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# Functional capacities of gill mitochondria in oyster *Crassostrea gigas* during an emersion/immersion tidal cycle

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**Abstract –** Sessile animals that live on the foreshore undergo tidal cycles, and have to face variations in physical and chemical parameters such as oxygen concentration. During emersion, availability of dissolved oxygen can be lowered for bivalves, which have only a small reserve of seawater inside their closed shell. Differences in oxygen concentration are thus expected to lead to modifications of the metabolism, including changes in mitochondrial activity. Previous studies investigated air exposure under extreme conditions, which do not always reflect environmental conditions these invertebrates have to cope with. In this study, oxidative capacities of gill mitochondria of the oyster *Crassostrea gigas* were studied during a tidal cycle period, by comparing oysters collected after emersion and immersion. Only minor differences were found in state 3 (oxidative phosphorylation) or state 4 (non-phosphorylating oxygen consumption) rates between the two conditions. Similarly, no difference was observed in cytochrome *c* oxidase activity or in oxygen consumption related to maximal electron flux through complexes I-IV, II-IV and IV. While capacities of substrate oxidation were maintained in both emersion and immersion conditions, capacity of mitochondria to produce adenosine triphosphate (ATP) was significantly lower in oysters sampled during emersion. These results suggest that although *C. gigas* could maintain aerobic metabolism during emersion period within a tidal cycle in its environment, energy producing mechanisms are affected.

**Keywords:** Mitochondria / oxygen consumption / ATP production / respiratory chain inhibitor / *Crassostrea gigas*

## 1 Introduction

Sessile organisms inhabiting the intertidal zone such as bivalves have to cope with variations in physical and chemical parameters in their environment, especially because of tidal cycles. Periodical oxygen fluctuations are an important environmental stressor in intertidal and coastal habitats. Benthic sessile animals face large variations in oxygen concentration between emersion and immersion phases. Bivalves, which are filter feeders animals, open their shells during immersion to filter water and take up oxygen directly from the seawater. During emersion, the amount of dissolved oxygen available decreases because bivalves have only a small reserve of seawater in their pallial cavity, as they close shell valves to prevent desiccation (Newell 1979; Grieshaber et al. 1994).

Intertidal molluscs are hypoxia-tolerant animals successfully dealing with fluctuating oxygen levels with a suite of physiological, biochemical and metabolic adaptations. Major effects of air exposure reported in bivalves are reduced oxygen uptake (Coleman 1973; Widdows et al. 1979; Mingoa-Licuanan 1993; Huang and Newell 2002), decreased hemocyte number and activity as well as reduced hemolymph pH (Pampanin et al. 2002; Allen and Burnett 2008), alterations of lysosomal membrane integrity in hemocytes (Zhang et al. 2006). Air exposure also affects metabolism leading to accumulation of anaerobic endproducts (Michaelidis et al. 2005), altered activities of antioxidant enzymes, increased levels of lipid peroxidation and DNA damage (Almeida et al. 2005), and reduced adenosine triphosphate (ATP) concentration (Wijsman 1976; Widdows et al. 1979; Nicchitta and Ellington 1983; Moal et al. 1989; Sukhotin and Pörtner 1999). While emersion could result in major variations in oxygen concentration of the seawater reserve within the pallial cavity, some bivalves can take up oxygen from the air thanks to shell gaping (Lent 1968). The intensity of shell gaping can vary among species and can explain differences in aerial rate of oxygen consumption (Widdows et al. 1979; Rafrafi and Uglow 2009). Rafrafi and Uglow (2009) showed lower weight loss and lower succinate accumulation in oysters *Crassostrea gigas* placed in emersion under humid air, as compared to those

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placed under nitrogen atmosphere. This indicated that oxygen uptake can be possible by air-gaping during emersion and that gaping behaviour adopted by oysters under air exposure could limit transition from aerobic to anaerobic metabolism. Such phenomenon would allow some intertidal bivalves to maintain ~40% of immersed metabolism when emersed (Shick et al. 1988).

The Pacific oyster *C. gigas* is a facultative anaerobe inhabiting intertidal area and capable of surviving prolonged periods with no or low oxygen. Oysters can detect fluctuations of ambient oxygen levels and show a high metabolic plasticity in response to these fluctuations (Le Moullac et al. 2007; Rafrafi and Uglow 2009). Among the biochemical mechanisms that drive metabolic controls, mitochondria play critical roles and show important plasticity in oysters which could allow coping with sharp oxygen fluctuations (Kurochkin et al. 2009; Sussarellu et al. 2013).

