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RESEARCH ARTICLE

Rapid mitochondrial adjustments in response to short-term hypoxia and re-oxygenation in the Pacific oyster, *Crassostrea gigas*

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SUMMARY

As oxygen concentrations in marine coastal habitats can fluctuate rapidly and drastically, sessile marine organisms such as the oyster *Crassostrea gigas* can experience marked and rapid oxygen variations. In this study, we investigated the responses of oyster gill mitochondria to short-term hypoxia (3 and 12 h, at 1.7 mg O_2 l^{-1}) and subsequent re-oxygenation. Mitochondrial respiratory rates (states 3 and 4 stimulated by glutamate) and phosphorylation efficiency [respiratory control ratio (RCR) and the relationship between ADP and oxygen consumption (ADP/O)] were measured. Cytochrome *c* oxidase (CCO) activity and cytochrome concentrations (*a*, *b*, c_1 and *c*) were measured to investigate the rearrangements of respiratory chain subunits. The potential implication of an alternative oxidase (AOX) was investigated using an inhibitor of the respiratory chain (antimycin A) and through gene expression analysis in gills and digestive gland. Results indicate a downregulation of mitochondrial capacity, with 60% inhibition of respiratory rates after 12 h of hypoxia. RCR remained stable, while ADP/O increased after 12 h of hypoxia and 1 h of re-oxygenation, suggesting increased phosphorylation efficiency. CCO showed a fast and remarkable increase of its catalytic activity only after 3 h of hypoxia. AOX mRNA levels showed similar patterns in gills and digestive gland, and were upregulated after 12 and 24 h of hypoxia and during re-oxygenation. Results suggest a set of controls regulating mitochondrial functions in response to oxygen fluctuations, and demonstrate the fast and extreme plasticity of oyster mitochondria in response to oxygen variations.

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INTRODUCTION

Marine organisms, and in particular sessile ones, can be confronted with a wide range of oxygen concentrations. Oxygen depletion in seawater is essentially caused by eutrophication. Plankton blooms and microbial degradation of organic matter can lead to hypoxia (Cloern, 2001; Gray et al., 2002). Eutrophication can also cause macrophyte blooms in shallow areas, resulting in low dissolved oxygen levels during the night due to algal respiration (Grieshaber et al., 1994). Oxygen deficiency can have fitness costs, such as reduced growth and reproduction, or even be rapidly lethal for sensitive organisms (Wu, 2002). Conversely, benthic animals can experience hyperoxic conditions when macrophyte photosynthesis leads to high oxygen production during the daytime (Bridges et al., 1984; Grieshaber et al., 1994). Moreover, re-oxygenation after low tide can cause an oxidative burst, similar to that experienced during reperfusion-ischemia injury (Storey, 1996). Hyperoxic conditions can potentially lead to over-production of reactive oxygen species (ROS) and oxidative stress (Turrens, 2003; Massabuau, 2001). Dealing with fluctuating environmental oxygen levels requires a suite of physiological and biochemical adaptations. Survival of hypoxia typically requires depression of metabolic rate (hypometabolism), and the use of anaerobic fermentative pathways to produce ATP (Larade and Storey, 2002; Grieshaber et al., 1994). Conversely, the avoidance of oxidative bursts during re-oxygenation is mainly achieved by maintenance of constitutive high levels of antioxidants (Storey, 1996), by increasing antioxidant defences during hypoxia/anoxia to minimize oxidative damage during recovery (Storey, 1996) or by energy-dissipating mechanisms that avoid increased ROS production (Buttemer et al., 2010).

The biochemical mechanisms that drive metabolic depression during hypoxia and avoid oxidative injury during re-oxygenation are still poorly understood in marine invertebrates, but mitochondria are likely to play important roles. The ability to adjust mitochondrial oxidative capacity is crucial to controlling energy production under changing environmental conditions (Guderley, 2004; Bremer and Moyes, 2011). In mammals, hypoxic conditions reduce mitochondrial respiration and oxidative phosphorylation (Schumacker et al., 1993). The decrease of mitochondrial respiration may implicate several mechanisms such as decreasing the area of the inner mitochondrial membrane, slowing down electron flux through the electron transport system, and inhibiting ATP synthase activity (Bishop et al., 2002; Duerr and Podrabsky, 2010). Studies on reversible decrease of respiration during moderate hypoxia in isolated rat hepatocytes, revealed a role of cytochrome *c* oxidase (CCO) in cellular oxygen sensing and in the reversible suppression of oxidative phosphorylation (Chandel et al., 1995; Chandel et al., 1996;

Chandel et al., 1997). Adjustments of energy dissipation through decreased proton leak have been proposed to preserve aerobic energy production during metabolic depression (St-Pierre et al., 2000a). This proton conductance of the inner membrane of mitochondria allows a leak of protons back into the matrix, and results in oxidative phosphorylation being less than fully coupled (Brand, 2000). This energy-dissipating proton leak can involve different components of the mitochondrial membrane such as adenine nucleotide translocase or uncoupling proteins, which have been recently characterized in oysters (Sokolova and Sokolov, 2005; Kern et al., 2009).

Besides systems controlling the respiratory chain, recent studies show the presence of an alternative oxidase (AOX) in hypoxia-tolerant marine invertebrates (Tschischka et al., 2000; Buchner et al., 2001; Pichaud et al., 2012). AOX gene sequences were found in many invertebrates and in particular in bivalves (McDonald and Vanlerberghe, 2004; McDonald et al., 2009). The AOX, a mitochondrial inner-membrane protein, branches off the classical respiratory chain, reducing oxygen without pumping protons in the inter-membrane space and therefore uncoupling respiration from ATP production. This alternative pathway in marine invertebrates is thought to protect cells against oxidative injury during hypoxic and hyperoxic events by preventing increased rates of ROS production (Abele et al., 2007).

