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Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions

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The molecular response to hypoxia stress in aquatic invertebrates remains relatively unknown. In this study, we investigated the response of the Pacific oyster *Crassostrea gigas* to hypoxia under experimental conditions and focused on the analysis of the differential expression patterns of specific genes associated with hypoxia response. A suppression subtractive hybridization method was used to identify specific hypoxia up- and downregulated genes, in gills, mantle and digestive gland, after 7–10 days and 24 days of exposure. This method revealed 616 different sequences corresponding to 12 major physiological functions. The expression of eight potentially regulated genes was analysed by RT-PCR in different tissues at different sampling times over the time course of hypoxia. These genes are implicated in different physiological pathways such as respiration (carbonic anhydrase), carbohydrate metabolism (glycogen phosphorylase), lipid metabolism (delta-9 desaturase), oxidative metabolism and the immune system (glutathione peroxidase), protein regulation (BTF3, transcription factor), nucleic acid regulation (myc homologue), metal sequestration (putative metallothionein) and stress response (heat shock protein 70). Stress proteins (metallothioneins and heat shock proteins) were also quantified. This study contributes to the characterization of many potential genetic markers that could be used in future environmental monitoring, and could lead to explore new mechanisms of stress tolerance in marine mollusc species.

In the last few decades, marine hypoxia has become one of the major ecological concerns in the world, because of the increase of excessive anthropogenic input of nutrients and organic matter into coastal seawater [1]. Benthic communities are the most sensitive parts of the coastal ecosystem to eutrophication and resulting hypoxia [2]. High production in stratified waters results from nutrient enrichment and can cause hypoxic or anoxic bottom waters because of the subsequent deposition of algal biomass [3]. Marine organisms are directly affected by hypoxia at various levels of organization and behavioural, biochemical and physiological responses to limited availability of oxygen have been well studied in fish and marine invertebrates [4]. Most of the invertebrate species that inhabit the intertidal zone, and especially sedentary ones, have developed mechanisms for surviving twice-daily oxygen deprivation at low tide. Depression of metabolic rate can be considered as one of the most important adaptations for hypoxia endurance [5,6]. Many marine molluscs do indeed show reversible protein phosphorylation to limit the activity of many enzymes and functional proteins during anoxia [5,7]. The same response to hypoxia has already been

Abbreviations
GPx, glutathione peroxidase; HIF-1, hypoxia-inducible factor-1; HSP, heat shock protein; MT, metallothionein; SSH, suppression subtractive hybridization.

described at the cellular level in turtle hepatocytes associated with a global decline in protein biosynthesis [8]. Moreover, adaptations to anaerobiosis in marine invertebrates resulting from hypoxia or anoxia include the maintenance of large reserves of fermentable fuels such as glycogen or aspartate, and the production of alternative end products of fermentative metabolism, to increase ATP yield [6]. Hypoxia also favours a decrease in the generation of reactive oxygen species, and thus a decrease in the activity of antioxidant enzymes [9]. The modulation of enzyme activity by hypoxia or anoxia has been extensively studied in marine invertebrates [10–12]. Nevertheless, although the modulation of gene expression by oxygen is widely recognized at a cellular level, molecular responses of marine animals to hypoxia remain largely unknown [13]. Many studies have been carried out on molecular mechanisms of anoxia tolerance in mammals and insects. Induction of hypoxia-sensitive genes by hypoxia-inducible factor-1 (HIF-1) has been demonstrated [14–17]. For example, in mice, four isozyme genes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase family (PFKFB-1–4) were shown to be responsive to *in vivo* hypoxia in different organs [18]. Hypoxia-induced gene expression profiling has also been studied in fish using cDNA microarrays revealing tissue-specific patterns of expression [19]. In invertebrates, specific RNA transcripts have been found that are upregulated during anoxia exposure: a novel gene named *fau* in *Drosphila melanogaster* [20], ribosomal protein L26 [21] and novel genes named *kvn* [22] and sarp-19 [23] in the marine snail *Littorina littorea*. The *d*ADAR gene, that plays a role in the sensitivity to low levels of oxygen, has also been identified in *Drosophila melanogaster* [24]. In marine benthic fauna, we can underline moreover the recent studies of Brouwer *et al*. [25] who used macroarrays and suppression subtractive hybridization to assess gene expression modulation in response to hypoxia in the blue crab *Callinectes sapidus*. However, very few studies have been conducted on patterns of gene expression in conditions of hypoxia in marine molluscs and in particular in bivalves.

The Pacific oyster *Crassostrea gigas* is a bivalve mollusc well distributed along the West European coast. As it can inhabit the intertidal zone, *C. gigas* is submitted to oxygen deprivation during emersion phases, and therefore we can suppose that it has developed strategies to endure the diminution of oxygen availability. However, to our knowledge, there is a lack of studies on hypoxia tolerance of this species at both the molecular and the physiological level. Studies on oysters belonging to the same genus, *C. virginica*, showed regulation of metabolic enzyme activities with hypoxia, suggesting metabolic adaptations of oysters to hypoxia [11,12].

In this study, we report genes involved in the stress response induced by hypoxia in *C. gigas*. First we determined the inhibited and induced genes after 7–10 days and 24 days of hypoxia exposure, using a suppression subtractive hybridization (SSH) method. Then we used RT-PCR to analyse the expression of some particular genes and an ELISA test to quantify two stress-related proteins-heat shock proteins 70 family (HSP70), and metallothioneins (MTs).

Results

Identification of hypoxia regulated genes

Suppression subtractive hybridization libraries were made from pooled digestive glands, gills and mantle of *C. gigas* after 7–10 and 24 days of exposure. The search for homology using the BLASTX program revealed 616 different sequences, with 354 sequences (about 57%) unidentified. Four tables list the sequences obtained from the various SSH libraries: 7–10-days upregulated (122 sequences, Table 1); 7–10 days downregulated (111 sequences, Table 2); 24-days upregulated (186 sequences, Table 3); and 24-days downregulated (196 sequences, Table 4). These results indicate that hypoxia exposure up- and downregulated genes associated to 12 major cellular physiological functions during the experiment: reproduction, stress proteins, protein regulation (including protein synthesis and degradation), nucleic acid regulation (including transcription, cell cycle regulation, and metabolism of nucleic acid components), respiratory chain, structure (including cellular matrix and cytoskeleton), lipid metabolism, cell communication (including immune system and membrane receptors), energetic metabolism (including digestive enzymes), xenobiotic detoxification, metabolism of amino acids and development. Several ribosomal proteins encoding transcripts were also detected in both forward and reverse libraries.

Expression of hypoxia regulated genes

The time-dependent expression of hypoxia regulated genes encoding carbonic anhydrase, glutathione peroxidase (GPx), myc homologue, glycogen phosphorylase, delta-9 desaturase, BTF3, a putative metallothionein and Heat Shock Protein 70, was analysed by RT-PCR using gills, mantle and digestive glands of oysters after 0, 3, 7, 10, 14, 17, 21 and 24 days of hypoxia exposure. Results are summarized in Table 5 and Fig. 1. The

Table 1. Upregulated genes identified after 7–10 days of hypoxia exposure. G, Gills; M, mantle; Dg, digestive gland.

