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Metabolic adjustments in the oyster *Crassostrea gigas* according to oxygen level and temperature.

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Abstract

The purpose of this study was to examine the responses of the oyster *C. gigas* to oxygen levels at subcellular and whole-organism levels. Two experiments were carried out. The first experiment was designed to measure at 15°C, 20°C and 25°C for 20 hours the clearance (CR) and oxygen consumption (OC) rates of oysters exposed at different concentrations of oxygen. The goal of this first part was to estimate the hypoxic threshold for oysters below which their metabolism shifts toward anaerobiosis, by estimating the oxygen critical point (PcO_2) at 15, 20 and 25°C. The second experiment was carried out to evaluate the metabolic adaptations to hypoxia for 20 days at three temperatures: 12°C, 15°C and 20°C. The metabolic pathways were characterized by the measurement of the enzymes pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) and the alanine and succinate content and the adenylate energy charge (AEC). The respiratory chain functioning was estimated by the measurement of the activity of the electron transport system (ETS). The values of PcO_2 were 3.02±0.15, 3.43±0.20 and 3.28±0.24 mg O_2 L^{-1} at 15, 20 and 25°C, respectively. In whole oysters, hypoxia involved the inhibition of PK whatever the temperature but PEPCK was not stimulated. Succinate accumulated significantly only at 12°C and alanine at 12 and 15°C. A negative relationship between the PK activity and the alanine content was found only in hypoxic oysters. Lastly, hypoxia increased significantly the activity of ETS. With high PcO_2 values, the metabolic depression occurred quickly showing that oysters had a low capacity to regulate their respiration when oxygen availability is reduced, particularly in summer.

Keywords: Oysters, hypoxia, temperature, pyruvate kinase, phosphoenolpyruvate carboxykinase, alanine, succinate.

Introduction

Important mortality outbreaks have been reported in *Crassostrea gigas* populations on the French coasts over the past 15 years (Goulletquer et al., 1998; Soletchnik et al., 1999). The syndrome is known as summer mortality and is generally associated with temperatures above 20°C and coincides with the period of gonad maturation. Some authors suggest that mortalities occurring in the Pacific oyster are the result of multiple factors, including elevated temperatures, physiological stress associated with gonadal maturation, genetics, aquaculture practices, pathogens and pollutants (Samain et al., 2007; Degremont et al., 2005; Labreuche et al., 2006). Sediment nearness has been shown to affect both growth and survival of oysters (Soletchnik et al, 1999, 2006), and could result from a respiratory stress, indeed sediment is often hypoxic but the highest oxygen demand can be in the water close to the bottom (Diaz and Rosenberg, 1995).

Marine molluscs can experience oxygen limitations during emersion in the intertidal zone and during hypoxic periods, on sediment or due to algal blooming. However, most marine molluscs tolerate low oxygen levels. In bivalves, this tolerance is first based on a mechanism of water pumping/ventilation (Tran et al., 2000) which makes more oxygen available to the gills and improves its distribution to the tissues, helping to maintain the rate of oxygen consumption (OC). This mechanism remains functional until an oxygen threshold is reached below which bivalves cannot maintain their rate of OC. This threshold, around 2 mg O_2 L^{-1}, defines the hypoxic threshold (Diaz and Rosenberg, 1995; Bricker et al., 1999). The reduction of feeding activity and OC is a commonly observed response to hypoxia in bivalves (Sobral and Widdows, 1997; Hicks and McMahon, 2002).

Aquatic animals can be subjected to moderate or even severe hypoxia and are characterized by the adaptation of their OC. One distinguishes the oxyregulators from the oxyconformers. The former maintain an OC independent of the DO concentration (at least until a certain level) whereas in the latter, OC decreases as the oxygen partial pressure of the water declines (Dejours, 1981; Pörtner et al, 1985). The basic principles that allow regulators to maintain their oxygen uptake under variable conditions of DO from hyperoxia to hypoxia have been studied in many aquatic invertebrates (Bayne, 1971a, Taylor and Brand, 1975a, 1975b; Tran et al 2000). The concept of critical oxygen point (PcO_2) was introduced to characterize the threshold of DO below which an oxyregulator cannot maintain its rate of oxygen uptake (Grieshaber et al, 1988). This concept includes metabolic changes towards an anaerobic pathway of energy production (Pörtner and Grieshaber, 1993).