The Pacific oyster *Crassostrea gigas* Thunberg 1793 is well known for its tolerance to environmental oxygen fluctuations, making it an interesting model for studying mitochondrial modifications during hypoxia. To date, studies on hypoxia responses were performed at the levels of the transcriptome (David et al., 2005; Sussarellu et al., 2010) and energy metabolism (Le Moullac et al., 2007a) on oysters exposed to long-term hypoxia (30% of oxygen saturation during 3 weeks). The biochemical mechanisms by which oysters respond to a short-term hypoxia (24 h) and subsequent re-oxygenation are less well known. Oxygen fluctuations in seawater often occur on short time scales and the response of hypoxia-tolerant systems to oxygen depletion occurs in two defence and rescue phases (Hochachka et al., 1996; Hochachka and Lutz, 2001). Thus, it is very important to understand the early mechanisms of hypoxia responses. Recently we studied antioxidant and energetic adjustments in *C. gigas* occurring in response to short-term oxygen variations (Sussarellu et al., 2012). Short-term hypoxic conditioning did not cause a switch towards anaerobic metabolism nor an oxidative stress, but AOX mRNA levels showed rapid modifications on a short time scale, suggesting a role in redox balance of *C. gigas*.

The aim of the present study was to investigate functional changes of *C. gigas* gill mitochondria during short-term hypoxia and re-oxygenation, and to evaluate the implication of AOX during such oxygen variations. The main goals were to determine whether mitochondrial capacities are affected by a short-term hypoxia stress, and in which way. For this purpose, mitochondrial capacities were analyzed in oyster gills after 3 and 12 h of hypoxia ($1.7\pm0.14\,mg\,l^{-1}$) and subsequent re-oxygenation. We sought to identify potential mechanisms by which mitochondrial oxidative capacities changed by examining phosphorylation efficiency, the activity of CCO and the concentrations of cytochromes (*a* representing the concentration of complex IV-CCO of the respiratory chain; *b* that of complexes II and III; c_1 that of complex III; and cytochrome *c*). The presence of an AOX pathway and its adjustments in the mitochondrial respiratory chain were estimated using antimycin A to inhibit complex III of the respiratory chain and through gene expression analysis in gills and digestive gland.

MATERIALS AND METHODS

Animal conditioning and sampling

Three-year old oysters (66–85 g, 8–10 cm) were obtained in May 2010 from an oyster farm in the Bay of Brest (Plougastel, France). Immediately after arrival, oysters were placed in a 500 l flow-through seawater tank and were allowed to acclimate for 1 week before experiments. During the 1 week of acclimation, no food was added, but the flow-through seawater system uses a sand filter, likely allowing oysters to feed on natural phytoplankton brought in by the pumped seawater. Throughout the experiments, salinity and temperature were 34 and 13°C, respectively. After 1 week, oysters were placed in six tanks with an effective volume of 40 l, each containing 30 animals. Three tanks were continuously supplied with fully oxygenated seawater ($9.24\pm0.54\,mg\,O_2\,l^{-1}$) to serve as the normoxic controls. In the three other tanks, oxygen levels were reduced to $1.7\pm0.14\,mg\,O_2\,l^{-1}$ for 24 h using the oxygen depletion system described by Pichavant et al. (Pichavant et al., 2000). Hypoxic O_2 concentrations were reached by bubbling nitrogen in seawater in a reservoir tank supplying the three hypoxic tanks. Surface gas exchange was limited by positioning the water outflow under the water surface and covering tanks with transparent plastic bags. After 24 h of hypoxia, nitrogen bubbling was stopped and fully oxygenated water was delivered to the tanks. To speed up re-oxygenation, air pumps were placed in each tank and the plastic bags were removed. The oxygen concentration 1 h after stopping nitrogen bubbling was $8.58\pm0.31\,mg\,O_2\,l^{-1}$. Water flow rates were 0.8 and $1\,l\,min^{-1}$ under hypoxic and normoxic conditions, respectively. Oxygen concentrations were monitored using a SevenGo DuoPro Mettler Toledo oxymeter (Greifensee, Switzerland), and were adjusted when necessary to keep hypoxia constant throughout the experiment. Water parameters of the experimental setup (oxygen concentration and temperature) are provided in supplementary material Table S1. The level of hypoxia chosen ($<2\,mg\,O_2\,l^{-1}$) was justified by the findings of Le Moullac et al. (Le Moullac et al., 2007a), who determined the critical partial pressure of O_2 (P_{O_2}) for oysters at 15°C to be $3.02\pm0.15\,mg\,O_2\,l^{-1}$.

For mitochondria extraction, gills were dissected after 3 and 12 h of hypoxia and 1 h after re-oxygenation. Oysters from normoxic tanks were used as control. At each sampling time nine oysters were sampled, and three pools containing gills of three individuals were used (each pool contained 3.7±0.40 g of gill tissue). Gill pools were immediately processed for mitochondrial extraction.