An important limitation of some of the earlier studies is that bivalves have been subjected to unrealistic conditions of exposure to air which did not reflect situations that bivalves have to face in their natural environment. To prevent oxygen uptake from air-gaping, some authors have closed oyster shells with a rubber band and duration of the oxygen-limiting/anoxia conditions were reaching several days which is more typical of the long-term hypoxia in coastal "dead zones" but far exceeds the typical duration of tidal cycle (Kurochkin et al. 2009; Ivanina et al. 2010a; Ivanina et al. 2010b; Piontkivska et al. 2011). Modifications of mitochondrial functional capacities during air exposure in a natural tidal cycle have not been extensively studied in oysters or other bivalves.

The present study aimed to investigate responses of oyster mitochondria to oxygen variability upon emersion and immersion periods during a natural tidal cycle. Respiratory capacities and maximal oxygen consumption due to flux through electron transport chain (ETC) complexes, as well as ATP production capacities were measured in isolated gill mitochondria and compared in animals sampled in the field after tidal immersion and emersion periods.

## 2 Materials and methods

All chemicals were purchased from Sigma (Saint Quentin Fallavier, France), except otherwise mentioned.

### 2.1 Experimental procedures

Adult Pacific oysters *Crassostrea gigas*, of 6 to 11 cm shell length (8.5 ± 0.2, mean value ± standard error) were collected on January 2012 on a site named "Grand Dellec" (48° 21′N, 4° 34′W), located ~10 min from the laboratory. Water temperature was 12 °C. Eighty oysters, still cemented to their rock, were moved and placed in the intertidal zone where they would undergo about 4 h of immersion and 8 h of emersion. This corresponded to the upper limit of oysters' distribution on the intertidal zone, with a maximum emersion time for the oysters at this site. After four weeks, oysters were opened and dissected on-the-spot at the end of immersion or emersion periods. Samplings were conducted within a week, 3 days for emersion period and 2 days for immersion period. Fifteen oysters per day were sampled, divided into 3 pools of 5 oysters. Gills were excised, transferred into 50 ml plastic tubes held on ice and immediately brought to the laboratory for subsequent preparation of mitochondrial suspension. Gill pools contained in average 3.2 ± 0.2 g of gill tissue.

### 2.2 Mitochondrial analysis

Gills were chosen for isolation of mitochondria because they represent major sites of oxygen uptake and have important metabolic function in regulating ion exchange (Sokolova et al. 2005; Piontkivska et al. 2011).

#### *Isolation of mitochondria*

Procedures of mitochondria isolation and assays were adapted from Kraffe et al. (2008). All manipulations were carried out on ice and centrifugations were performed at 4 °C, taking about 1 h to be completed. Oyster gills were dried with paper towels, weighted, and initially chopped with scissors and rinsed two times on 80 $\mu$m mesh with 2 ml of isolation buffer containing 300 mM sucrose, 30 mM HEPES, 100 mM KCl, 8 mM EGTA and 1% of protease inhibitor cocktail (Sigma), pH 7.5. On the day of the experiment, 0.5% fatty acid-free bovine serum albumin (BSA) was added to the buffer. The minced gills were then homogenized with three series of three passes in a motorised Potter tissue grinder (Heidolph, Kelheim, Germany) with a loosely fitting pestle, in 10 volumes of ice-cold isolation buffer. The homogenate was centrifuged at 900 × g at 4 °C for 10 min. The supernatant was collected, filtered on 80 $\mu$m mesh, and again centrifuged at 900 × g at 4 °C for 10 min. The resulting supernatant, considered free of unbroken cells or cell debris, was centrifuged at 10 000 × g. The mitochondrial pellet was re-suspended in reaction buffer (400 mM sucrose, 10 mM $KH_2PO_4$, 30 mM HEPES, 90 mM KCl, 50 mM taurine, 50 mM $\beta$-alanine, pH 7.5, with 0.5% fatty acid-free BSA added on the day of the experiment), corresponding to one-tenth of the mass of gill used (i.e. 100 $\mu$l of buffer per each gram of gill tissue used for isolation), and oxygen uptake and ATP production were measured immediately on the fresh mitochondrial preparations. A subsample was stored at –80 °C for subsequent assays of enzymatic activities.