Hypoxia and anoxia bring about a metabolic depression due to the switch from aerobic to anaerobic energy production leading to a lower ATP yield per unit glucose (Shick et al., 1986; De Zwaan et al, 1991). Metabolic rate depression is a concordant decline in energy supply and energy demand and thus involves downregulation of many ATP-dependant cellular process. This metabolic switch is located at the end of glycolysis at the level of phosphoenolpyruvate (PEP). In aerobic mode, PEP is the substrate of pyruvate kinase (PK) for the production of pyruvate. PEP can also be the substrate of gluconeogenesis which is essentially rate limited by Phosphoenolpyruvate carboxykinase (PEPCK) (Schein et al., 2004). The metabolic switch from aerobic to anaerobic pathways is controlled by the reversible phosphorylation of PK and allosteric regulations that leads to a reduction in its activity (Storey and Storey, 1990, Simpfendörfer et al, 1997). Pyruvate cannot be oxidized so that it is converted into alanine. When this occurs, PEP becomes mainly a substrate for PEPCK contributing to the formation of succinate (Zammit and Newsholme, 1978). Another mechanism of enzyme control is changes in the amount of enzyme present in tissues via modification of the enzyme turnover. This control used for the long-term metabolic changes of seasonal adjustment could have a role in adaptation to prolonged hypoxia lasting several days. Recently, the response of *C. gigas* to hypoxia has been investigated focusing on the analysis of the differential expression pattern of specific genes associated showing down-regulations in energy metabolism and up-regulations in respiratory chain (David et al., 2005).

Although the response to hypoxia and anoxia in aquatic invertebrates has been widely studied, there is a lack of information on hypoxia tolerance of *C. gigas*. The objective of the present study was to study the sensitivity of oysters to hypoxia with the temperature. A first experiment was carried out to characterize the behaviour of *C. gigas* under variable conditions of DO: (1) Is *C. gigas* a regulator or a conformer? If it is a regulator, what is the PcO_2 value? (2) Is there a relationship between temperature and PcO_2? The second experiment was designed to observe the effect of prolonged hypoxia on cellular functioning. The metabolic pathways were evaluated by assessing PK and PEPCK enzyme activities as well as succinate and alanine accumulation. The adenylate energy charge (AEC) and the activity of the respiratory chain were evaluated by measuring the electron transport system (ETS) activity.

Materials and methods

Experiment 1: effect of dissolved oxygen on CR and OC

The oysters were collected in the Aber Wrach (Finistère, France) in August 2005 when temperature was around 17°C. Before each experiment, the oysters were adapted over a period of 3 weeks to the experimental temperature in open flow tanks and fed with microalgae. Three temperatures were tested: 15°C, 20°C and 25°C. The algae (*I. galbana*) were injected into sea water to give a concentration of algae of approximately 50000 cells mL^{-1}. The oxygen level was controlled by injection of nitrogen in sea water. The lowest dissolved oxygen concentration reached was 1.69, 1.27 and 1.54 mg O_2 L^{-1} at 15°C, 20°C and 25°C respectively (Table 1). The nitrogen flow was controlled by a flowmeter.

Ecophysiological measurement system

The laboratory of Argenton is fitted with an ecophysiological system, which allows the simultaneous monitoring of the clearance rate (CR, $L.h^{-1}$) and oxygen consumption rate (OC, mg $O_2.h^{-1}$) in individual open-flow chambers, for 7 individuals. Individuals were placed, successively, in one of the 7 individual chambers of the system and kept undisturbed in their respective chamber for at least 20h. The first chamber was kept empty and used as a control. Flow rates (FR) in the individual chambers were constant and equal to 100 mL min^{-1}. This value was chosen after a series of tests: the flow rate had to be low enough to allow us to detect filtration or respiration activity; and had to be high enough to prevent the bivalve from removing too much algae, and to allow us to detect accurately the time of variations in filtration and respiration activity. The out-flowing water of each chamber was analysed for phytoplankton (fluorescence) and oxygen concentrations which were recorded each for 5 min continuously during 20h.