For gene expression analysis, gills and digestive gland were sampled in hypoxic and normoxic tanks after 0, 1, 3, 6, 12 and 24 h of hypoxia, and 1 h after re-oxygenation. For each condition and each time, eight animals were sampled. Tissues were dissected, immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Mitochondrial isolation

Procedures of mitochondrial isolation and assay were adapted from Kraffe et al. (Kraffe et al., 2008). Mitochondrial isolation were performed immediately after dissection; all manipulations were carried out on ice, and centrifugations were performed at 4°C and took approximately 1 h. Gill pools were initially crumbled with scissors, and rinsed with isolation buffer on a mesh (80 µm). The minced tissues were then homogenized with a motorized potter tissue grinder (Heidolph, Kelheim, Germany; three passes on ice) in 10 volumes of isolation buffer containing sucrose $300\,mmol\,l^{-1}$, HEPES $30\,mmol\,l^{-1}$, KCl $100\,mmol\,l^{-1}$, EGTA $8\,mmol\,l^{-1}$, pH 7.5 at 25°C, and 0.5% bovine serum albumin (BSA) (fraction V fatty acid free,

Sigma A6003, Sigma-Aldrich, St Louis, MO, USA; added the day of experiment). The homogenate was centrifuged at 900 ***g*** for 10 min and the supernatant was filtered on an 80 µm mesh and re-centrifuged at 900 ***g*** for 10 min. The resulting supernatant, considered free of unbroken cells or cell debris, was centrifuged at 9000 ***g*** for 10 min and the pellet was re-suspended in a volume of reaction buffer (containing sucrose 400 mmol l^{-1}, KH_2PO_4 10 mmol l^{-1}, HEPES 30 mmol l^{-1}, KCl 90 mmol l^{-1}, taurine 50 mmol l^{-1}, β-alanine 50 mmol l^{-1} and 0.5% BSA, pH 7.5 at 25°C) corresponding to one-tenth of the initial mass of gills used.

Mitochondrial respiration

Mitochondrial oxygen consumption was measured in a water-jacketed respiration chamber using a Clark-type electrode OX1LP-1mL Dissolved Oxygen Package (Qubit Systems, Kingston, ON, Canada). Temperatures of chambers were maintained at 13°C with a circulating refrigerated bath. For each assay, around 600 µg of mitochondrial protein was added to 400 µl of reaction buffer. Glutamate was added to a final concentration of 40 mmol l^{-1} as substrate. The maximal rate of oxidative phosphorylation (state 3) was obtained by the addition of ADP to a final concentration of 600 µmol l^{-1}. Preliminary experiments showed that glutamate alone stimulates respiration through complex I in *C. gigas* gill mitochondria, as previously found in *Crassostrea virginica* (Burcham et al., 1983). Glutamate was also oxidized at higher rates than other substrates (data not shown), as in *C. virginica* (Kurochkin et al., 2009) and *Placopecten magellanicus* (Kraffe et al., 2008). Non-phosphorylating rates (state 4) were measured after ADP depletion. Measurements were performed in triplicate in three respiration chambers simultaneously. Inhibition of complex III of the respiratory chain was obtained after adding antimycin A to a final concentration of 20 µmol l^{-1}. Antimycin A was added 3 min after ADP addition (state 3 lasts on average 6 min using ADP at 600 µmol l^{-1}). Inhibited rates were then measured and compared with non-inhibited rates (state 3 rates) measured on the same sample before antimycin A addition. Respiratory control ratio (RCR) values are defined as the ratio between state 3 and state 4. Oxidative phosphorylation efficiency (relationship between ADP and oxygen consumption) was calculated using the ADP/O ratio according to Chance and Williams (Chance and Williams, 1956).

Cytochrome *c* oxidase activity

CCO activity measurement on mitochondrial preparations was adapted from Bouchard and Guderley (Bouchard and Guderley, 2003) and Kraffe et al. (Kraffe et al., 2008). Briefly, the mitochondrial preparation was submitted to two cycles of freezing and thawing. The disrupted mitochondria were diluted 100 times in the assay buffer containing NaH_2PO_4 and Na_2HPO_4 50 mmol l^{-1} pH 7.8 at 25°C. Bovine cytochrome *c* (cyt *c*, C2037 Sigma-Aldrich) was employed as substrate to stimulate the maximal CCO activity. Reduced cyt *c* solution was prepared by adding a few grains of sodium dithionite. To avoid dithionite excess, small amounts of a stock solution of reduced cyt *c* were added to a cyt *c* oxidized solution and the absorbance was followed at 550 nm to obtain 95% of the absorbance of the reduced cyt *c* stock solution. The reaction was performed in microplates at 25°C using an initial cyt *c* concentration of 60 µmol l^{-1} reduced at 95%. The decrease in absorbance of cyt *c* was followed at 550 nm for 10 min and all assays were run in triplicate. Activities were calculated using an extinction coefficient of 19.1 mmol l^{-1} cm^{-1} for cyt *c* and are expressed as µmol cyt *c* transformed min^{-1} (U_{CCO}, first-order reaction).

Cytochrome concentrations

The concentrations of cytochromes *a*, *b*, c_1 and *c* from the electron transport chain were quantified by difference spectra according to Leary et al. (Leary et al., 2003). Six hundred micrograms of mitochondrial proteins (40 µl of mitochondrial suspension) were diluted in 960 µl of phosphate buffer (NaH_2PO_4 and Na_2HPO_4 50 mmol l^{-1} pH 7.8 at 25°C) without Triton-X. The cytochromes were reduced by adding a few grains of sodium dithionite and incubated for 15 min at room temperature. The reduced samples were read against air-oxidized samples between 400 and 630 nm. We used the solution to the simultaneous equations (Williams, 1964) to assess individual cytochrome concentrations.