#### *Measurement of oxygen consumption*

Mitochondrial oxygen consumption was measured polarographically using a water-jacketed $O_2$ monitoring system (Qubit System, Kingston, Ontario, Canada). Temperature was controlled at 10–11 °C by a circulating refrigerated water bath. The oxygen probes were calibrated with air-saturated reaction buffer and corrected for assay temperature and local atmospheric pressure. For each assay, around 0.8 mg of mitochondrial protein (40 $\mu$l mitochondrial preparation) was added to 0.4 ml reaction buffer containing 400 mM sucrose,

30 mM HEPES, 90 mM KCl, 10 mM $KH_2PO_4$, 50 mM taurine and 50 mM $\beta$-alanine, pH 7.5. On the day of the experiment, 0.5% BSA was added to the assay medium. For measurement of maximal oxidative capacities, glutamate (40 mM) or succinate (20 mM) was added to fuel oxygen consumption through complex I or II, respectively. The maximal respiration rate (state 3) was obtained after addition of adenosine diphosphate (ADP) to a final concentration of 600 $\mu$M. Preliminary experiments showed that glutamate alone stimulates respiration through complex I as previously found in *Crassostrea virginica* (Burcham et al. 1983). Our pilot studies also showed that 40 mM for glutamate and 20 mM for succinate are saturating concentrations under the assay conditions of this study (data not shown). Non-phosphorylating oxygen consumption (state 4) rate was measured after adenosine diphosphate (ADP) depletion. Subsequently, 6.3 $\mu$g ml$^{-1}$ oligomycin (final concentration), an inhibitor of the $F_oF_1$-ATP synthase, was added to evaluate oxygen consumption in the absence of oxidative phosphorylation (state $4_{oligo}$) (Nesci et al. 2012). Each measurement was performed in duplicates. Respiratory control ratio (RCR) was defined as the ratio between state 3 and state 4. Oxidative phosphorylation efficiency (relation between ADP added and oxygen consumption) was calculated with ADP/O ratio according to Estabrook (1967).

*Polarographic assessment of maximal flux through electron transport chain (ETC) complexes*

Oxygen consumption related to maximal electron flux through complexes I-IV, II-IV and IV was measured polarographically on the day of mitochondrial isolation using the same water-jacketed $O_2$ monitoring system. Temperature was also controlled at 10–11 °C. For these measurements, around 0.8 mg of mitochondrial protein (40 $\mu$l mitochondrial preparation) was added to 0.4 ml of the reaction buffer. Oxygen consumption related to electron flux through various segments of the ETC was assessed using specific chemical inhibitors (Fig. 1).

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 20 $\mu$M) was used to uncouple mitochondrial respiration from ATP production providing maximal electrons flux capacity through the ETC complexes (Oellermann et al. 2012). CCCP disrupts mitochondrial membrane potential via stimulation of proton leak, but does not directly affect the substrate oxidation of the ETC (Ivanina et al. 2012). No ADP was added for these measurements. Oxygen consumption due to flux through complexes I-IV was estimated from oxidation rates of glutamate (40 mM).

After steady state rates were obtained, rotenone (2 $\mu$g ml$^{-1}$) was added to the chamber to inhibit complex I, and subsequently succinate (20 mM) was added to stimulate flux through complexes II-IV.

Malonate (1 mg ml$^{-1}$) was then added to inhibit complex II, and ascorbate (10 mM) and *N*, *N*, *N*′, *N*′-tetramethyl-*p*-phenylene-diamine (TMPD, 2 mM) were added to determine flux through complex IV cytochrome *c* oxidase (CCO) (Martin et al. 2012). Adding potassium cyanide, KCN (32 $\mu$g ml$^{-1}$) fully inhibited oxygen consumption by CCO, demonstrating that no auto-oxidation of TMPD occurred in the presence of ascorbate.