Homologue (protein)	BLASTX value	GenBank accession number	Organ
Cytoskeleton,structure, matrix			
Proximal thread matrix protein 1	7e-08	CX069115	G/M
Thymosin beta 4 chromosome X	3e-12	CX069117	G/M
Matriline 1	3e-14	CX069120	G/M
Actin	2e-82	CX069121	G/M
Alpha-tubulin 2	2e-49	CX069159	Dg
Respiratory chain			
Cytochrome *c* oxidase subunit III	4e-78	AF177226	Dg
Cytochrome *b*	1e-20	AF177226	G/M
NADH dehydrogenase subunit 6	3e-12	AF177226	G/M
NADH dehydrogenase subunit 4	0	AF177226	Dg
NADH dehydrogenase subunit 3	3e-39	AF177226	G/M
NADH dehydrogenase subunit 5	0	AF177226	Dg
Nucleic acid regulation			
Chain A human reconstituted DNA topoisomerase I	9e-14	CX069118	G/M
Myc homologue	2e-5	CX069136 CX069141	G/M Dg
High mobility group protein 1; HMG1	7e-23	CX069137	G/M
Xenobiotique detoxification			
Glutathione *S*-transferase	2e-26	CB617447	G/M
Amino acids metabolism			
Glutamine synthetase	7^{e}-10	CG1753	Dg
Energetic metabolism			
Ran protein	5e-18	CX069126	G/M
Cellulase	2e-21	CX069160	Dg
Carbonic anhydrase	6e-05	CX069170	G/M
Protein regulation			
F box protein FBL5	1e-06	CX069124	G/M
Elongation factor 1 delta	4e-44	CX069125	G/M
Eukaryotic translation elongation factor 2	1e-25	CX069127	G/M
BTF3a	2e-24	CX069131	G/M
Cystatin B	7e-18	CX069133	G/M
Elongation factor 1-alpha	4e-22	CX069156	Dg
RNA polymerase III 53 kDa subunit RPC4	5e-13	CX069158	Dg
Cellular communication, membrane receptor and Immune system			
Calmodulin	1e-51	CX069134	G/M
Low-affinity IgE receptor CD23	4e-15	CX069142	Dg
Glutathion peroxidase	4e-50	CX069146	Dg
Guanine nucleotide-binding protein beta subunit- like protein (receptor for activated protein kinase C)	2e-26	CX069147	Dg
Ribosomal proteins			
Ribosomal protein large subunit	4e-25	CX069116	G/M
Ribosomal protein L6	1e-52	CX069132	G/M
Ribosomal protein L7	2e-71	CX069138	Dg
Ribosomal protein L10a	5e-38	NC_003076	G/M
Ribosomal protein L12	2e-62	CX069140	Dg
Ribosomal protein L15	4e-51	CX069143	Dg
Ribosomal protein L18	1e-67	AJ563457	G/M
Ribosomal protein L19	0	AJ563476	Dg
Ribosomal protein L22	9e-20	CX069149	Dg
Ribosomal protein L31	5e-58	AJ563466	G/M
Ribosomal protein L27A	9e-10	CF369246	G/M Dg
Ribosomal protein S3a	3e-98	CF369245	G/M
Ribosomal protein S4	2e-50	CX069145	G/M
Ribosomal protein S5	2e-74	CB617370	G/M
40S ribosomal protein S18	4e-27	CX069129	G/M

Table 1. (Continued).

Homologue (protein)	BLASTX value	GenBank accession number	Organ
Ribosomal protein S20	2e-44	AJ563463	G/M
Ribosomal protein S27-1	0	AJ563471	Dg
Ribosomal protein S30	9e-30	CX069152	Dg
40S ribosomal protein	1e-21	CX069154	Dg
Unknown function			
Unnamed protein product	2e-18	CX069148	Dg
Hypothetical protein	4e-52	MGC73053	G/M
Hypothetical protein AN8152.2	7e-09	CX069155	Dg
Unknown genes (70 sequences)		CX068761 to CX068830	

carbonic anhydrase revealed a peak of mRNA expression compared to the control between 7 and 10 days, significant in gills ($z = -2.61$; $P = 0.009$; Fig. 1A), mantle ($z = -1.98$; $P = 0.047$) and digestive gland ($z = -2.45$; $P = 0.014$); then expression decreased between 14 and 17 days below the control in gills ($z = -2.40$; $P = 0.016$) and digestive gland ($z = -2.40$; $P = 0.016$); it finally reached a maximum value in the mantle and the digestive gland at 24 days ($z = -2.33$; $P = 0.020$ and $z = -2.45$; $P = 0.014$, respectively). The expression of GPx revealed a more progressive increase to a maximum value reached at 24 days in the three tissues ($z = -2.40$; $p = 0.016$ in gills; $z = -2.61$; $P = 0.009$ in mantle, Fig. 1B, $z = -2.20$; $P = 0.027$ in digestive gland compared to time zero) with, however, a peak at 14 days in mantle ($z = -2.61$; $P = 0.009$; Fig. 1B) and digestive gland samples ($z = -2.14$; $P = 0.133$) compared to control. The expression of the Myc homologue did not show strong variations with hypoxia exposure. After a slight increase in digestive gland at 10 days compared to the control ($z = -1.98$; $P = 0.047$), we can detect a decrease in gills after 17 days ($z = -2.61$; $P = 0.009$; Fig. 1C) and in digestive gland after 14 days of exposure, compared to the control ($z = 2.61$; $P = 0.009$). BTF3 showed a peak of expression between 10 and 14 days of exposure in the gills in comparison to time zero ($z = -2.33$; $P = 0.020$), after 17 days in the mantle compared to time zero and to control ($z = -2.15$; $P = 0.032$; Fig. 1D), and at 10 days in the digestive gland compared to time zero ($z = -2.94$; $P = 0.003$). Expression in digestive glands of exposed oysters was below that of the control at 3 days ($z = 2.61$; $P = 0.009$). The glycogen phosphorylase expression showed a decrease between the third and seventh day of exposure in gills compared to the control and to time zero (respectively $z = 2.61$, $P = 0.009$ and $z = 2.94$, $P = 0.003$) and digestive gland ($z = 3.06$; $P = 0.002$ in comparison to time zero), but increased significantly after 24 days in digestive gland ($z = -2.45$; $P = 0.014$; Fig. 1E). Delta-9 desaturase showed a strong induction between 10 and 17 days of exposure in gills ($z = -2.45$; $P = 0.014$), mantle ($z = -2.61$; $P = 0.009$; Fig. 1F) and digestive gland ($z = -2.20$; $P = 0.027$), in which it then declined after 24 days of exposure ($z = 2.12$; $P = 0.034$). The expression of the putative metallothionein revealed important fluctuations with time exposure. Expression remained under the control level until 7 days of exposure in the three tissues ($z = 2.26$; $P = 0.024$ in gills; $z = 3.06$; $P = 0.002$ in mantle, Fig. 1G, $z = 2.26$; $P = 0.024$ in digestive gland), then it increased in gills and mantle ($z = -2.45$, $P = 0.014$ and $z = -2.61$, $P = 0.009$, respectively, in comparison to the control), before a decrease in mantle (Fig. 1G) and digestive gland ($z = 2.82$, $P = 0.005$ and $z = 2.26$, $P = 0.024$, respectively) at 24 days. HSP70s mRNA levels stayed similar in exposed oysters than in control oysters in the three tissues, until 10 days of exposure in digestive gland when it dropped ($z = 2.45$, $P = 0.014$), and until an increase of expression at 14 days in gills ($z = -2.61$, $P = 0.009$, Fig. 1G). In gills and mantle (Fig. 1H), expression of HSP70 gene decreased after 21 days of exposure ($z = 2.45$, $P = 0.014$ and $z = 2.24$, $P = 0.025$, respectively). However, a peak of expression was observed at 17 days in digestive gland ($z = -2.61$, $P = 0.009$).

The expression of genes involved in hypoxia response showed that this response started very early after the onset of exposure (7 days) and continued until day 24.

Quantification of HSP70 and MTs

Quantification by ELISA showed a significant increase in HSP70 expression in the digestive gland of exposed oysters after 17 days ($z = -2.61$; $P = 0.009$) and after 24 days ($z = -2.61$; $P = 0.009$) of exposure compared to the control (Fig. 2A). The same trends were

Table 2. Downregulated genes identified after 7–10 days of hypoxia exposure. G, Gills; M, mantle; Dg, digestive gland.