Protein concentration

Aliquots of 20 µl of fresh mitochondrial preparations were suspended in the reaction buffer without BSA and centrifuged for 10 min at 12,000 ***g*** at 4°C. The supernatant was discarded and the pellet was re-suspended, washed and centrifuged once more to remove the BSA. Pellets were re-suspended with a known volume of ultra-pure water and frozen at −80°C until protein analysis. The protein concentration was determined with the RC-DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as standard.

mRNA extraction

Total RNA was isolated using 1 ml of Extract-all Reagent (Eurobio, Courtaboeuf, France) per 100 mg of tissue according to manufacturer's instructions. RNA quality was assessed using an Agilent Bioanalyzer 2100 and an RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. RNA concentrations were measured at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). For both gills and digestive gland, the reference sample used for real-time PCR inter-run calibration was one of the normoxic samples at time 0. Four micrograms of RNA samples were first treated with 0.5 U RQ1 RNase-Free DNase (Promega, Madison, WI, USA) according to manufacturer's instructions, precipitated in 3 mol l^{-1} 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 an 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, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions, on 1 µg of total RNA using random hexamer primers to start the reaction.

Real-time PCR analysis

The levels of AOX mRNA transcripts were measured in gills and digestive gland by real-time PCR using specific primers designed using Primer Express software V 2.0 (Applied Biosystems, Foster City CA, USA) on oyster AOX mRNA sequence (BQ426710), AOX_forward 5′-GAA TGG TGG CAG CGA TGA C-3′, AOX_reverse 5′-CCG TGA TCC CGC TTG AGT-5′. Two different reference genes were screened: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with primers designed by Sussarellu et al. (Sussarellu et al., 2010), and elongation factor 1 alpha (EF1) with primers designed by Fabioux et al. (Fabioux et al., 2004). The choice of these two reference genes was justified by the fact that they were both already tested in previous hypoxic experimentation on *C. gigas* and resulted as the least variable (Sussarellu et al., 2010; Sussarellu et al., 2012). Furthermore, GAPDH was one of the best

reference gene over the ensemble of the tissues in *C. gigas* by a screening of seven different reference genes (Dheilly et al., 2011). Variation coefficients for EF1 and GAPDH were <5% in both gills and digestive gland.

The primers' PCR efficiency (E) was estimated for each primer pair on both tissues by serial dilutions (from 1/20 to 1/640) of the reference cDNA sample (used for inter-run calibration). The primer efficiencies were determined by the slope of the standard curves by the following formula: $E=10^{(-1/\text{slope})}$ (Ståhlberg et al., 2003). E was between 93 and 105% for the references genes and AOX in both tissues.

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 70 nmol l^{-1} of each primer in a 7300 Real-Time PCR System (Applied Biosystems). Runs started with 15 min activation of the Thermo-Start DNA Polymerase at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. A melting curve program was performed from 95 to 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 inter-run control, a no-template control and a water control. Negative reverse-transcription controls on DNase-treated mRNA were run in order to assess the absence of genomic contaminations.

Reference gene expression stability (geNorm algorithm and coefficient of variation) and relative gene expression were calculated with the qbase^{plus} software (biogazelle) (Hellemans et al., 2007). The geometric mean of the two reference genes was employed to normalize gene expression. Specific amplification efficiencies on each tissue were employed for each primer pair (90%<E<110%). Inter-run calibration was employed to normalize inter-run variations within a gene between samples in order to remove the run-to-run difference. The difference between samples and the inter-run calibration sample were calculated in each run.

Statistical analysis

Sample normality was checked using the Shapiro–Wilk test. Homoscedasticity of variances was tested with the Bartlett test. ANOVA was performed if the assumptions of normality and homogeneity of variances were met. ANOVA were followed by *post hoc* Fisher's least significant difference (LSD) tests. As data from real-time PCR analysis were not normally distributed, non-parametric Kruskal–Wallis ANOVA was performed, followed by Mann–Whitney U-tests on each time of sampling between conditions (normoxia/hypoxia). For each test, the significance threshold was 5%. All analyses were performed with STATISTICA 9 software (StatSoft, Tulsa, OK, USA).

RESULTS

Mitochondrial properties

Mean RCR values were between 6.4 and 7.3 and did not vary significantly between different conditions (Table 1), indicating that mitochondria were maintained tightly coupled. The ADP/O ratio increased significantly after 12 h of hypoxia and remained at the same level 1 h after re-oxygenation (Table 1). The maximal capacity of glutamate oxidation (state 3 rates, Fig. 1A) decreased markedly in oyster gill mitochondria exposed to hypoxia. State 3 rates tended to decrease after 3 h of hypoxia (−25% compared with normoxia) and were significantly reduced after 12 h of hypoxia (−57.5% compared with normoxia). State 3 rates 1 h after re-oxygenation remained as low as in mitochondria after 12 h of hypoxia (−58.1% compared with normoxia). Non-phosphorylating rates (state 4, Fig. 1B) decreased in oysters exposed to hypoxia, following the same pattern as observed for state 3 rates. State 4 rates in oyster gill mitochondria exposed to 3 h of hypoxia were significantly lower than in the normoxic samples (−33%), and decreased further in oysters exposed to 12 h of hypoxia (−62.6% compared with normoxia). State 4 rates from oysters sampled 1 h after re-oxygenation remained similar to those measured after 12 h of hypoxia (−60.2% compared with normoxia).

Table 1. Phosphorylation capacity of mitochondria expressed as respiratory control ratio (RCR, state 3/state 4), and oxidative efficiency of mitochondria (ADP/O, ADP added per nmol of consumed oxygen)

	RCR	ADP/O
Controls	6.38±0.50	2.18±0.03[a]
3 h hypoxia	7.31±0.77	2.04±0.08[a]
12 h hypoxia	7.05±0.56	2.86±0.29[b]
1 h reoxygenation	6.84±0.90	2.96±0.26[b]

Differences between groups (indicated by superscripted letters) were tested for significance using Fisher's LSD test. Values are means ± s.e.m. (N=3).