CR, an indicator of feeding activity, is defined as the volume of water cleared of suspended particles per unit time. In this study, CR was estimated by using fluorescence recordings. CR was calculated as follows: $(C1-C2) \times FR$. *C1* was the fluorescence level of the control chamber, *C2* was the fluorescence of the experimental chamber containing one oyster. FR was the flow rate. The OC rate is defined as the quantity of oxygen consumed by the animal per unit time (mg $O_2.h^{-1}$). Differences in the oxygen concentration between reference and experimental chambers can be used to calculate the OC rate, as follows: $(O2-O1) \times FR$. *O1* was the oxygen level of the control chamber, *O2* was the oxygen level of the experimental chamber containing one oyster. CR and OC rate were thus calculated and an average was calculated for each animal. In order to compare CR and OC rates on the same basis, it was necessary to correct for weight differences. At the end of each experiment, oysters were freeze dried for 72 h and their dry weight obtained. Physiological rate was then converted to a "standard" animal of 1 g tissue weight using the following equation: $Ys = (Ws/We)^b \times Ye$, where Ys is the physiological rate for an animal of standard weight, Ws the standard weight (1g), We the observed weight of the animal (g), Ye the uncorrected (measured) physiological rate, and b the weight exponent for the physiological rate function. The average b weight exponents are 0.66 and 0.75 for CR and OC respectively in bivalves (Savina and Pouvreau, 2004).

Experiment 2: Effect of prolonged hypoxia

Three experiments were carried out in order to distinguish the differences of responses to hypoxia with the temperature. Experiments were conducted in winter, in summer and in autumn when the sea water temperatures were respectively about 12°C, 20°C and 15°C. Site for oyster collection were chosen for their temperature at the time of collection in order to reduce the acclimation duration to the experimental temperature in laboratory. The oysters came from north Brittany for the experiments during winter and autumn and south Brittany for the summer experiment.

Experimental design

The experiments were performed at the Ifremer laboratory in Argenton (Finistère, France). Oysters were placed in 300-L experimental tanks with 20µm-filtered running seawater. The oysters were first adapted for one week to the laboratory conditions at the experimental temperatures of 12°C, 15°C and 20°C and fed with an algal diet composed of *Isochrysis* at a daily ration close to saturation level. The microalgae ration was standardized and calculated to supply continuously an algae biomass of 12% of the oyster biomass in dry weight.

Two different conditions were tested for 3 weeks: (1) normoxia, where oysters were kept in oxygen saturated seawater and fed; (2) hypoxia, where oysters were maintained immersed in hypoxic seawater and fed. Hypoxia was obtained by bubbling nitrogen through the seawater which allowed the

oxygen level to be controlled at 2 mg O_2 L^{-1}. Each tank was connected to a system that provided measurements every 2h30min to control dissolved oxygen and chlorophyll levels (Blain et al, 2004). Sampling were made on day 10 and day 20 to assess PK, PEPCK, ETS activities and the content of alanine, succinate and the AEC of both normoxic and hypoxic oysters. After each sampling, water renewal and algal distribution were adjusted to maintain the same environmental conditions.