Homologue (protein)	BLASTX value	GenBank accession number	Organ
Cytoskeleton,structure, matrix			
Collagen protein	5e-08	CX069163	G/M
Thymosin beta-4 precursor	2e-12	CX069192	Dg
Tubulin, beta polypeptide paralogue	4e-32	CX069204	Dg
Peritrophin	4e-07	CX069206	Dg
Respiratory chain, respiration			
NADH dehydrogenase subunit 4	8e-89	AF177226	G/M
Cytochrome *c* oxidase subunit II	4e-73	AF177226	G/M
Cytochrome *b*	0	AF177226	Dg
NADH dehydrogenase subunit 1	0	AF177226	Dg
Stress proteins			
Putative ethylene-inducible protein	7e-08	CX069189	Dg
Heat shock protein 70	6e-70	CX069205	Dg
Xenobiotique detoxification			
Cytochrome P450 1A1	6e-27	CX069165	G/M
Amino acids metabolism			
Glutamine synthetase 2	7e-10	CX069169	G/M
Energetic metabolism			
Lipopolysaccharide and beta-1,3-glucan binding protein	4e-31	CX069184	Dg
Threonine 3-dehydrogenase	1e-16	CX069187	Dg
Putative 28 kDa protein, partner of Nob1	3e-82	CX069208	Dg
ATP synthase alpha subunit	1e-17	CX069210	Dg
Protein regulation			
Translation elongation factor 1-alpha	5e-80	CX069182	Dg
Elongation factor 2	0	CX069197	Dg
Reproduction			
Vitellogenin precursor	5e-04	CX069172	G/M
Cellular communication, membrane receptor and immune system			
Cavortin	4e-22	CF369147	G/M
Sodium-coupled ascorbic acid transporterl	2e-15	CX069171	G/M
Voltage dependent anion selective channel protein 2	2e-54	CX069174	G/M
Tumor-specific transplantation antigen P198 homologue p23	5e-44	CX069179	Dg
Calmodulin-related protein	5e-13	CX069181	Dg
Translocon associated protein gamma subunit	5e-36	CX069186	Dg
Dopamine-beta-hydroxylase	9e-04	CX069193	Dg
Perlucin	3e-05	CX069194	Dg
Insulin-like growth factor I	6e-05	CX069196	Dg
Solute carrier family 3, member 1	9e-20	CX069198	Dg
Steroid dehydrogenase-like	2e-04	CX069203	Dg
Peroxisomal membrane protein 3	3e-14	CX069207	Dg
Ribosomal proteins			
Ribosomal protein L7a	1e-29	CX069162	G/M
Ribosomal protein L9	2e-28	CX069161	G/M
Ribosomal protein L14	2e-19	CX069164	G/M
Ribosomal protein L17a	7e-44	AJ563474	G/M Dg
Ribosomal protein L15	4e-04	CX069175	G/M
Ribosomal protein L22	2e-19	CX069173	G/M
Ribosomal protein 19-prov protein	3e-18	CX069176	G/M
Ribosomal protein S17	2e-56	CF369144	Dg
Ribosomal protein S10	3e-30	AJ561117	Dg
Ribosomal protein S14	2e-29	CX069188	Dg
Ribosomal protein S3a	e-112	CF369245	Dg
60S acidic ribosomal protein P1	3e-20	CX069191	Dg

Table 2. (Continued).

Homologue (protein)	BLASTX value	GenBank accession number	Organ
Ribosomal protein L28	6e-17	CX069200	Dg
Ribosomal protein L8	3e-63	CX069201	Dg
Ribosomal protein S4	2e-51	CX069209	Dg
Unknown function			
Unnamed protein product	2e-04	CX069167	G/M
Unnamed protein product	1e-11	CX069178	G/M
Hypothetical protein	5e-04	CX069168	G/M
Expressed protein F10B6.29	2e-05	CX069180	Dg
Expressed protein	1e-05	CX069183	Dg
Unknown, protein for image:3343149	4e-42	CX069190	Dg
ENSANGP00000012031	3e-05	CX069195	Dg
Hypothetical protein	1e-56	CX069199	Dg
Unnamed protein product	4e-75	CX069202	Dg
Unknown genes (56 sequences)		CX068831 to CX068887	

observed in gills but were not significant ($z = -1.10$, $P = 0.270$ and $z = -1.71$, $P = 0.086$ after 17 and 24 days, respectively) (Fig. 2B).

Expression of MTs measured by ELISA revealed a significant increase in digestive gland of exposed oyster after 17 days ($z = -1.97$, $P = 0.048$) and 21 days of exposure ($z = -2.45$, $P = 0.014$) before decreasing at 24 days of exposure to the level observed in the control (Fig. 3A). In gills, a nonsignificant increase was observed between 3 and 14 days ($z = -0.18$, $P = 0.854$) in exposed oysters (Fig. 3B).

Discussion

Despite the increase of hypoxia events in coastal ecosystems, only few studies have focused on gene expression patterns of marine organisms subjected to this particular stress. In this paper, we characterized the molecular response to hypoxia exposure under experimental conditions of a marine mollusc, the oyster *C. gigas*. Using a SSH method, we obtained 616 different partial sequences of cDNA, encoding proteins involved in the stress response induced by hypoxia in oysters after 7–10 days and after 24 days of exposure. This approach was previously used to assess the response of aquatic molluscs to various contaminants: pesticides [26] and hydrocarbons [27] in *C. gigas*, or different contaminants in zebra mussel *Dreissena polymorpha* [28].

The method we used allowed us to have an outline of the main physiological functions affected by hypoxia exposure in *C. gigas*, and to understand the regulation process involved in the response to hypoxia. Several physiological pathways have been shown to be regulated by hypoxia stress and among the different genes characterized, several genes appeared to encode proteins involved in oxidative metabolism, confirming a close relationship between hypoxia and reactive oxygen species [29,30]. The same physiological functions were affected in similar studies carried out on the effects of other stresses on *C. gigas*, such as hydrocarbon exposure [27], infection by parasites [31] or exposure to herbicides [26].

Response to hypoxia stress seems to cause a cascade of molecular and physiological processes. Precisely, Hochachka *et al.* [8] described different phases of response to oxygen lack in hypoxia tolerant systems. The authors constructed their theory based on observations in anoxia-tolerant aquatic turtle cells. They suggested that hypoxia-sensing and signal transduction systems are first mobilized to cause a series of molecular processes. Among these processes, they underlined a global decline in protein biosynthesis and a decline in membrane permeability. Larade and Storey [32] observed a reduction of protein synthesis in the periwinkle *Littorina littorea* digestive gland after 30 min of anoxia. The SSH libraries made in this study showed that hypoxia exposure affected mainly genes involved in cell communication and immune system and in protein regulation. Concerning the immune system response, the shrimps *Palaemonetes pugio* and *Peneus vannamei* showed lower survival when injected with *Vibrio* and held under 30% air saturation compared with control held in well-aerated water [33]. This study suggests that the innate immune system is depressed in hypoxia, and can contribute to animal mortality.

Table 3. Upregulated genes identified after 24 days of hypoxia exposure. G, Gills; M, mantle; Dg, digestive gland.