CCO activity was expressed using both the amount of protein (mg) in the mitochondrial preparations and the amount of cyt *a* (nmol) (Fig. 2). As cyt *a* is located in complex IV (CCO) of the respiratory chain, activity of CCO expressed per cytochrome *a* unit reflects the catalytic activity of the enzyme. CCO activity expressed per cytochrome *a* increased significantly in mitochondria of oysters exposed to 3 and 12 h of hypoxia when compared with normoxia (Fig. 2B). The concentrations of cytochromes *a*, *b*, c_1 and *c* expressed per milligram of mitochondrial protein did not show significant differences with sampling time (Table 2). However, when cytochrome ratios were calculated, cyt *a*/cyt c_1 was significantly higher 1 h after re-oxygenation than under normoxic or hypoxic conditions (Table 2). These differences appear to be mainly due to a decrease in the amount of cyt c_1.

Alternative oxidase pathway

Inhibition with antimycin A reduced state 3 rates of glutamate oxidation by ~85–90% and did not vary significantly with sampling time (Fig. 3). Approximately 10–15% of the total oxygen consumption could thus be attributed to the AOX pathway. Albeit not significant (ANOVA, P=0.087), the proportion of oxygen consumed by the alternative pathways tended to increase in mitochondria isolated 1 h after re-oxygenation.

Expression of AOX mRNA was significantly affected by hypoxia in gills and digestive gland (Kruskal–Wallis ANOVA, P<0.001), showing similar patterns for both tissues. In gills, AOX mRNA levels were significantly higher in hypoxia than in normoxia at 12 h of hypoxia and 1 h after re-oxygenation (Mann–Whitney U-test, P<0.05; Fig. 4A). In digestive gland, AOX mRNA levels were significantly higher in normoxia than after 1 h of hypoxia (Mann–Whitney U-test, P<0.001; Fig. 4B). However, AOX mRNA levels were more elevated in hypoxia than in normoxia at 12 h, 24 h of hypoxia and 1 h after re-oxygenation (Mann–Whitney U-test, P<0.05; Fig. 4B).

DISCUSSION

Overall, this study indicates that hypoxia induces rapid modifications of mitochondrial functions in oyster gills. Maximal oxidative capacities stimulated by the addition of glutamate (state 3) and non-phosphorylating respiration (state 4) drastically

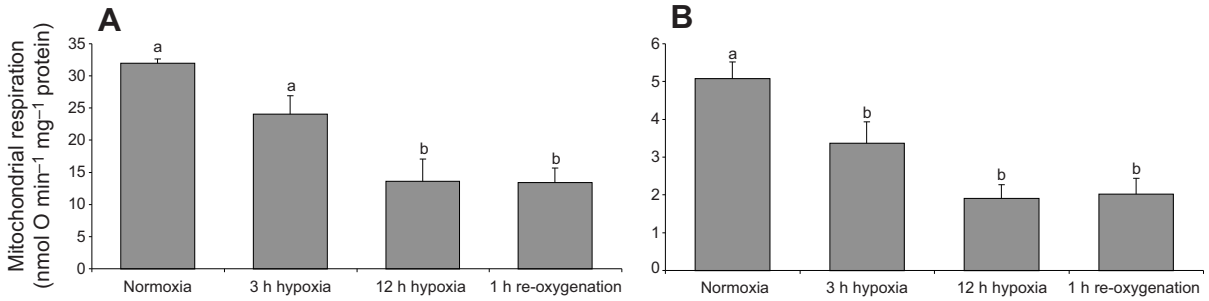

Fig. 1. State 3 (A) and state 4 (B) rates of mitochondrial respiration (nmol O min^{-1} mg^{-1} total mitochondrial protein) in the Pacific oyster. Differences between groups were tested for significance using Fisher's LSD test. Values are means ± s.e.m. (*N*=3).

decreased during hypoxia. Rates of states 3 and 4 were reduced by approximately 60% after 12 h of hypoxia. Only after 3 h of hypoxia, a decrease was apparent for state 3 and was significant for state 4. Decreasing respiratory rate is one of the first modifications during the onset of metabolic depression of hypoxia-tolerant organisms and is crucial in the prevention of the onset of fatal oxygen depletion (Chandel et al., 1997; St-Pierre et al., 2000b; Bishop et al., 2002; Abele et al., 2007). Studies on hibernating amphibians suggest that a decrease in substrate oxidation and electron transport in the respiratory chain is the main mechanism reducing mitochondrial respiration (St-Pierre et al., 2000a). Results of the present study suggest that functional modifications of mitochondrial capacity could reflect rapid metabolic depression in oysters during hypoxia. The marked decline in state 3 respiration rates during hypoxia was matched by that of state 4 respiration, and RCR remained stable during the duration of the experiment, indicating that mitochondria were well coupled. Oxygen consumption in state 4 is considered as an estimate of proton leak through the inner mitochondrial membrane (St-Pierre et al., 2000a). The relative stability of RCR could therefore provide an indication that proton leak, and the relative respiration that does not contribute to phosphorylation, was controlled in oyster mitochondria during hypoxia. Decrease of proton leak during hypoxia is a consequence of reductions in electron transport chain activity and in membrane potential (St-Pierre et al., 2000a). Such adjustments have been proposed to preserve or even increase the efficiency of aerobic energy production during metabolic depression in the frog *Rana temporaria* (St-Pierre et al., 2000a). At the mitochondrial level, reduction in proton leak could therefore result in significant energy savings under conditions of low ATP turnover during hypoxia. Optimization of ATP production during limited oxygen exposure could also be another strategy and may explain the increase in phosphorylation efficiency, commonly estimated using the ADP/O ratio (Chance and Williams, 1956), in oyster mitochondria after 12 h of hypoxia. Increase in the ADP/O is still difficult to explain as it may involve different controls that may function upstream and downstream of the electron transport chain. Nevertheless, the higher ADP/O during hypoxia suggests a tighter coupling of oxygen consumption that is used to drive the futile cycle of proton pumping used by ATP synthase to produce ATP.