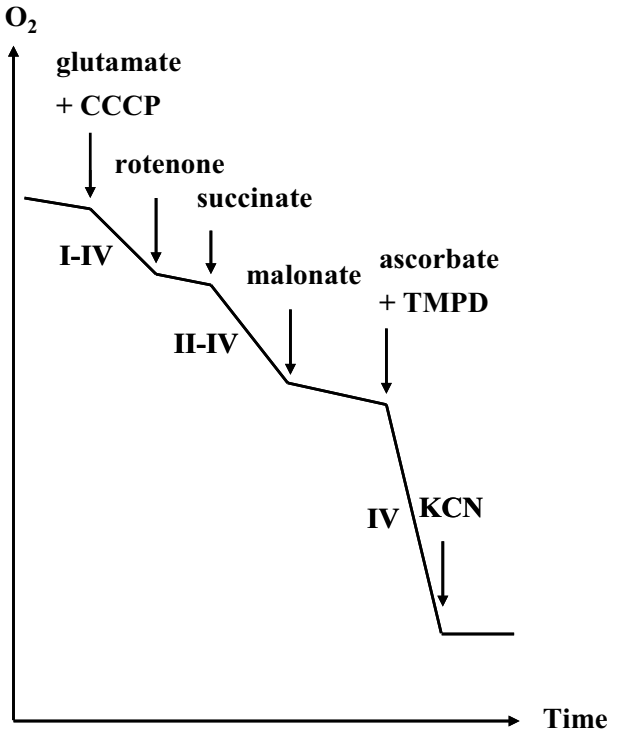


**Fig. 1.** Respiratory profile of mitochondria isolated from gill tissue of *Crassostrea gigas* in response to substrate or inhibitor additions. Glutamate, succinate and a mixture of ascorbate + TMPD allow to measure oxygen consumption related to maximal electron flux through complexes I-IV, II-IV and IV, respectively. Rotenone, malonate and KCN are specific inhibitors of complexes I, II and IV, respectively.

### 2.3 ATP production

ATP production was determined using the ATP luminescent assay (ATP LITE-M 300 Assay Kit, 6016943 Perkin Elmer). Mitochondrial suspensions held on ice were firstly pre-incubated for 1 min with or without 10 mM malonate in Eppendorf vials. Subsequently, 20 mM succinate was added. We used malonate as a specific inhibitor of complex II. Titration with malonate leads to an inhibition of mitochondrial membrane potential ($\Delta\psi_m$, the driving force of ATP synthesis through $F_oF_1$-ATP synthase) via inhibition of the substrate oxidation subsystem (complex II) but does not affect phosphorylation subsystems (Hinkle et al. 1991; Ivanina et al. 2012). We found a >90% of inhibition of oxygen consumption under state 3 (ADP 0.6 mM) for malonate (10 mM) in mitochondrial preparations when succinate 20 mM was used as substrate.

Subsequently, in a 96-well microplate, 100 $\mu$l of mitochondrial suspensions (with or without malonate) was added to 50 $\mu$l of reaction buffer and to 50 $\mu$l of substrate solution provided with the assay Kit. Luminescence was first read at 25 °C every 2 min for 8 min to confirm the absence of ATP production at this step, then ADP (0.6 mM) was added to start oxidative phosphorylation and luminescence was read every 50 s for 15 min. In order to convert relative luminescence units

into ATP contents, a calibration curve of ATP was constructed by preparing a series of dilutions of a stock solution furnished with the assay kit, except that mammalian cell lysis solution was replaced by mitochondrial reaction buffer. The calibration curve was linear in the range of ATP concentrations tested and ATP levels were determined by interpolation from the calibration curve. All assays (standard curve and samples) were run in triplicates. ATP production rate of the samples was determined after ADP addition in the linear part of the curve. The ATP production rate measured without malonate referred to total ATP production and was determined per milligram of mitochondrial proteins per minutes. The remaining ATP production in the presence of malonate was defined as non-$F_oF_1$-ATP synthase related mechanisms, and corresponds to ATP production by other mitochondrial or non-mitochondrial contaminant ATP-producing systems. The ATP production through oxidative phosphorylation was determined as the difference between total ATP production rate and non-$F_oF_1$-ATP synthase related ATP production rate.

### 2.4 Enzymatic activities

Cytochrome *c* oxidase activity was measured in mitochondrial preparations according to Bouchard and Guderley (2003) and from Kraffe et al. (2008). Mitochondrial preparations were submitted to two cycles of freezing and thawing. The disrupted mitochondria were diluted in the assay buffer containing $NaH_2PO_4$ and $Na_2HPO_4$ 50 mM pH 7.8 at 25 °C. Bovine cytochrome *c* (cyt *c* C2037 Sigma) was employed as substrate to stimulate maximal CCO activity. Reduced cyt *c* solution was prepared by adding 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. Absorbance was followed at 550 nm to obtain 95% absorbance of the reduced cyt *c* stock solution. The reaction was performed in microplates at 25 °C in the presence of an initial cyt *c* concentration of 60 $\mu$M. The decrease in absorbance was followed at 550 nm for 10 min and all assays were run in triplicate. Activities were calculated using an extinction coefficient of 19.6 mmol $L^{-1}$ $cm^{-1}$ for cyt *c* (in $\mu$mol cyt *c* transformed $min^{-1}$), $U_{CCO:}$ $\mu$mol cytochrome *c* reduced $min^{-1}$, first order reaction.