Homologue (protein)	BLASTX value	GenBank accession no	Organ
Cytoskeleton,structure, matrix			
Thymosin beta 4 X chromosome	3e-12	CX069216	G/M
Hemicentin, fibulin 6	1e-05	CX069229	G/M
Actin, cytoplasmic 2	2e-35	CX069237	Dg
Alpha-tubulin	7e-06	CX069245	Dg
Beta-actin 3	3e-49	CX069247	Dg
Respiratory chain, respiration			
NADH dehydrogenase subunit 5	7e-21	AF177226	G/M
NADH dehydrogenase subunit 3	2e-38	AF177226	G/M
Cytochrome c oxidase	2e-04	AF177226	G/M
Cytochrome oxidase subunit 1	0	AF177226	Dg
Detoxification proteins			
Polyamine N-acetyltransferase (spermidine)	3e-10	CX069230	G/M
Spermidine synthase	6e-21	CX069283	Dg
Laccase 2	3e-17	CX069275	Dg
Stress protein			
Metallothionein	3e-06	CX069233	G/M
Heat shock protein 25, isoform b	5e-09	CX069265	Dg
Energetic metabolism			
Glycogen phosphorylase	7e-63	CX069214	G/M
Arginine kinase	0	BAD11950.1	Dg
Sdhb-prov protein	2e-50	CX069267	Dg
Endo alpha-1,4 polygalactosaminidase precursor	1e-30	CX069284	Dg
Lipid metabolism			
Delta-9 desaturase	2e-34	CX069227	M
Fatty acid binding protein 7	3e-16	CX069274	Dg
Protein regulation			
Ubiquitin conjugating enzyme	3e-34	CX069212	G/M
Histone acetyltransferase HPA2	3e-07	CX069224	G/M
CG31019-PA (RNA binding motif prot 5)	3e-04	CX069232	G/M
Translation elongation factor eEF-1 delta-2 chain	2e-27	CX069234	G/M
Elongation factor 1-alpha	0	BAD15289.1	Dg
Alpha-1-inhibitor III precursor	2e-07	CX069244	Dg
Eukaryotic translation initiation factor 6	4e-46	CX069250	Dg
Proteasome 26S non-ATPase subunit 1	4e-06	CX069258	Dg
Homologue of ES1	1e-45	CX069263	Dg
Putative calcium dependent protein kinase	2e-04	CX069266	Dg
Eukaryotic translation initiation factor 3 subunit 6 interacting protein	7e-31	CX069268	Dg
Apopain precursor (Caspase-3)	3e-3	CX069273	Dg
Carboxypeptidase B	3e-57	CX069279	Dg
Cathepsine L-like cysteine protease	3e-44	CX069282	Dg
Protein disulfide-isomerase A6 precursor	1e-32	CX069278	Dg
Cellular communication, membrane receptor and immune system			
Translocon associated protein gamma	7e-21	CX069236	G/M
Chrysoptin precursor	4e-06	CX069239	Dg
Cavortin	0	CF369147 AAP12558.1	Dg
Putative apical iodide transporter	1e-48	CX069249	Dg
Hemagglutinin/hemolysin-related protein	3e-3	CX069251	Dg
Alph-2-macroglobulin, N-terminal and alpha-2-macroglobulin family member	5e-21	CX069254	Dg
Ependymin related protein-1 precursor	1e-14	CX069256	Dg
Prosaposin	1e-10	CX069257	Dg
Calmodulin	7e-04	CX069260	Dg
Sialic acid binding lectin	5e-14	CX069269	Dg

Table 3. (Continued).

Homologue (protein)	BLASTX value	GenBank accession no	Organ
Nucleic acids regulation			
Adenosylhomocysteinase	3e-16	CX069215	G/M
Myc homologue	1e-04	CX069221 CX069261	M
Putative HMG-like protein	0	CAD91447.1	Dg
ENPP4 protein	6e-23	CX069280	Dg
Development, differentiation			
SHG precursor	9e-04	CX069240	Dg
Apextrin	8e-21	CX069241	Dg
Putative sphingosine-1-phosphate lyase	7e-17	CX069242	Dg
DEC-1	2e-05	CX069259	Dg
Ribosomal proteins			
Ribosomal protein	3e-10	CX069211	G/M
Ribosomal protein S11	3e-28	AJ563454	G/M
60S ribosomal protein L37A	1e-23	CX069222	G/M
Ribosomal protein S5	5e-73	AJ563480	G/M
Ribosomal protein L35A	2e-13	CX069238	Dg
Ribosomal protein S6	5e-76	CX069246	Dg
Ribosomal protein L30	4e-22	CX069248	Dg
Ribosomal protein S14A	2e-47	CX069188	Dg
Ribosomal protein S8	0	AJ563461	Dg
Unknown function			
Unnamed protein product	1e-76	CX069223	G/M
ENSANGP00000024201	2e-43	CX069213	G/M
Expressed protein	2e-11	CX069252	Dg
Riken cDNA 1200003O06	2e-07	CX069253	Dg
Hypothetical protein FG01274.1	7e-04	CX069255	Dg
Hypothetical 18K protein	3e-05	CB617354	Dg
MGC64292 protein	4e-16	CX069264	Dg
Zgc: 56211	2e-24	CX069270	Dg
Unknown (protein for IMAGE: 5139212)	2e-55	CX069271	Dg
SnoK-like protein	6e-05	CX069272	Dg
Unnamed protein product	4e-7	CX069276	Dg
CG3051-PC	2e-8	CX069277	Dg
Hypothetical protein	1e-32	CX069285	Dg
Unknown genes(109 sequences)		CX068888 to CX068996	

Our results suggest that energetic metabolism could be affected by exposure to long-term hypoxia in oysters. An upregulation of glycogen phosphorylase mRNA after 24 days of hypoxia exposure was observed. This enzyme is involved in glycogen degradation during glycogenolysis and often activated by hypoxia [34,35]. Taken together, these results suggest that activation of glycogen phosphorylase and of transcription, i.e. expression of this enzyme could thus aim to sustain energy supply in stress situation in oysters. Therefore, despite the decrease in O_2 cell supply induced by hypoxia, ATP production could be maintained in oysters by increasing carbohydrate catabolism and therefore anaerobic metabolim as previously reported in other species [6]. Often, this increase is then replaced by a suppression of the rates of ATP production and of ATP utilization, in order to reduce metabolic rate and ATP turnover rates, and thus to save energy by maintaining ATP supply demand balance [8]. Our results revealed a regulation of expression of genes encoding enzymes of the respiratory chain. In particular, an ATP synthase subunit appeared to be downregulated after 7–10 days of hypoxia exposure. The fact that we observed a downregulation of ATP synthase earlier than an upregulation of glycogen phosphorylase suggests that the series of regulation of these enzymes may be more complex at the trancriptional level than at the level of activity.

Furthermore, still in order to maintain ATP supply demand balance, hypoxia exposure modifies the hierarchy of energy-consuming processes in cells [6]. To

Table 4. Downregulated genes identified after 24 days of hypoxia exposure. G, gills; M, mantle; Dg, digestive gland.

Homologue (protein)	BLASTX value	GenBank accession no	Organ
Cytoskeleton,structure, matrix			
Fibrillin	5e-22	CX069292	G/M
Proximal thread matrix protein 1	5e-7	CX069293	G/M
Myosin subunit essential light chain	5e-25	CX069307	G/M
Alpha-3 collagen type VI	2e-17	CX069310	G/M
Collagen protein	2e-4	CX069315	G/M
Actin 1	1e-23	CX069341	Dg
Cofilin	1e-13	CX069339	Dg
Respiratory chain			
Cytochrome c oxidase subunit II	9e-38	AF177226	G/M
Stress protein			
Superoxide dismutase	8e-5	CX069299	G/M
HSP 70	2e-19	CAC83009	G/M
Y-box factor homologue (APY1)	8e-16	CX069347	Dg
Energetic metabolism			
Alcohol dehydrogenase class III chain	1e-53	CX069325	Dg
Lipid metabolism			
Putative enoyl-CoA hydratase/isomerase family protein	1e-15	CX069345	Dg
Protein regulation			
Ubiquitin	1e-22	CX069287	G/M
Elongation factor 1-alpha	7e-43	BAD15289	G/M
Proteinase inhibitor	1e-9	CX069295	G/M
Eef2-prov protein	9e-61	CX069231	G/M
Translation elongation factor 1-gamma	1e-33	CX069306	G/M
Translation elongation factor 1-delta	3e-30	CX069309	G/M
Ubiquitin/ribosomal L40 fusion protein	2e-63	CX069286	G/M
Hepatopancreas kazal-type proteinase inhibitor	4e-5	CX069319	Dg
Eukaryotic translation initiation factor 4 A, isoform 1	2e-5	CX069326	Dg
Protein kinase, calcium-dependent (EC 2.7.1)	1e-4	CX069337	Dg
Ubiquitin conjugating enzyme	4e-34	CX069340	Dg
Elongation factor 1-delta	7e-33	CX069343	Dg
PP2A inhibitor	4e-49	CX069354	Dg
Amino acid metabolism			
Glutamine synthetase	1e-44	CX069291	G/M
Reproduction			
Male sterility domain containing 1	3e-10	CX069303	G/M
Cellular communication, membrane receptor and immune system			
Calreticulin	2e-10	CX069289	G/M
CAP, adenylate cyclase-associated protein 1	3e-41	CX069294	G/M
Prohormone convertase 1	6e-36	CX069297	G/M
Vertebrate gliacolin C1Q	2e-7	CX069305	G/M
Prothrombinase FGL2 (fibrinogen like 2)	4e-42	CX069318	G/M
Precerebellin-like protein	2e-3	CX069029	G/M
Complement receptor-like protein 3	3e-7	CX069321	Dg
Scavenger receptor cysteine-rich protein type 12	4e-11	CX069350	Dg
Nodulin	2e-15	CX069323	Dg
T-cell activation protein phosphatase 2C	6e-49	CX069356	Dg
Nucleic acids regulation			
Histone protein Hist2h3c1	4e-15	CX069324	Dg
Chain A, human reconstituted DNA polymerase I noncovalent	5e-16	CX069118 CX069353	Dg
Esophageal cancer associated protein	1e-6	CX069352	Dg

Table 4. (Continued).