PK and PEPCK activities

The activity of PK in oyster tissues was determined as described by Greenway and Storey (1999). Extracts for enzyme assay were prepared using ground up, frozen, pooled tissues. Then, using a 1:5 w/v ratio for all tissues, the aliquots were homogenized in a buffer composed of 50 mM imidazole–HCl buffer (pH 7.2), 100 mM Na_3F, 5 mM EDTA, 5 mM EGTA and 15 mM 2-mercaptoethanol. A few crystals of phenylmethylsulfonyl (PMSF) were added immediately prior to homogenization. The homogenates were centrifuged for 20 min at 20 000 *g* at 5°C. Assays were conducted at room temperature (20°C) and initiated by mixing the enzyme preparation, of 250 µl total microplate well volume, with the homogenate. The preparation for Pyruvate kinase (PK; EC 2.7.1.40) was as follows: 100 mM imidazole–HCl buffer (pH 7.2), 50 mM KCl, 5 mM $MgCl_2$, 10 mM phosphoenol pyruvate, 2 mM ADP, 0.15 mM NADH, 0.2% 2 (v/v) rotenone-saturated ethanol, and 1 U.mL^{-1} LDH. For Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) the preparation was: 100 mM imidazole–HCl buffer (pH 6.6), 30 mM 2-mercaptoethanol, 5 mM phosphoenolpyruvate, 50 mM $NaHCO_3$, 1.25 mM IDP, 1 mM $MnCl_2$, 0.15 mM NADH, and 2.5 U.mL^{-1} MDH. Blanks were run and subtracted. Changes in NAD(P)H absorbance at 340 nm were monitored using a Biotek Microplate Reader.

Electron transport system activity

ETS activity was determined following the method of Owens and King (1975), where INT-tetrazolium is reduced to the optically active INT-Formazan when substituted for oxygen as the terminal electron acceptor. Reagents were freshly made and kept on ice and all assay procedures were carried out in an ice bath. The powdered tissue was removed from liquid nitrogen storage, placed in ETS B solution (75µM $MgSO_4$ $7H_2O$, 1.5 mg mL^{-1} polyvinylpyrrolidone, and 0.2% (v:v) Triton X-100 in 0.1 M phosphate buffer pH 8.5) and sonicated. Then samples were centrifuged for 15 min (4°C, 3000g). 150µL supernatant was transferred in another tube to which 400 µL substrate solution (1.2 mg mL^{-1} NADH, 0.2 mg mL^{-1} 1 NADPH in ETS B solution) and 250 mL INT solution (2 mg mL^{-1} INT-tetrazolium in double distilled water pH 8.5) was added. Samples were incubated for 20 min at room temperature (20°C) after which the reaction was stopped with 400 µL quench solution (50% formalin, 50% 1M H_3PO_4). The absorbance of each sample was then measured spectrophotometrically at 490 nm. The absorbance value was corrected using a turbidity blank, which consisted of 150 µL samples, 400 µL ETS B, 250 µL INT solution and 400 µL quench solution. ETS was then calculated by the following equation given by Garcia-Esquivel et al. (2001):

$$\text{ETS } \mu M\ O_2\ h^{-1}\ g\ ww^{-1} = \{(E_{corr} \times V_{hom} \times 60/T \times V_{rxn})/(V_{inc} \times ww \times 31.8)\}$$

Ecorr = measured absorbance, corrected with the buffer and reagent blank, Vhom = volume of the total homogenate (mL), 60 = factor used for to express the activity per hour, T = time of incubation (min), Vrxn= final volume in the assay (mL), Vinc = volume of the homogenate used in the reaction assay (mL), ww : wet weight of the sample in the total homogenate (g), 31.8 E490nm cm-1: the molar extinction coefficient of INT-formazan at 490 nm is 15.9 mM-1INT cm-1 assuming that 2 molecules of INT-formazan are formed per molecule of oxygen.

Adenylate energy charge and metabolite determination

Metabolites and nucleotides were extracted from 200 mg of the powdered oyster tissue prepared above with 2 ml of trichloro-acetic acid (TCA), neutralized with 1.2 ml of amine freon trioctylamine/trifluoro-trichloro-ethane, v:v 1:5). Adenylate energy charge (AEC) analyses were conducted according to Moal et al. (1989). Extracted nucleotides were analysed by high-performance liquid chromatography on a reverse phase column with a counter-ion (tributylamine). AEC was calculated as follows: (ATP+0.5 ADP)/(ATP+ADP+AMP) (Ivanovici, 1980). Alanine was measured enzymatically according to standard procedures as described respectively by Williamson (1974). Succinate was quantified with the succinate determination kit of R-Biopharm Inc (Marshall, MI, USA).