### 2.5 Protein concentration

Aliquots of 20 $\mu$l of 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 re-suspended, washed and centrifuged two times again to remove the BSA. Pellets were maintained in 0.5 ml of ultra-pure water and frozen at −80 °C until protein analysis. The protein concentration was determined with the RC DC Protein Assay Kit (BIORAD) using BSA as standard.

### 2.6 Statistical analysis

MANOVA followed by post hoc Fisher's least significant difference (LSD) test was performed to compare oxygen uptake related to electron flux through complexes I-IV, II-IV and IV (factor 1) between oysters sampled during emersion or immersion (factor 2). Non-parametric Mann-Whitney test was performed on other parameters (oxygen consumption, enzymatic activities and ATP production). Significant threshold was $p < 0.05$. All analyses were performed with the Statgraphics software, version Plus 5.1. (Manugistics Inc, Dallas, USA).

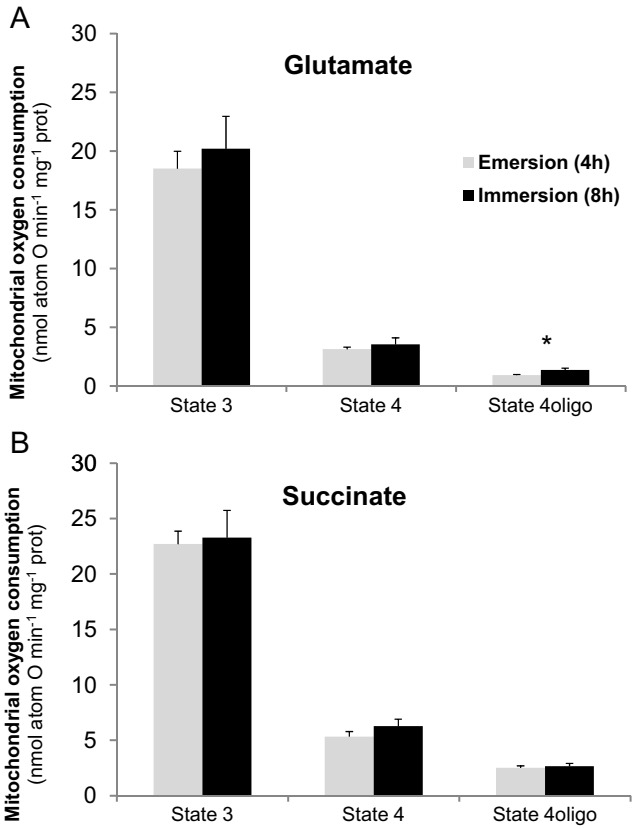


**Fig. 2.** Oxidative phosphorylation (state 3), non-phosphorylating oxygen consumption (state 4) and oligomycin-inhibited state 4 (state $4_{oligo}$) with glutamate (A) or succinate (B) on mitochondria isolated from gills of oysters sampled after 4 h of emersion or after 8 h of immersion. Assay temperature was 10 °C. Values are mean ± SE ($n$ = 9 pools of 5 oysters for emersion and $n$ = 6 pools of 5 oysters for immersion). (⋆): Star indicates values that differ between conditions ($p < 0.05$).

## 3 Results

### 3.1 Mitochondrial oxidative capacities

Maximal state 3 respiration of oyster gill mitochondria fuelled with glutamate was not different from state 3 with succinate ($p$ = 0.083 and 0.411 for oysters sampled during emersion and during immersion, respectively) (Fig. 2). However, non-phosphorylating rates (state 4) were significantly higher with succinate for substrate when compared to rates obtained with glutamate ($p < 0.05$). Respiratory control ratio and ADP/O ratio were significantly higher with glutamate as substrate compared to succinate ($p < 0.05$, Table 1). RCR is indicative of the degree of mitochondrial coupling. The significantly lower RCR obtained with succinate as substrate would

**Table 1.** Respiratory control ratio (RCR) and oxidative phosphorylation efficiency (ADP/O) with glutamate or succinate used as substrate for isolated mitochondria from gills of oysters sampled after 4 h of emersion or after 8 h of immersion. Values are mean ± SE ($n$ = 9 pools of 5 oysters for emersion and $n$ = 6 pools of 5 oysters for immersion).