Homologue (protein)	BLASTX value	GenBank accession no	Organ
Development, differentiation			
TGF beta-inducible nuclear protein 1 (LNR42)	2e−32	CX069355	Dg
Ribosomal proteins			
Ribosomal protein S27-1	1e-41	CAD91436	G/M
60S ribosomal protein L14	2e-19	CX069164	G/M
Ribosomal protein L9	2e-27	CX069161	G/M
Ribosomal protein L18	2e-67	CAD91422	G/M
Ribosomal protein L	4e-69	CX069300	G/M
40S ribosomal protein S14	1e-46	CX069313	G/M
Ribosomal protein L10	1e-36	CX069316	G/M
Ribosomal protein S28	7e-8	CX069317	G/M
Ribosomal protein L7a	6e-29	CX069327	Dg
Ribosomal protein S1	2e-42	CX069330	Dg
Ribosomal protein L10a	1e-34	CX069331	Dg
Ribosomal protein L32	6e-34	CX069333	Dg
Ribosomal protein S2	5e-45	CX069157	Dg
Ribosomal protein L4	2e-53	CX069335	Dg
Unknown function			
Riken cDNA E330026B02	7e-13	CX069288	G/M
Hypothetical protein CBG01956	4e-5	CX069296	G/M
Unnamed protein product	3e-71	CX069301	G/M
Hypothetical protein FG05763.1	2e-12	CX069302	G/M
Hypothetical protein CBG17384	3e-17	CX069312	G/M
Unnamed protein product	1e-5	CX069314	G/M
Hypothetical 18K protein	1e-3	CB617354	G/M
CG6770	4e-8	CX069320	Dg
ENSANGP00000005322	5e-7	CX069322	Dg
ENSANGP00000012272	2e-54	CX069329	Dg
ENSANGP00000010808	8e-64	CX069328	Dg
ENSANGP00000021803	3e-5	CX069332	Dg
Hypothetical protein	4e-5	CX069334	Dg
Unnamed protein product	7e-4	CX069336	Dg
ENSANGP00000021720	4e-18	CX069338	Dg
Unnamed protein product	7e-23	CX069342	Dg
Unnamed protein product	7e-4	CX069344	Dg
ENSANGP00000020091	8e-50	CX069346	Dg
MGC23908 protein	1e-23	CX069348	Dg
Unnamed protein product	5e-13	CX069349	Dg
MGC84748	4e-4	CX069351	Dg
Unknown genes(118 sequences)		CX068997 to CX069114	

sustain ATP supply, transcription rates and protein synthesis drop dramatically. So we analysed the expression of a transcription factor named BTF3. This general transcription factor was initially purified and described from HeLa cells [36]. The protein has been shown to bind to RNA polymerase II, in order to form a transcriptionally active complex. BTF3 was thus supposed to be required for initiation of transcription at several class II promoters but this need is now under discussion [37]. Two isoforms have been described, BTF3a and BTF3b [38]. We focused on BTF3a which is the transcriptionally active isoform. We observed a strong induction of BTF3a mRNA in oysters after 10 days in the three tissues analysed. If hypoxia generally leads to reduced gene transcription, genes whose protein products are likely to play a very important role in anoxia have upregulated transcription during the lack of oxygen [22]. This could explain the BTF3 mRNA upregulation observed in exposed oysters in relation to the transcriptional increase with other specific hypoxia-related genes. Expression analyses of *myc* homologue gene that is involved in nucleic acid regulation showed a two-phase response. At 7 days of hypoxia exposure, we

Table 5. Summary of the results of expression studies in the three tissues. ns, Nonsignificant.

Gene	3	7	10	14	17	21	24
Carbonic anhydrase							
Gills	ns	ns	$+^{c,0}$	$+^{c}$	^{c}	$+^{0}$	ns
Mantle	ns	ns	$+^{c}$	$+^{c}$	$+^{0}$	ns	$+^{c,0}$
Digestive gland	$-^{c,0}$	$+^{c,0}$	$+^{c,0}$	ns	$-^{c}$	$+^{c,0}$	$+^{c,0}$
Glutathione peroxidase							
Gills	$+^{c}$	$+^{c}$	ns	$+^{c}$	ns	$+^{c}$	$+^{c}$
Mantle	$+^{c}$	$+^{c}$	ns	$+^{c}$	$+^{c}$	$+^{c}$	$+^{c}$
Digestive gland	ns	ns	ns	$+^{c}$	ns	$+^{c}$	$+^{0}$
Myc homologue							
Gills	ns	ns	ns	ns	$-^{c,0}$	$-^{0}$	$-^{c}$
Mantle	ns	ns	ns	ns	ns	ns	ns
Digestive gland	ns	ns	$+^{c}$	ns	$-^{cc}$	ns	ns
BTF3							
Gills	ns	$+^{c}$	$+^{0}$	ns	ns	ns	ns
Mantle	ns	ns	$+^{c}$	ns	$+^{c,0}$	ns	ns
Digestive gland	$-^{c}$	$+^{c,0}$	$+^{0}$	ns	$+^{c,0}$	ns	$+^{c}$
Glycogen phosphorylase							
Gills	$-^{c}$	$-^{c}$	ns	ns	ns	$-^{c}$	ns
Mantle	ns	$-^{c}$	^{c}	ns	ns	ns	ns
Digestive gland	$-^{0}$	ns	ns	ns	ns	ns	$+^{c}$
Delta-9 desaturase							
Gills	ns	ns	ns	$+^{c,0}$	$+^{c}$	ns	ns
Mantle	ns	$-^{c,0}$	ns	$+^{c}$	$+^{c}$	ns	ns
Digestive gland	ns	ns	$-^{c}$	ns	$+^{c}$	ns	$-^{c}$
Putative metallothionein							
Gills	$-^{c}$	$-^{c}$	$-^{c,0}$	$+^{c}$	ns	ns	ns
Mantle	$-^{0}$	$-^{c,0}$	ns	$+^{c}$	$-^{c}$	$+^{c}$	$-^{c,0}$
Digestive gland	$-^{0}$	$-^{c,0}$	ns	ns	$-^{c,0}$	$-^{c,0}$	$-^{c}$
HSP70							
Gills	ns	ns	ns	$+^{c,0}$	$+^{0}$	$-^{c,0}$	$-^{c,0}$
Mantle	$+^{0}$	ns	ns	ns	ns	$-^{c}$	ns
Digestive gland	ns	ns	$-^{c}$	ns	$+^{c}$	ns	ns

– Significant decrease at 5%. + Significant increase at 5%. ^{c} Significant difference from corresponding control. ^{0} Significant difference from time zero).

observed an increase of expression in gills, followed by a drop after 17 days in the three tissues. The *myc* homologue belongs to the proto-oncogene family and is involved in the control of cell division; it is also able to elicit the adverse process, programmed cell death [39]. To our knowledge, little is known about the *myc* homologue in molluscs, although it appeared upregulated in *C. gigas* after 21 days of hydrocarbon exposure [27]. Under stress conditions such as hypoxia, early *myc* homologue overexpression could be explained by a reaction of cell protection, and the observed decrease may be due to the efficiency of the resistance system to the response to hypoxia. Mazure *et al.* [40] reported a reduction of c-myc mRNA and protein amounts in human hepatoma cells growing under hypoxic conditions. They concluded to a possible competition between HIF-1 and c-*myc* to modulate the transcriptional activity of hypoxia responsive genes. As HIF-1 has not previously been described in oysters, this inhibition may be due to competition with another regulation element inducible by hypoxia. We actually showed a reduction of *myc* homolog gene expression after 17 days in gills and digestive gland.