Statistics

The effect of DO on OC and clearance rates was analysed using a one way ANOVA. The post hoc protected least squares difference (PLSD) Fisher's test was used to determine which means differed significantly. PcO_2 was determined as the crossing point of the regression lines of the 2 slopes of the relationship between the OC and the value of DO, the horizontal segment and the steeply sloping segment (Cochran and Burnett, 1996). The PcO_2 means were then compared 2 to 2 by the Student T test. Two-way ANOVA with replication was carried out to test for differences in PK activity, PEPCK activity, ETS activity, alanine, succinate and AEC between seasons and oxygen level. Post hoc comparison (PLSD Fisher) tests were used to determine which means differed significantly. The relationship between the alanine content and the PK activity was tested using the critical value Table for Pearson's Correlation Coefficient. Statistical analyses were performed using Statview (SAS).

Results

Experiment 1: effect of dissolved oxygen on CR and OC

The changes in CR and OC corresponded to the decrease in DO. At each temperature, the oysters maintained their CR and OC until a threshold where they fell significantly (Fig 1, 2). This threshold corresponds to a value of DO that expresses the PcO_2. The PcO_2 occurred at 3.02±0.15, 3.43±0.20 and 3.28±0.24 mg O_2 L^{-1} at 15°C, 20°C and 25°C respectively. At 20° C, the PcO_2 was significantly higher than at 15°C (Table 2). When PcO_2 was expressed as oxygen saturation level, there was a positive relationship with temperature (Table 2).

Experiment 2: metabolic adjustments with the temperature during prolonged hypoxia

Enzyme activities

The PK activity differed significantly with temperature ($F=21.01$, $P<0.001$) (Fig 3a). The Fischer PLSD test revealed that the PK at 12°C was significantly lower than at 15 and 20°C. The PK activities at 15 and 20°C were not significantly different. Hypoxia significantly inhibited PK activity ($F=21.15$, $P<0.001$). The PEPCK activity did not change significantly with the temperature ($F=0.98$, NS) and in hypoxia ($F=0.22$, NS). But a significant interaction ($F=4.18$, $P<0.05$) between temperature and oxygen revealed that at 20°C the PEPCK activity in hypoxic oyster was significantly lower than in normoxic oyster (Fig 3b). The ratio of PK and PEPCK activities is considered to be an index of the anaerobic capacity of succinate production, where a lower ratio is associated with a higher anaerobic potential (Simpfendörfer et al., 1995). The anaerobic capacity decreased with an increase of temperature, the PK/PEPCK ratio increased with the temperature ($F=39.42$, $P<0.0001$). In hypoxic oysters, the PK/PEPCK ratio was significantly lower $F=10.94$, $P<0.01$) (Fig 3c). ETS activity increased significantly with the temperature ($F=30.16$, $P<0.0001$) and hypoxia stimulated significantly ETS activity ($F=9.02$, $P<0.01$) (Fig 4).

Metabolite and AEC

There were significant changes in alanine concentration depending on the temperature ($F=14.20$, $P<0.0001$). The alanine content was maximal at 12°C and was significantly higher under hypoxic conditions than at 15°C and 20°C (Fig 5a). Hypoxia involved significant accumulation of alanine ($F=5.39$, $P<0.05$) especially at 12°C (Fig 5a). Succinate content changed with temperature ($F=23.36$, $P<0.0001$) and was the highest at 12°C under hypoxia (Fig 5b). Hypoxia resulted in a significant increase ($F=4.98$, $P<0.05$) of succinate concentration at 12°C (Fig 5b). There was a significant change of AEC with the temperature ($F=35.74$, $P<0.0001$). AEC was the lowest at 12°C and maximal at 15°C (Fig 5c). Hypoxia involved a significant decrease of AEC ($F=10.66$, $P<0.01$) except at 20°C where the level of AEC was similar to that in normoxia (Fig 5c).

Regulation of PK by alanine

There was a significant negative relationship between the alanine content and the PK activity only in hypoxic oyster ($r = -0.356$, $P<5\%$, $df = 37$), at $df = 37$, the null hypothesis was rejected when $r = 0.314$ at the alpha level of 5%. In normoxic oysters, this relationship between alanine content and PK activity did not appear ($r = 0.031$, NS, $df = 37$).