| Ratio | Emersion | | Immersion | | |
|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | |
| RCR glutamate | 5.9 | 0.2 | 5.1 | 0.4 | NS |
| RCR succinate | 4.4 | 0.4 | 4.0 | 0.2 | NS |
| ADP/O glutamate | 1.4 | 0.0 | 1.5 | 0.1 | NS |
| ADP/O succinate | 1.1 | 0.0 | 1.2 | 0.1 | NS |

NS: no significant difference

indicate that succinate metabolism is less coupled. This was confirmed by a lower ADP/O ratio with succinate (Table 1). Nevertheless, RCR > 5.1 for glutamate and >4.0 for succinate indicated mitochondrial preparations with tightly coupled mitochondria. Maximal state 3 rates and state 4 rates of glutamate and succinate oxidation were not affected by air exposure (Fig. 2). State 4-respiratory rate after adding oligomycin (State $4_{oligo}$) was significantly higher in oysters sampled during immersion than in those collected during emersion when glutamate was used as substrate but not with succinate. RCR and ADP/O were not modified (Table 1).

### 3.2 Mitochondrial maximal electron flux

Oxygen consumption related to maximal electron flux through complexes I-IV, II-IV and IV were similar between emersion and immersion conditions. However, oxygen consumption due to maximal electron flux through complexes I-IV, II-IV and IV were different (Fig. 3). After adding CCCP, maximal electron flux through complexes II-IV led to a significantly higher oxygen consumption rate, as compared to flux through complexes I-IV ($p < 0.05$). Maximal electron flux through complex IV measured with ascorbate + TMPD led to two and three times higher oxygen consumption as compared to flux through complexes II-IV and I-IV, respectively ($p < 0.05$).

### 3.3 Cytochrome *c* oxidase activity

CCO activity in mitochondrial suspension, expressed in U $mg^{-1}$ mitochondrial protein, was not different between emersion and immersion conditions (Table 2, mean value 0.36 ± 0.02).

### 3.4 ATP production

Total ATP production rate, ATP production rate related to $F_oF_1$-ATP synthase and non- $F_oF_1$-ATP synthase related ATP production rate (in nmol $min^{-1}$ $mg^{-1}$ mitochondrial proteins) were significantly higher in oysters sampled during immersion as compared to oysters sampled during emersion (45%, 52%, and 39% higher, respectively) ($p < 0.05$, Fig. 4). ATP produced by other mechanisms than $F_oF_1$-ATP synthase (non-$F_oF_1$-ATP synthase related ATP production) accounted for about 50% of total ATP production rates in gills mitochondrial preparations of oysters sampled during emersion and immersion.

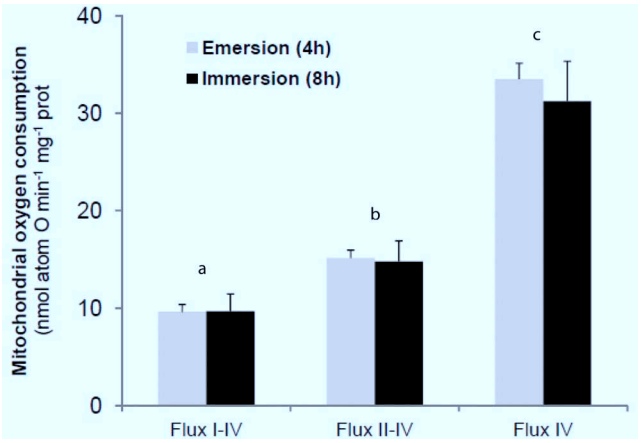


**Fig. 3.** Oxygen uptake related to electron flux through complexes I-IV, II-IV and IV of isolated mitochondria from gills of oysters sampled after 4 h of emersion or after 8 h of immersion. Assay temperature was 10 °C. Values are mean ±SE ($n$ = 9 pools of 5 oysters for emersion and $n$ = 6 pools of 5 oysters for immersion). Different superscript letters indicate values that differ between flux. Maximal flux through complexes of the ETC did not differ statistically between groups ($p < 0.05$).

**Table 2.** Cytochrome *c* oxidase (CCO) activity expressed in U per mg of mitochondrial proteins, of isolated mitochondria from gills of oysters sampled after 4 h of emersion or after 8 h of immersion. Assay temperature was 25 °C. Values are mean ± SE ($n$ = 9 pools of 5 oysters for emersion and $n$ = 6 pools of 5 oysters for immersion).

| | Emersion | | Immersion | | |
|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | |
| CCO activity | 0.35 | 0.02 | 0.38 | 0.04 | NS |

NS: no significant difference.