As the supply of ATP by the respiratory chain relies on O_2 consumption, genes implicated in respiration and more generally in gas fluxes were expected to be affected by hypoxia exposure. In our libraries, we identified the carbonic anhydrase as being upregulated. This enzyme has been well studied in vertebrates [41]. It has also been more recently described in a symbiotic marine invertebrate, *Riftia pachyptila* [42]. Carbonic anhydrase is involved in the transfer of proton to CO_2 leading to bicarbonate [43]. This enzyme can play a role in gas exchange during respiration, permitting a shorter CO_2 transfer time, and also in ion and fluid exchanges and intra- and extracellular pH regulation. It also plays a role in calcification in molluscs. Among the different isoforms of carbonic anhydrase described, some (generally involved in tumours) are known to be inducible by hypoxia via HIF-1 [44,45]. In this study we observed an upregulated carbonic anhydrase mRNA expression, which is in accordance with a high CO_2/O_2 exchange efficiency needed during hypoxia exposure.

Genes encoding enzymes that need oxygen to be active could also be regulated by hypoxia exposure. We studied expression of the delta-9 desaturase gene that is involved in lipid metabolism. This enzyme catalyses the reaction of formation of monounsaturated fatty acids and requires acyl-CoA, NADH, NADH-reductase, cytochrome b5, phospholipid and oxygen as cofactors [46]. Delta-9 desaturase has been extensively studied in mammals, chicken, fish and insects [47]. The degree of unsaturation of fatty acids resulting from delta-9 desaturase action affects physical properties of membrane phospholipids. Moreover, metabolites of polyunsaturated fatty acids act as signalling molecules in many organisms [48]. To our knowledge, less is known about delta-9 desaturase in molluscs, although it appeared to be downregulated in *C. gigas* after 7 days of hydrocarbon exposure [27]. In the yeast *Saccharomyces cerevisiae*, Vasconcelles *et al.* [49] observed an induction of mRNA expression of OLE1 gene encoding delta-9 desaturase in hypoxia and in transition metal exposure. In *C. gigas*, we observed an upregulation of delta-9 desaturase mRNA expression after 10 days of hypoxia exposure. This induction may be a response to the limitation of O_2 as a substrate [49].

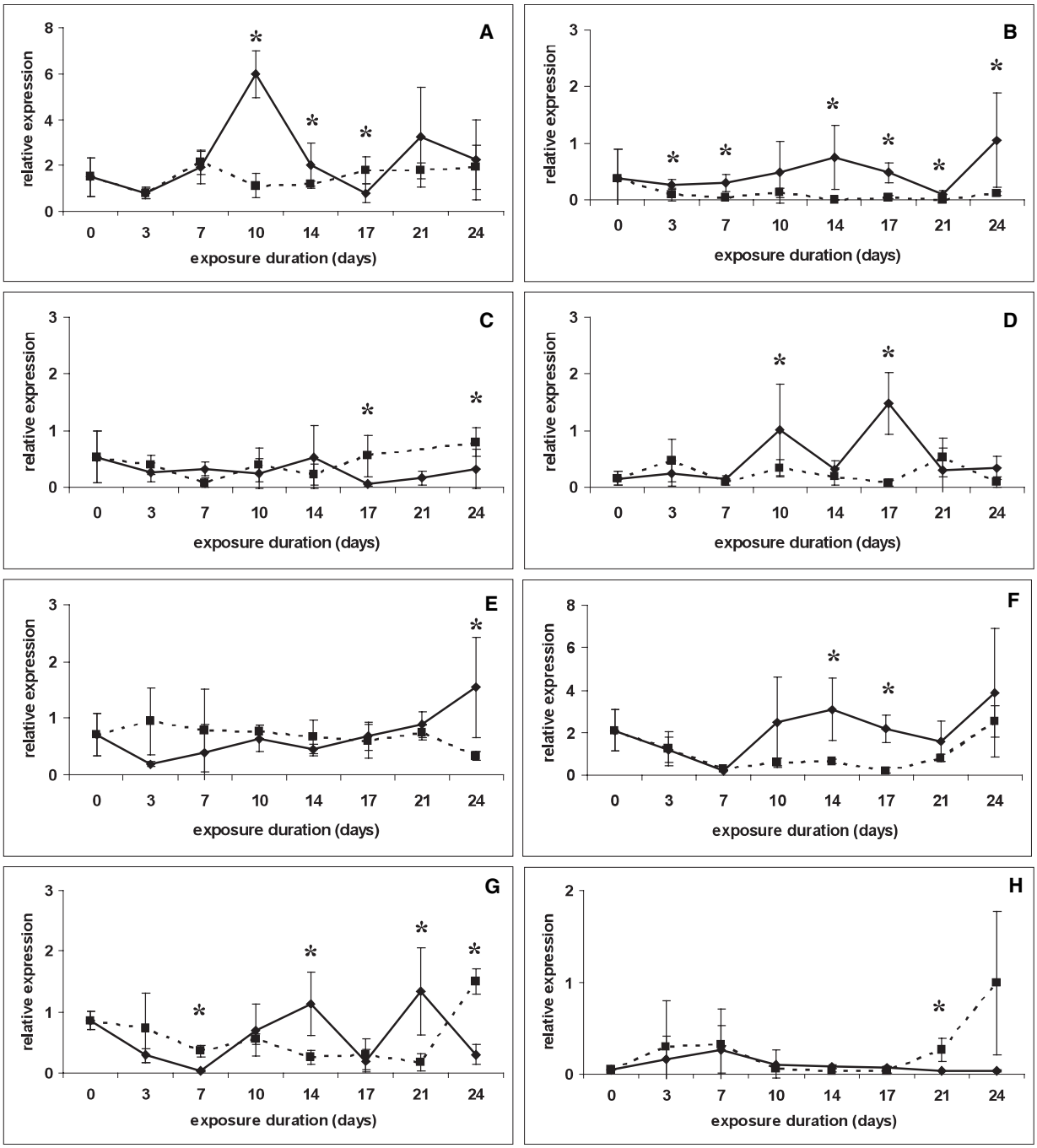

Fig. 1. Analysis of differential expression of up- and downregulated genes in *C. gigas* exposed to hypoxia. Gene expression is presented as the calculated ratio Do_{gene}/Do_{28S} after RT-PCR. For each gene, the dotted line represents control samples, the full line the experimental samples, and the error bars correspond to the SD for the five samples at the sampling time considered**.** *Significant difference between control and hypoxic samples. (A) Expression of carbonic anhydrase in gills. (B) Expression of glutathione peroxidase in mantle. (C) Expression of myc homologue in gills. (D) Expression of BTF3 in mantle. (E) Expression of glycogen phosphorylase in digestive gland. (F) Expression of delta-9 desaturase in mantle. (G) Expression of putative metallothionein in mantle. (H) Expression of HSP70 in mantle.

Some products of the enzyme could also play an important role in hypoxia tolerance by signal transduction.