Discussion

Effect of temperature

The relationship between temperature and maximum OC obtained in this experiment supports the model established by Bougrier et al. (1995) in *C. gigas*. This relationship is common for numerous temperate and tropical bivalves (Haure et al., 1998; Saucedo et al., 2004).
In invertebrates, the overall glycolytic capacity is increased in summer (Greenway and Storey, 1999, 2001). The influence of temperature on the glycolytic capacity was related to the seasonal cycle of storage and mobilization of energetic reserves, especially glycogen but also stored lipids, and is correlated with the annual reproductive cycle (Bacca et al., 2005, Berthelin et al., 2000). This study has confirmed the importance of the temperature on the functioning of PK and PEPCK in *C. gigas* as in other invertebrates (Wieser and Wright, 1979; Michaelidis and Storey, 1990; Sokolova and Portner, 2001). Seasonal adjustments are mediated with the amounts of enzymes and/or with change of the type of isoforms present. In *C. gigas* muscle, regulation of PK can be achieved at different steps from the kinetic control via the allosteric effectors to the transcriptional level while regulation of PEPCK seems essentially to be at transcriptional level (Le Moullac et al., 2007). The increase of PEPCK activity with temperature which has a gluconeogenic role, could also suggest a higher rate of amino acid catabolism from dietary sources (Greenway and Storey, 2001). The functionally linked enzymes PK and PEPCK were used to estimate the potential anaerobic capacity. These two enzymes are likely to compete for a common substrate, PEP, channelling it to aerobic (PK) or anaerobic (PEPCK) pathways, so that a low PK/PEPCK ratio is indicative of a relative higher anaerobic capacity. We found in the oyster *C. gigas* that the PK/PEPCK ratio differed consistently in normoxic oysters with the temperature suggesting that the capability of oysters to survive anaerobiosis would be lower at 15°C and 20°C than at 12°C (Sokolova and Pörtner, 2001). ETS activity was used to estimate the potential respiratory capacity of marine zooplankton (Owens and King, 1975). In bivalves, ETS activity has been used also to assess the oxygen demand to evaluate seasonal changes (Fanslow et al., 2001) and pH (Simcic and Brancelj, 2006). Our results showed in the oyster *C. gigas* for the first time the relationship of ETS activity with temperature while in this species, previous studies already shown that oxygen consumption and feeding regulate ETS activity (Garcia Esquivel et al, 2001, 2002).

Effect of hypoxia

The bivalves are adapted to survive periodic hypoxia the occurrence of which increases in estuaries, bays and lagoons. Faced with a decrease of oxygen tension, the organisms exhibit various models for the adaptation of their OC. Certain species called regulators seek to maintain their OC constant over a wide range of DO. Others reduce their OC with the decrease of DO and are called conformers (Prosser, 1973). Our results showed that the oyster *C. gigas* acts as a regulator for OC. The mechanism which allows the bivalves to adopt this behaviour is carried out by an adjustment of the ventilatory activity that is the key of the adaptation to avoid changes of blood oxygenation (Bayne, 1971b; Taylor and Brand, 1975a, 1975b; Tran et al., 2000). But this system of adaptation finds its limit at the oxygen threshold below which the organisms can no longer maintain their OC. The PcO_2 represents the limit of regulatory ability (Grieshaber et al., 1988). The concept of PcO_2 includes the setting of an anaerobic energy metabolism (Pörtner and Grieshaber, 1993). The relationship between the temperature and the PcO_2 relies only on the oxygen solubility depending on the water temperature. Nevertheless, the oysters were weak regulators, strongly dependent of oxygen and could regulate their OC over only a limited range of oxygen availability.
At a cellular level, hypoxia led to a metabolic depression in *C. gigas*. The PK activity was inhibited in hypoxic oysters; this inhibition was related to the alanine content. The molecular basis of PK activity suppression in response to a lack of oxygen is the same in many species of marine molluscs (Storey, 1993; Greenway and Storey, 2000). Phosphorylation of PK reduces its affinity for PEP, reduces sensitivity to the allosteric activator fructose-1,6-biphospahte (F1,6BP) and greatly increases enzyme inhibition by alanine from anaerobic metabolism (Plaxton and Storey, 1984; Storey, 1993; Greenway and Storey, 2000; Greenway and Storey, 2001). Recently we have shown in *C. gigas* that the adaptation to hypoxia of muscle PK was first allosteric by the content of alanine and then later at transcriptional level after 10 days of hypoxia by decreasing the PK mRNA level. In the same time, regulation of PEPCK in hypoxic muscle occurred at gene level by significantly increasing the PEPCK mRNA level (Le Moullac et al., 2007). Accumulation of anaerobic end products in hypoxia such as alanine and succinate occurred only at 12°C showing that the highest anaerobic capacity of oyster was at low temperature. Hypoxia did not induce significant changes of PEPCK activity especially at