## 4 Discussion

Functional capacities of gill mitochondria from *C. gigas* were compared between oysters sampled after emersion and immersion periods, at a natural tidal time scale. Although mitochondrial capacities measured at the different levels of organisation (oxidative capacities, flux through ETC complexes and CCO activity) appeared to be maintained between emersion and immersion conditions, a significant impact on ATP production capacities of mitochondria was observed.

Although dissolved oxygen availability likely change between immersion and emersion, oxygen consumption in phosphorylating and non-phosphorylating states (state 3 and 4, respectively) were similar after immersion and emersion regardless of the substrate used (glutamate or succinate). Only

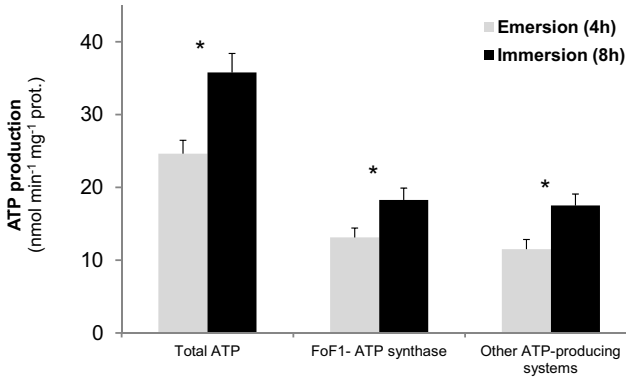


**Fig. 4.** ATP production rate of mitochondria isolated from gills of oysters sampled after 4 h of emersion or after 8 h of immersion. Rates are expressed as nmol $min^{-1}$ $mg^{-1}$ mitochondrial proteins, and determined with succinate 20 mM, ADP 0.6 mM, and with or without malonate 10 mM. Assay temperature was 25 °C. Values are mean ±SE ($n$ = 6 pools of 5 oysters). (*) Stars indicate values that differ between conditions ($p < 0.05$). ATP production of samples incubated with malonate should correspond to ATP produced by other mechanisms than $F_oF_1$-ATP synthase (Other ATP-producing systems). ATP produced by $F_oF_1$-ATP synthase was evaluated as the difference between total ATP production and other ATP-producing systems (see materials and methods).

state $4_{oligo}$ with glutamate as substrate was lower in oysters sampled after emersion compared to their counterparts sampled during immersion.

That mitochondrial properties and capacities were maintained for oysters following emersion strongly suggests that emersion does not involve mitochondrial functional responses to a drop of oxygen. The present results contrast with those obtained when oysters are immersed in seawater at 20% oxygen saturation, which induce rapid and significant modifications (only after 3 h hypoxia) of functional properties of mitochondria in the Pacific oyster (Sussarellu et al. 2013). One explanation could come from the air-gaping activity of *C. gigas* when emersed (Rafrafi and Uglow 2009). This behavior possibly maintains oxygen partial pressure ($pO_2$) of seawater in the pallial cavity (Rafrafi and Uglow 2009). This may be sufficient to avoid the onset of down regulation of mitochondrial capacity in oysters during emersion within a tidal cycle in its environment.

Maintenance of oxidative capacities after emersion may reflect regulatory mechanisms between ETC complexes or other systems that could buffer changes in individual protein activities. Thereby, regulations at the level of individual enzymes of the ETC were explored by measuring flux through the different ETC complexes. Use of CCCP permitted to evaluate capacity of electrons flux through ETC complexes uncoupled to ATP production by $F_oF_1$-ATP synthase (Oellermann et al. 2012; Ivanina et al. 2012). Oxygen consumption fueled by glutamate therefore represents ETC maximal flux from complexes I to IV. Rotenone blocks complex I, with succinate dehydrogenase providing FADH2 to complex II, so that succinate oxidation represents flux from complexes II to IV. With complex II inhibited by malonate, TMPD donates electrons to cytochrome *c*, so TMPD oxidation represents flux through complex IV (cytochrome *c* oxidase). In oyster gill mitochondria, flux through complex IV alone was two to three times higher than flux through the entire ETC. This apparent excess capacity over mitochondrial state 3 respiration rate would reflect that in gills mitochondria of oysters complex IV is typically present at much higher levels in the inner mitochondrial membrane than either complex I or II (Blier and Lemieux 2001; Pichaud et al. 2012; Muleme et al. 2006; Sussarellu et al. 2013). This excess catalytic capacity of CCO would be required to ensure an efficient thermodynamic gradient and free access of electrons to the ETC (Blier and Lemieux 2001). Nevertheless, no change between emersion and immersion conditions was observed for maximal electron flux through complexes I-IV, II-IV or through complex IV. The latter finding was also confirmed by the measurements of CCO activity by spectrophotometry. This suggests that oyster mitochondria undergo no functional alteration after short-term emersion typical for the middle intertidal zone and that no signal of hypoxia/anoxia occurs in the ETC of oysters during these periods of emersion in the field.

