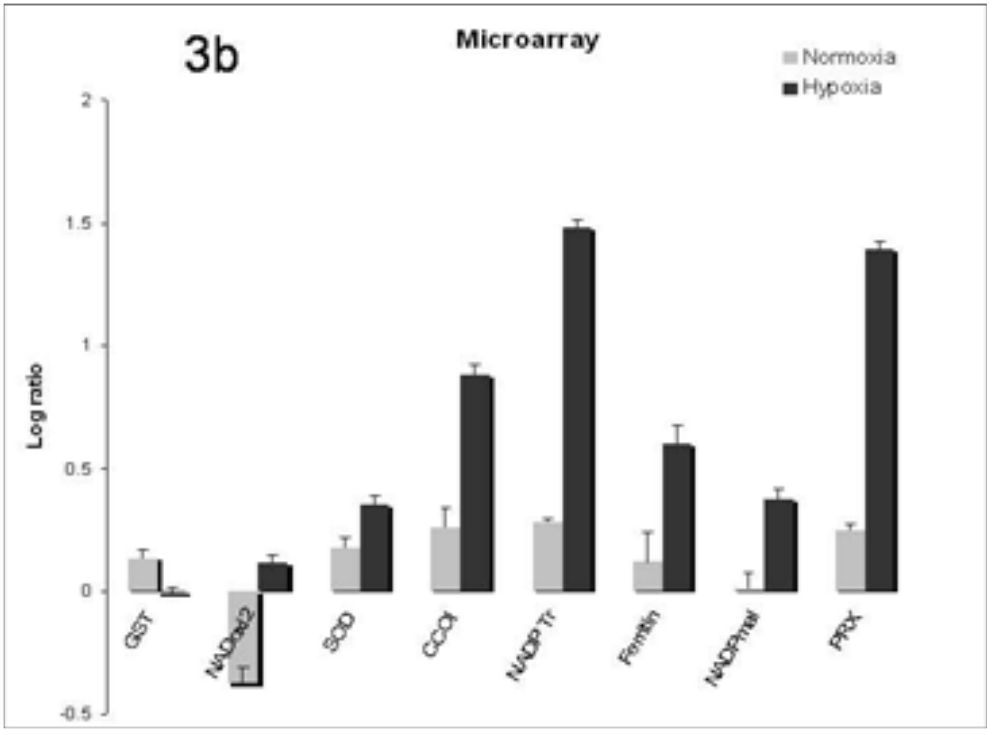
2
0
-2
GST
NADHd2
SODE
CCOI
NADP Tr
Ferritin
NADPmal
PRX


3b
Microarray
Normoxia
Hypoxia
Log ratio
2
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1
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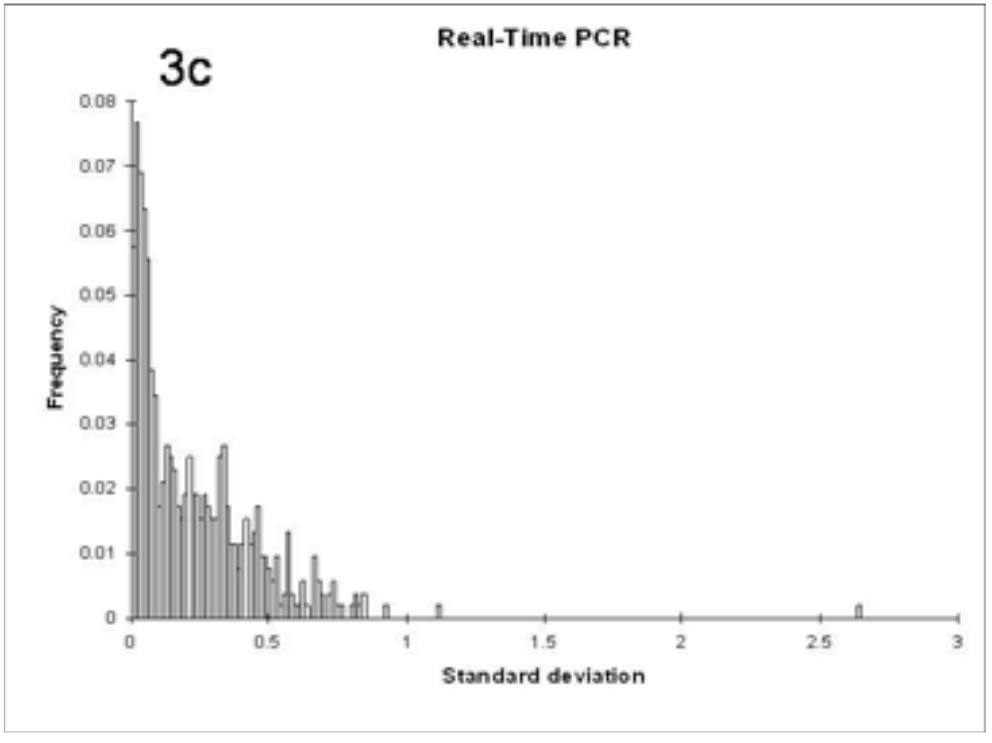
-0.5
GST
NADHd2
SOD
CCOI
NADP Tr
Ferritin
NADPmal
PRX