The maintenance of at least 40% of normoxic respiration capacities and the increased efficiency after 12 h of hypoxia suggest that oysters did not switch towards anaerobic ATP production, but rather depressed their metabolic rate and optimized the residual aerobic metabolism. This would be confirmed by the onset of anaerobic pathways in *C. gigas* muscle observed only after 10 days of hypoxia [1.96 mg O_2 l^{-1} (Le Moullac et al., 2007b)], and that genes involved in the switch toward anaerobic pathways were not activated during short-term hypoxia [2.6 mg O_2 l^{-1} for 24 h (Sussarellu et al., 2012)]. In addition, oysters seem to use different mechanisms to adjust their metabolism and mitochondrial functionality when hypoxia is experienced in seawater and when it occurs during air exposure. When *Crassostrea virginica* are exposed to anoxia under air exposure (Kurochkin et al., 2009), state 4 rates of gill mitochondria remained at near normoxic values throughout anoxia while state 3 rates were decreased. Thus, RCR decreased throughout the 6 days of air exposure, suggesting a decrease of mitochondrial efficiency. Moreover, accumulation of products of fermentation signalled the onset of anaerobic metabolism (Kurochkin et al., 2009). Hence, air exposure seems to decrease mitochondrial efficiency and initiate anaerobic energy production, whereas short-term hypoxia

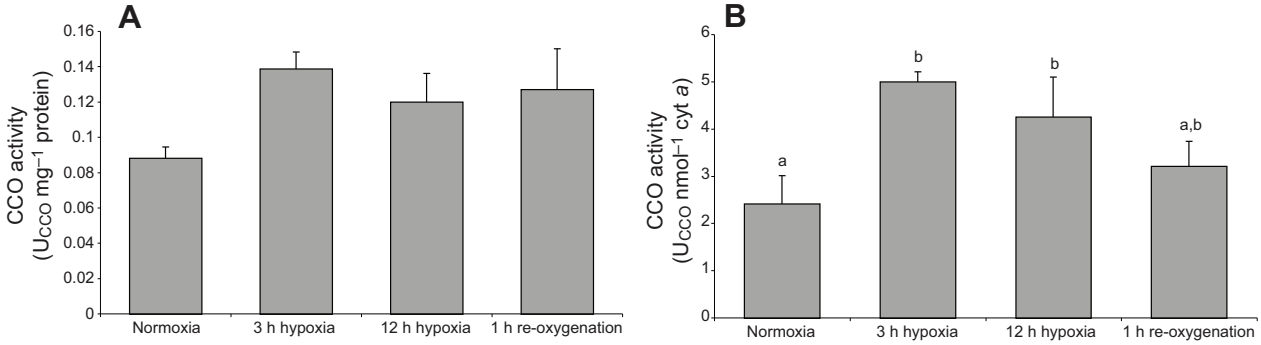

Fig. 2. Cytochrome *c* oxidase (CCO) activity expressed in terms of total mitochondrial protein (A) and cytochrome *a* (B) in the Pacific oyster. U_{CCO}, µmol cytochrome *c* reduced per minute. Differences between groups are defined using Fisher's LSD test. Values are means ± s.e.m. (*N*=3).

Table 2. Concentrations of mitochondrial cytochromes *a*, *b*, c_1 and *c* expressed as nmol cytochrome mg^{-1} total mitochondrial protein, and relative cytochrome ratios using c_1 as denominator

	Normoxia	3 h hypoxia	12 h hypoxia	1 h re-oxygenation
Concentration				
a	0.04±0.01	0.03±0.00	0.03±0.00	0.04±0.01
b	0.19±0.02	0.22±0.05	0.18±0.01	0.14±0.02
c_1	0.11±0.01	0.09±0.01	0.09±0.02	0.06±0.01
c	0.06±0.00	0.06±0.02	0.05±0.01	0.05±0.01
Relative cytochrome ratio				
a/c_1	0.31±0.04[a]	0.22±0.04[a]	0.29±0.05[a]	0.54±0.01[b]
b/c_1	1.70±0.05	2.02±0.20	1.82±0.10	2.12±0.01
c/c_1	0.45±0.07	0.41±0.08	0.41±0.03	0.61±0.05

Superscripted letters indicate differences between groups tested for significance using Fisher's LSD test. Values are means ± s.e.m. (*N*=3).

in seawater decreases aerobic metabolic rate and maintains mitochondria coupling to optimize oxygen use for energy production.