Contrastingly, total ATP production rate was found to be 31% lower in mitochondrial preparations of oysters sampled after emersion as compared to those sampled after immersion. Decrease of ATP level in bivalves exposed to air was previously reported (Wijsman 1976; Widdows et al. 1979; Nicchitta and Ellington 1983; Sukhotin and Pörtner 1999), including *C. gigas* in a similar experiment (Moal et al. 1989). The latter study interpreted these results as an interruption of feeding processes and a reduction of oxidative phosphorylation during emersion time. Interestingly, the proportion of ATP produced by $F_oF_1$-ATP synthase represents only around 50% of the total ATP produced. This suggests that half of the ATP production seemingly came from other ATP-producing systems, although mitochondria are generally considered as the major aerobic ATP producers. The phosphorylation subsystems to ATP production in oyster gill mitochondria preparations are still unresolved. One possibility would be the presence of an active adenylate kinase (AK) that can catalyse the reversible conversion of 2 molecules of ADP in 1 molecule of ATP and 1 molecule of AMP. In isolated mitochondria from plants, it has been shown that ATP production due to mitochondrial AK activity can represent two to four times the maximum activity of $F_oF_1$-ATP synthase (Roberts et al. 1997). ATP production rate related to $F_oF_1$-ATP synthase activity was significantly affected by emersion (–28% as compared to values of oysters sampled after immersion). Regulation of ATP synthesis related to mitochondrial respiration still remains incompletely described in marine bivalves. It should be stressed that ATP production could be underestimated due to the potential ATP hydrolysis by ATPases in mitochondrial preparations. It is not excluded that $Na^+$, $K^+$-ATPase or other non-mitochondrial ATPases were present in our mitochondrial preparations, even if it has been shown that these enzymes have a very low activity in similar preparations from rat kidney or rat brain (Malis and Bonventre 1986; Zheng and Ramirez 1999). Non-mitochondrial ATPases can be inactivated by EDTA (Rustin et al. 1994), but we chose not to add this product in the reaction buffer as our preliminary results showed an uncoupling of mitochondrial respiration in response to EDTA. Moreover, $F_oF_1$-ATP synthase can also display a bifunctional rotating

molecular mechanism capable of re-energizing the membrane by ATP hydrolysis (Nesci et al. 2012). A small part of ATP hydrolysis could be due to this ATPase activity and proton conductance as we observed that oligomycin inhibited state 4 respiration rate by about 50% (Hinkle et al. 1991).

The present results indicate that an adjustment of mitochondrial ATP synthesis may be due to the inhibition of $F_oF_1$-ATP synthase and/or reduced ADP transport into the mitochondria during emersion rather than changes in the activity or coupling of the ETC. Indeed, ADP-stimulated respiration, phosphorylation efficiency (estimated through ADP/O) and flux through ETC complexes were not changed in oysters in response to emersion.

## 5 Conclusion

This study has revealed a high stability of mitochondrial oxidative capacities in the oyster gills between emersion and immersion, suggesting that oysters preserve mitochondrial capacities during tidal air exposure. This contrasts with the sharp responses of gill mitochondria to immersed hypoxia. Maintaining the capacity of substrate oxidation during emersion-immersion at a tidal cycle scale could be a means for oysters to preserve aerobic capacity and protect mitochondrial integrity. However, the decrease in ATP production in oysters during emersion showed that air exposure has an influence on energy production by decreasing the activity of ATP-producing systems. Although no data are currently available about the effects of emersion on the ATP turnover rates in bivalve mitochondria, present data suggest that onset of metabolic rate depression can occur on a short-time scale of emersion in response to lower oxygen availability. The mechanisms involved in the regulation of mitochondrial ATP production during the tidal cycle remain unclear and need further studies.

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