3c
Real-Time PCR
Frequency
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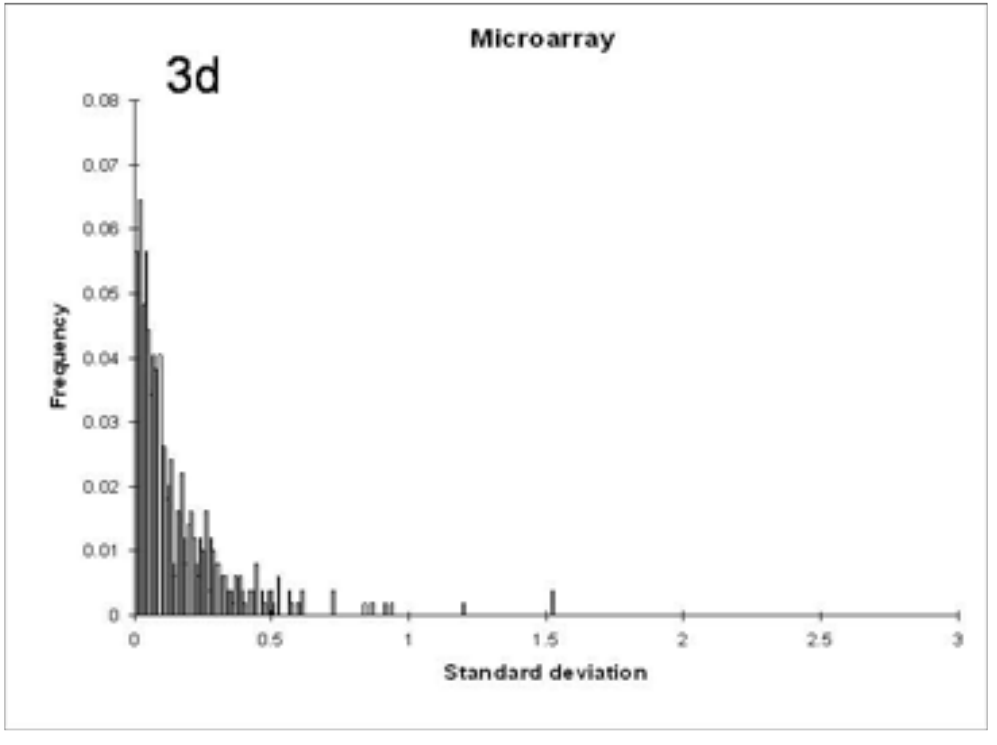
3
Standard deviation


3d
Microarray
Frequency
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3
Standard deviation

Biological process	N of Seq	Score %
Cellular process	82	51.1
Cellular component organization and biogenesis	36	10.1
Cell Communication	22	7.1
Cell proliferation	11	8.6
Cell cycle	7	2.7
Microtubule-based process	5	2.7
Actin filament-based process	5	2.7
Multicellular organismal process	43	20.6
Multicellular organismal development	38	22.4
System process	13	3.5
Developmental process	46	16.6
Anatomical structure development	30	14.0
Cellular developmental process	21	4.1
Death	15	2.4
Response to stimulus	29	16.6
Response to stress	16	10.7
Response to abiotic stimulus	11	2.9
Response to chemical stimulus	8	7.8
Response to endogenous stimulus	9	4.2
Response to external stimulus	7	3.4
Metabolic process	59	15.0
Cellular metabolic process	56	18.8
Primary metabolic process	55	16.8
Macromolecule metabolic process	49	13.2
Biosynthetic process	26	7.8
Catabolic process	8	1.5
Biological regulation	41	12.5
Regulation of biological process	36	13.4
Regulation of biological quality	14	5.7
Regulation of molecular function	8	0.8
Localization	36	9.0
Establishment of localization	29	8.7
Cellular localization	17	2.9
Localization of cell	13	5.5
Macromolecule localization	12	2.1
Growth	14	6.6
Reproduction	18	4.7
Reproductive process	13	2.9
Sexual reproduction	7	1.9
Immune system process	4	1.2