Regulation of respiration rates, coupling and phosphorylation efficiency during short-term hypoxia are presumably controlled by several adjustments of the respiratory chain. Reduction of state 3 could result from inhibition of the phosphorylation system (reactions that generate or utilize ATP), the substrate oxidation system (reactions responsible for generating proton motive force) or both (Gerson et al., 2008). The increase of CCO activity during hypoxia in oysters (reported per nmol of cytochrome *a*) is unlikely a candidate for respiratory inhibition but suggests an involvement of this complex in response to hypoxia. CCO is the terminal oxidase of cellular respiration catalyzing the transfer of electrons to molecular oxygen and shows significant control of respiration rates in isolated mitochondria (Groen et al., 1982) and an apparent excess capacity over mitochondrial state 3 respiration rates (Blier and Lemieux, 2001; Pichaud et al., 2012). The increased capacity of CCO combined with the decreased mitochondrial oxidative capacity could be a strategy for higher oxygen affinity of the respiratory chain (Gnaiger et al., 1998). Such a mechanism was described in mammals and its role would be to provide a buffer against the inhibition of the respiratory chain when CCO becomes oxygen limited. This increase in CCO catalytic capacity would be required to ensure an efficient thermodynamic gradient and free

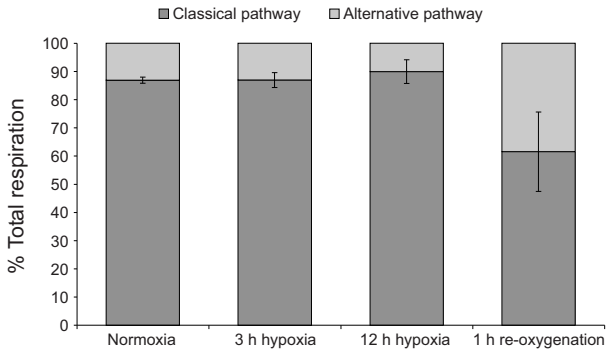

Fig. 3. Percentage contribution to mitochondrial oxygen consumption of the classical and alternative pathways in the Pacific oyster. Dark grey bars represent the percentage of inhibition of state 3 rates by antimycin A (20 μmol l^{-1}). Light grey bars represent the hypothetical percentage of alternative pathway respiration. Values are means ± s.e.m. (*N*=3).

access of electrons to the electron transport chain (Blier and Lemieux, 2001). CCO was also involved in changes in mitochondrial capacities during changes in environmental temperature and during metabolic depression in fish (Blier and Lemieux, 2001; Frick et al., 2010). Mechanisms regulating CCO activity can involve transcriptional, translational and allosteric regulation of the different CCO subunits as well as protein–phospholipid interactions (Stuart et al., 1998; Kraffe et al., 2007; Frick et al., 2010). In this context, cytochrome concentrations were expected to provide indications of whether structural modifications were involved in adjustments of mitochondrial functions, as differences in the relative levels or concentrations of electron transport chain complexes could contribute to variability of the catalytic capacity of the mitochondria (Sidell, 1983; Guderley et al., 2005). Levels of cytochromes were quite constant during hypoxia exposure, although the relative concentration of cytochrome *a* (cyt *a*/cyt c_1 ratio) was significantly increased during re-oxygenation (seemingly linked to a decrease in the amount of cyt c_1). Cytochrome *a* is one of the heme centres of subunit I of CCO, and cytochrome c_1 is a subunit of complex III. Thus, changes in their relative concentrations suggest that functional rearrangements of respiratory chain complexes may take place during re-oxygenation, which in turn could have reduced the capacity and activity of the classical respiratory pathway.

In hypoxia-tolerant marine invertebrates, electron transfer through an AOX was hypothesized to allow further control of the respiratory chain, to maintain cellular redox balance during oxygen fluctuations (Pörtner and Grieshaber, 1993; Abele et al., 2007). AOX branches off the classical respiratory chain before complex III, reducing oxygen without pumping protons in the inter-membrane space. Its role would be to maintain respiratory electron flux and cellular redox balance under metabolically downregulated conditions, or when oxygen levels within the cell start to rise because the classical respiratory chain is saturated with oxygen, minimizing the risk of oxidative stress (Tschischka et al., 2000). AOX activity has been detected in various marine invertebrates (Tschischka et al., 2000; Buchner et al., 2001; Pichaud et al., 2012), and the presence of its sequence was established in *C. gigas* and several bivalves (McDonald and Vanlerberghe, 2004; McDonald et al., 2009). In *C. gigas* an expressed sequence tag corresponding to the AOX sequence was first found in a hemocyte cDNA library coming from a bacterial challenge (Gueguen et al., 2003). Blast analysis on *C. gigas* AOX protein sequence (GenBank accession no. ACL31211) indicates the presence of the typical AOX domain (ferritin-like diiron-binding domain) found in plants, fungi and protists. In the present study, AOX mRNA levels showed the same pattern in gills and digestive gland, but expression levels were higher in gills. This could suggest an important role of AOX in gills as the first sensor responding to oxygen fluctuations and having a high metabolic importance in regulating ion exchange. AOX mRNA levels were overexpressed in both gills and digestive gland after 12 and 24 h of hypoxia and 1 h of re-oxygenation. Considering that AOX can be regulated at the transcriptional level (Vanlerberghe and McIntosh, 1997), these results support the hypothesis of a role of the AOX in fluctuating oxygen conditions in bivalves as proposed by Tschischka et al. (Tschischka et al., 2000).

The respective contribution to overall rates of mitochondrial oxygen consumption of the classical and the alternative respiratory pathways has been generally investigated using respiratory chain inhibitors (KCN and SHAM, specific inhibitors of CCO and AOX, respectively) (Vanlerberghe and McIntosh, 1997). However, even in very well studied plant models, it is difficult to quantify their