As oxygen is also at the basis of oxidative metabolism, genes encoding enzymes involved in the cellular regulation of oxidative stress, such as antioxidants, are

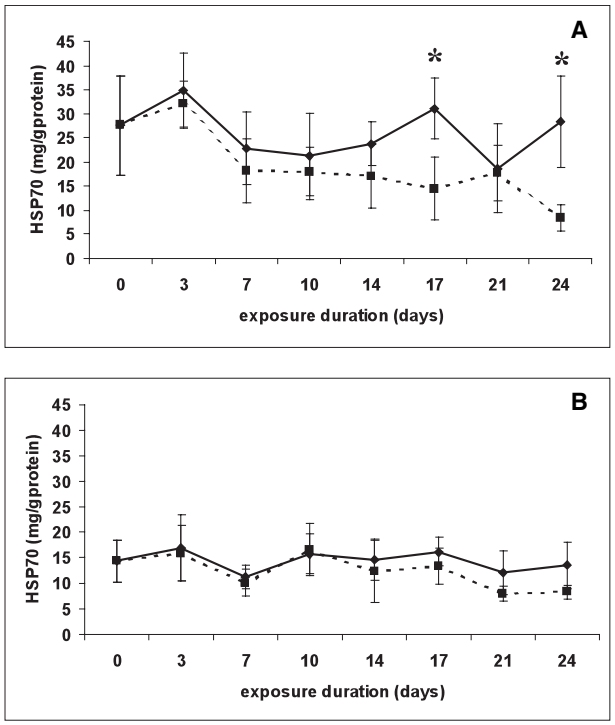

Fig. 2. Quantification of HSP70 in *C. gigas* exposed to hypoxia. The dotted line represents control samples, the full line the experimental samples, and the error bars correspond to the SD for the five samples at the sampling time considered. *Significant difference between control and hypoxic samples. (A) Quantification of HSP70 in digestive gland. (B) Quantification of HSP70 in gills.

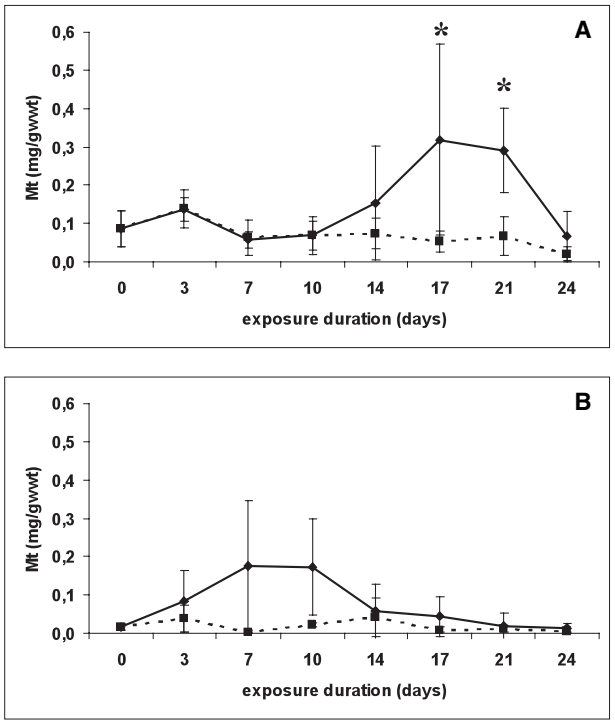

Fig. 3. Quantification of MTs in *C. gigas* exposed to hypoxia. The dotted line represents control samples, the full line the experimental samples, and the error bars correspond to the SD for the five samples at the sampling time considered. *Significant difference between control and hypoxic samples. (A) Quantification of MT in digestive gland. (B) Quantification of MT in gills.

consequently expected to be regulated by hypoxia. We studied the expression of GPx that is known to be directly involved in oxidative metabolism. Glutathione peroxidase is a selenium-dependent enzyme, which transforms H_2O_2 and various peroxides and requires reduced glutathione as a cosubstrate [50]. The classical form is cellular and dispersed throughout the cytoplasm, but GPx activity is also found in mitochondria [51]. Pannunzio and Storey [52] observed a suppression of GPx activity during anoxia exposure in the hepatopancreas of the marine gastropod *Littorina littorea*. On the other hand, hyperoxia increases the GPx mRNA level and activity in rat lung [53]. In our study, expression analysis of GPx mRNA revealed an upregulation with hypoxia exposure. Such an enhanced expression could aim to protect cells from reactive oxygen species that can be formed upon reoxygenation [54,55]. We also identified other potentially hypoxia-regulated genes known to participate in the oxidative stress response-the MTs. The sequence we obtained showed strong similarities with oyster MT genes (C-X-C patterns) but appeared to be a novel sequence. Metallothioneins are small, cysteine-rich, heat-stable proteins involved in the cellular regulation of essential metals, and in detoxification of heavy metals. Several MT isoforms such as Cg-MT2 have been described in *C. gigas* and have been shown to be inducible by metallic stress [56]. Metallothioneins also have diverse physiological functions including protection against oxidants [57]. Murphy *et al.* [58] reported activation of MT gene expression by hypoxia in human myoblasts. In the marine gastropod *Littorina littorea*, cDNA library differential screening allowed the identification of a sequence coding for a protein belonging to the MT family that appeared to be upregulated in foot muscle and digestive gland in response to anoxia stress [59]. The authors suggested that such an increase in MT expression could be explained by the antioxidant role of MT, a function that was previously demonstrated in mussels by Viarengo *et al.* [60]. This increase can be interpreted as a preparatory measure against oxidative stress that could occur during recovery from anoxia. In this study, we observed an induction of a putative MT after 14 days of hypoxia exposure in the mantle,

followed by a depression of expression. This induction occurred as an 'anticipatory response' to protect against the oxidative stress which occurs during reoxygenation. With exposure duration, MT gene expression became reduced, as reoxygenation did not occur. The same trends were observed by ELISA quantification of MTs in gills, revealing an increase of the level of this protein after 10 days of hypoxia exposure. In the digestive gland, however, induction occurs later (21 days), suggesting an organ-specific response. Quantification of another stress protein family, HSP70, revealed an induced expression of these proteins in hypoxia-exposed oysters compared to controls. These data indicate that hypoxia-exposed oysters were highly stressed by the exposure, but also suggest differential tissue-dependant time of response. Indeed, the HSP70 family is widely recognized to be induced by multiple stressors [61], and Delaney and Klesius [62] observed an induced HSP70 production by hypoxia in Nile tilapia. We emphasize, however, that HSP70 transcription appeared to be downregulated after 21 days of hypoxia. Reduced expression of HSP70 gene in response to hypoxia has been described in human microvascular HMEC-1 cells [63], associated with a reduction of HSP70 protein level, and the authors suggest that expression is cell type dependent and connected to hypoxia tolerance. However, our results show that during hypoxia HSP70 production increases in response to the stress. This increase in the enzyme quantity may be a consequence of signal transduction regulation, if a pool of mRNA is already present in cells, and perhaps of early transcriptional regulation in some tissues. These cells are therefore ready to react very quickly to any stress situation.

The results we report in this paper provide a preliminary basis for the comprehension of adaptive strategies developed by *C. gigas* in response to hypoxic conditions. Future efforts will focus on the expression of these regulated genes in wild populations of oysters submitted to various hypoxic stress intensities in marine estuaries, and on the search for functional polymorphisms in these genes.

Experimental procedures

Oyster conditioning and treatment

The experiment was performed in tanks with an effective water volume of 50 L. Tanks were supplied with a continuous flow of water at 15 °C and 34 ppt salinity. Adult oysters, collected from La pointe du Château (Britanny, France), were divided into two groups of 50 animals. They were fed three times a week with a microalgae suspension (containing *Isochrysis galbana*, *Pavlova lutheri* and *Dunaliella primolecta*). After a 7-day acclimatization period in tanks supplied with aerated 0.22 μm-filtered seawater, oysters were exposed for 24 days either to hypoxia [30% (v/v) O_2-saturation] or normoxia [100% (v/v) O_2-saturation, control group]. At day 0, the start of the experiment, O_2-concentration in the inflowing water was decreased to 30% O_2-saturation using an oxygen depletion system according to Pichavant *et al.* [64]. Briefly, before reaching the rearing tank, seawater flowed through a column where nitrogen was injected. Oxygen removal was controlled by nitrogen flow. Surface gas exchange in the rearing tank was limited by setting the water inflow under the water surface. The O_2 concentration in the tank was monitored using a WTW oxymeter and adjusted when necessary to keep hypoxia level constant all along the experiment. Normoxia was obtained by equilibrating seawater with air. Animals were fed throughout the experiment in the same way as during the acclimatization. No mortality was observed either in the control or in the hypoxia-exposed oysters.

For each experimental condition, animals were sampled at regular intervals (0, 3, 7, 10, 14, 17, 21 and 24 days). Digestive gland, gills and mantle were dissected, frozen in liquid nitrogen and stored until analyses.