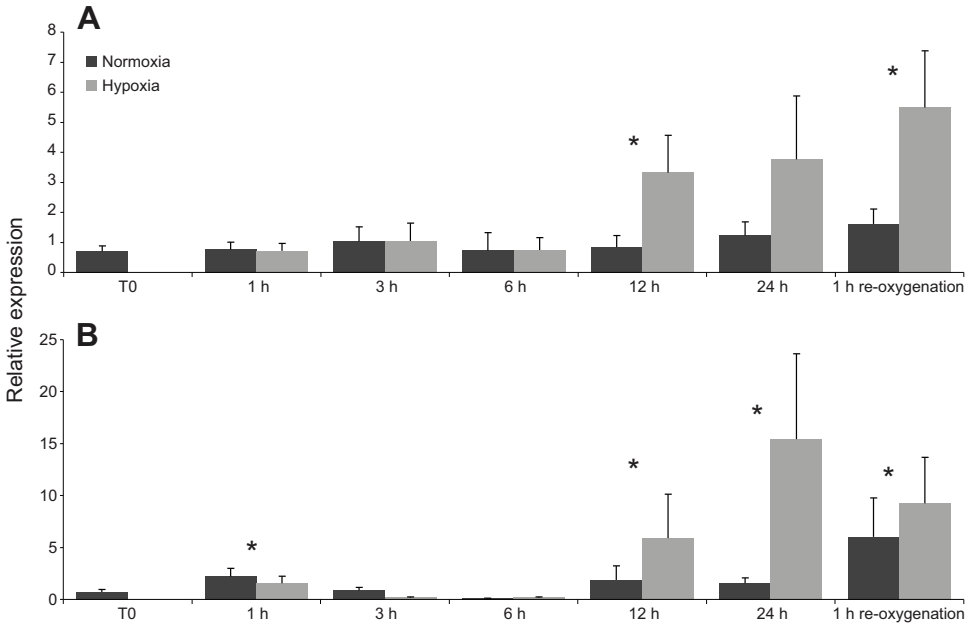

Fig. 4. Alternative oxidase mRNA relative expression for gills (A) and digestive gland (B) of the Pacific oyster. Results are expressed as the relative expression to the geometric mean of the two reference genes used. Asterisks indicate significant differences between hypoxic and normoxic samples within one time of sampling (Mann–Whitney *U*-test, $P<0.05$). Values are means ± s.e.m. ($N=8$).

relative contributions using these inhibitors (Njogu et al., 1980; Bingham and Stevenson, 1995). In preliminary trials, the use of SHAM to inhibit AOX in *C. gigas* was not conclusive, and might be due to a non-specific effect of SHAM on CCO (Tschischka et al., 2000), or to AOX in oysters that would be not sensitive to SHAM (Donaghy et al., 2012). In the present study, we blocked the respiratory chain at complex III with antimycin A to assess the potential contribution of AOX to mitochondrial respiration. This inhibitor has previously been used to investigate AOX oxygen consumption (Albury et al., 2010; Pichaud et al., 2012). In the present study, antimycin A inhibited approximately 85–90% of state 3 oxidation of glutamate in oyster gill mitochondria, suggesting that 10–15% of residual respiration could be attributed to AOX oxygen consumption. This observation is consistent with results observed in the sandworm *Nereis virens* (Pichaud et al., 2012). Furthermore, oxygen consumption by the 'classical' respiratory pathway showed a decrease, albeit not significant (ANOVA, $P=0.087$), in oyster mitochondria 1 h after re-oxygenation, indicating that AOX could be activated during re-oxygenation. AOX activity is controlled at the transcriptional level as well as by carbon availability, increased ROS production and redox potential (Vanlerberghe and McIntosh, 1997; Abele et al., 2007), and reversible covalent modification of specific carbon metabolites can also modify its activity (Vanlerberghe and McIntosh, 1997). In this context we can hypothesize that the increased AOX activity during re-oxygenation may be correlated to increased carbon availability, as the mitochondria are emerging from metabolic depression. Although our results suggest that an increased AOX activity 1 h after re-oxygenation can be associated with an increase in gene expression, mechanism of AOX regulations are not completely elucidated by this study, especially the relationship between increased AOX gene expression and higher AOX protein concentration. Moreover, we do not know whether gene expression after 24 h of hypoxia did reach its plateau. More time points in the hypoxic time series would help to answer this question. Furthermore, the increase of the alternative pathway 1 h after re-oxygenation may just be the result of a delay between gene expression increase during hypoxia and protein synthesis, therefore representing a carryover response of hypoxia. More time points in the re-oxygenation time series would help to answer this question. Although the evidence presented here suggests that AOX may play a role during hypoxia, the fact that AOX gene expression is only upregulated after 12 h of hypoxia indicates that AOX adjustments are not a short-term response to hypoxia but would rather be regulated on persisting hypoxic conditions.

Conclusions

The lower level of oxygen in water seems to be a strong stimulus for gills to downregulate mitochondrial capacities in the Pacific oyster. This study highlighted the plasticity of mitochondria, which appear to be optimized to cope with sharp oxygen fluctuations. Overall, our results indicate that adjustments in mitochondrial oxidative capacities in oyster gills during hypoxia and re-oxygenation occur very quickly and are likely related to regulation of electron transport chain enzymes. Oyster gill mitochondria showed a remarkable sensitivity to O_2 variation, as shown by the rapid decrease of their oxidative capacities (states 3 and 4 stimulated by glutamate) and CCO catalytic capacities. The analysis of AOX using measurements of both respiratory capacity and gene expression support the hypothesis of its existence in *C. gigas*, involved in the response to oxygen fluctuation. To the best of our knowledge, no studies so far have dealt with AOX gene expression and its activity in molluscs faced with oxygen fluctuations, but further elucidation of AOX regulation within the respiratory chain in oyster mitochondria is necessary.

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AUTHOR CONTRIBUTIONS

R.S., C.F., P.S. and E.K. conceived and designed the experiments; R.S., T.D. and E.K. performed the experiments; R.S. and E.K. analyzed the data; R.S. and E.K. wrote and drafted the paper; and P.S., C.R. and D.M. revised the article.

COMPETING INTERESTS

No competing interests declared.

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