Suppression subtractive hybridization

Total RNA was extracted from the digestive gland, gills and the mantle of 10 control and 10 exposed oysters after 7–10 and 24 days of exposure using RNAble (Eurobio, les Ulis, France) according to the manufacturer's instructions. Poly(A)$^+$ mRNA was isolated from total RNA using the PolyATtract®mRNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Forward and reverse subtracted libraries were made on 2 μg mRNA (1 μg mRNA from the gill, 1 μg mRNA from the mantle for one library; 2 μg mRNA from the digestive gland for the second library). A total of eight libraries (four forward, four reverse, Fig. 4) was constructed using: gills and mantle after 7–10 days, digestive gland after 7–10 days, gills and mantle after 24 days, digestive gland after 24 days. First and second strand cDNA synthesis, *Rsa*I endonuclease enzyme digestion, adapter ligation, hybridization, and PCR amplification were performed as described by the PCR-select cDNA subtraction manufacturer (Clontech, Palo Alto, CA, USA). Differentially expressed PCR products were cloned into pGEM-T vector (Promega). Two hundred white colonies per library were grown in Luria–Bertani medium (with 100 mg·L^{-1} ampicillin). The vector was extracted using an alkaline lysis plasmid minipreparation and screened by size after PCR amplification of the insert (performed in 2 mM $MgCl_2$ and 10 pmol of T7 and SP6 primers). A total of 1000 clones was sequenced using a Li-COR IR^2 (Sciencetech) and Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Bioscience, Uppsala,

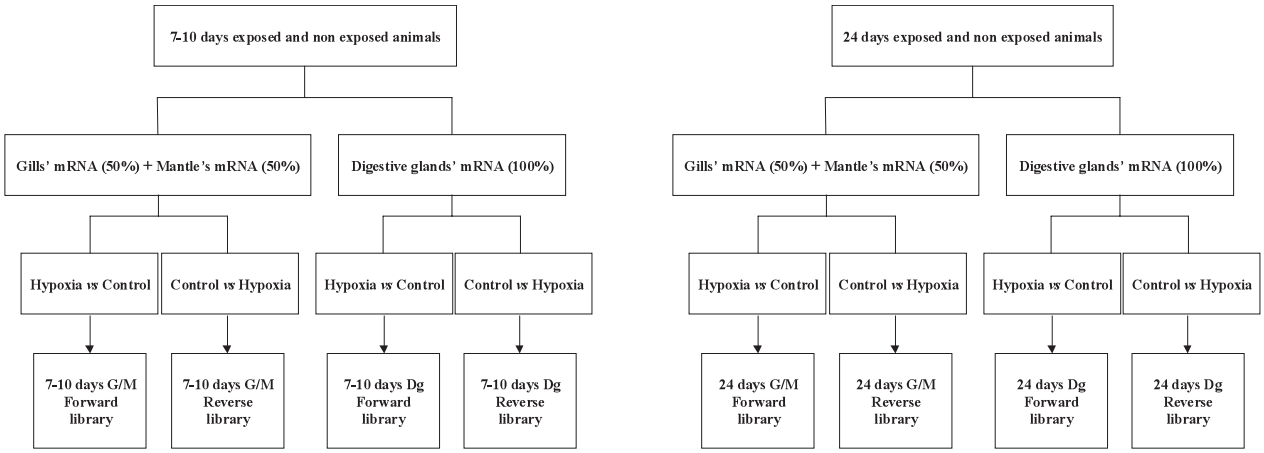

Fig. 4. Diagram of the different subtractions performed in *C. gigas* with SSH, after 7–10 days of hypoxia exposure and after 24 days of hypoxia exposure, and resulting libraries with corresponding tissues. G, Gills; M, mantle; Dg, digestive gland.

Sweden) and an AB3100 sequencer and Big Dye Terminator V3.1 Kit (both Perkin-Elmer, Wellesley, MA, USA). All sequences were subjected to a homology search through the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/).

Hypoxia response gene expression analysis by RT-PCR

Total RNA was extracted from the digestive gland, the gill and the mantle of control and oysters exposed to 0, 3, 7, 10, 14, 17, 21 and 24 days of hypoxia using a method based on extraction in guanidium isothiocyanate. For each sample, 20 μg RNA was submitted to reverse transcription using oligo dT anchor primer (5′-GACCACGCGTATCGA TGTCGACT$_{(16)}$V-3′) and Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega). The amplification of carbonic anhydrase, GPx, myc homologue, glycogen phosphorylase, delta-9 desaturase, BTF3, a putative metallothionein and HSP70 mRNA were performed in 2 mM $MgCl_2$ and 10 pmol of each primer. Combinations of primers we used are shown in Table 6. 28S ribosomal RNA was used as a PCR internal control under the same conditions with primers sense (5′-AAGGGCAGGAAAAGAAACT AAC-3′) and antisense (5′-GTTTCCCTCTAAGTGGTTT CAC-3′). The number of PCR cycles was 35 for carbonic anhydrase, glutathione peroxidase, BTF3, myc homologue and HSP70 expression, 40 for delta-9 desaturase, glycogen phosphorylase and putative metallothionein expression, and 25 for 28S amplification to avoid band intensity saturation for optical determination. The resulting PCR products were separated by electrophoresis through a 0.5 × TBE/1.5% agarose gel, and visualized with UV light after staining with ethidium bromide. Band intensities were quantified using the GENE PROFILER software (version 4.03, Scanalytics, Inc, Lincoln, NE, USA).

Table 6. Combinations of primers used in RT-PCR expression analysis.

Genes	Primer sequences
Carbonic anhydrase	5′-AAACAGGCGGGAAACCACAGTAACACGGT-3′
	5′-CACTGGACGCTTTCATAACAAGGGGGCGT-3′
Glutathione peroxidase	5′-GATGACGTCCCCAGTCATGAGGGGTGGTC-3′
	5′-TGGGGGATGGAGGGTAAGACCATACACTT-3′
Myc homologue	5′-TTCTATAACGGAACATTATACCAACAAGG-3′
	5′-CAACATTTACCTGGGGCAGGTGGGTTCAG-3′
BTF3	5′-AATCCAAAAGTGCAGGCCTCACTAGCAGC-3′
	5′-TTGCCGACTAATTCCGGGACTCCATCATC-3′
Glycogen phosphorylase	5′-CCGTCTTGCCAGAGTTTCTCCACCTCCTC-3′
	5′-GTCGTCAACAACGATCCTGACGTTGGGGA-3′
Delta-9 desaturase	5′-TACTGTCTTCTGCTAAACGCCAC-3′
	5′-GTCGTGATATTGAGGTGCCAGCC-3′
Putative metallothionein	5′-GCCCAGACGGGAAAATGCGTGTG-3′
	5′-CAGTTACACGATGCTTTGGCGCA-3′
HSP70	5′-GGAATAGATCTTGGAACCACATA-3′
	5′-TTGCCAAGATATGCTTCTGCAGT-3′

Protein extraction and quantification of HSP70 and MTs by ELISA

On days 0, 3, 7, 10, 14, 17, 21 and 24, samples of gills and digestive glands from exposed and control oysters ($n = 5$ for each sample) were collected, homogenized in protein extraction buffer (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM phenylmethanesulfonyl fluoride pH = 7.2) and centrifuged. Protein concentration was estimated with a D_c Protein Assay kit (Bio-Rad, Hercules, CA, USA) using BSA as the standard. Optical density was measured at 620 nm using a microplate reader. Microtiter plates were coated with 20 μg·well^{-1} of total proteins and incubated over night at 4 °C. HSP70 and MTs concentrations were estimated by ELISA using rabbit anti-*Cg*Hsc72 and anti-*Cg*Mt polyclonal

antibodies and recombinant *Cg*Hsc72 and *Cg*Mt, respectively, as standards, according to procedures previously described [65,66].

Statistical analysis

The variations in gene expression and in protein amount were analysed by the Mann–Whitney's U test using STATISTICA Software (Statsoft).

Acknowledgements

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