12°C while succinate accumulated suggesting that succinate was derived from the aspartate utilization (de Zwann et al., 1991; Isani et al, 1995).
The stimulation of the ETS activity, observed here for the first time in molluscs, could probably be linked to the up-regulation of some genes of the respiratory chain as occurs in anoxia tolerant vertebrates (Cai and Storey, 1996, Storey, 2004). Regulated genes of the respiratory chain were recently identified after 7-10 days of hypoxia exposure in C. gigas (David et al., 2005). In the carp, an increase in the activity of cytochrome c oxidase was observed in the muscle after seven days hypoxia (Zhou et al., 2000). The stimulation of ETS activity could be responsible for the maintenance of AEC during gametogenesis and of the modest decline of AEC at 12 and 15°C in hypoxic oyster. In order for cells to survive under prolonged hypoxia or anoxia, they must maintain their energy requirement by coupling effectively energy supply and demand. In bivalves, long term anoxia is characterized by the maintain level of AEC due to the phosphoarginine contribution and the reduction of ATP turnover (Isani et al., 1995). The maintenance of relatively constant concentrations of adenylates has also been reported in muscles from vertebrates showing anoxia-tolerance (Chew and Ip, 1992; Via et al., 1994; West and Boutilier, 1998).

Conclusion

This work has demonstrated that the oxygen level and temperature are important in the regulation of the energy metabolism of the oyster *C. gigas*. Though oysters acted as regulators they had a low capacity to regulate their oxygen uptake due to high PcO_2 values. The metabolic depression can occur frequently particularly in summer. This work proposed useful biomarkers such as the couple PK activity-alanine and the ETS activity to assess *in situ* the effects of environmental degradation such as hypoxia on metabolic alterations. Further studies should be carried out to clarify the role of trophic level on glycolytic enzymes and their regulation.

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Table 1: dissolved oxygen level tested for each temperature expressed as mg O_2 L^{-1} and % of oxygen saturation seawater.

15°C mg O_2 L^{-1} - % saturation	20°C mg O_2 L^{-1} - % saturation	25°C mg O_2 L^{-1} - % saturation
1.69 - 20	1.27 - 16	1.54 – 23
1.99 – 24	2.00 – 25	1.93 – 28
2.47 – 30	3.02 – 38	3.03 – 45
2.85 - 34	4.08 – 51	3.60 – 53
3.43 – 41	5.09 – 64	4.90 – 72
5.00 – 60	6.16 - 77	5.80 – 85
8.34 - 100	8.00 - 100	6.80 - 100

Table 2: maximal oxygen consumption and critical oxygen pressure (PcO_2)

temperature	OC max. mg O_2 $h^{-1}g^{-1}$	PcO_2 mg O_2 L^{-1}	PcO_2 % saturation
15°C	0.752 ± 0.047^{c}	3.02 ± 0.15^{b}	36.2
20°C	1.264 ± 0.062^{b}	3.43 ± 0.20^{a}	42.9
25°C	1.666 ± 0.113^{a}	3.28 ± 0.24^{ab}	48.2

a, b, c letter showing significant difference at the level of 5% within column

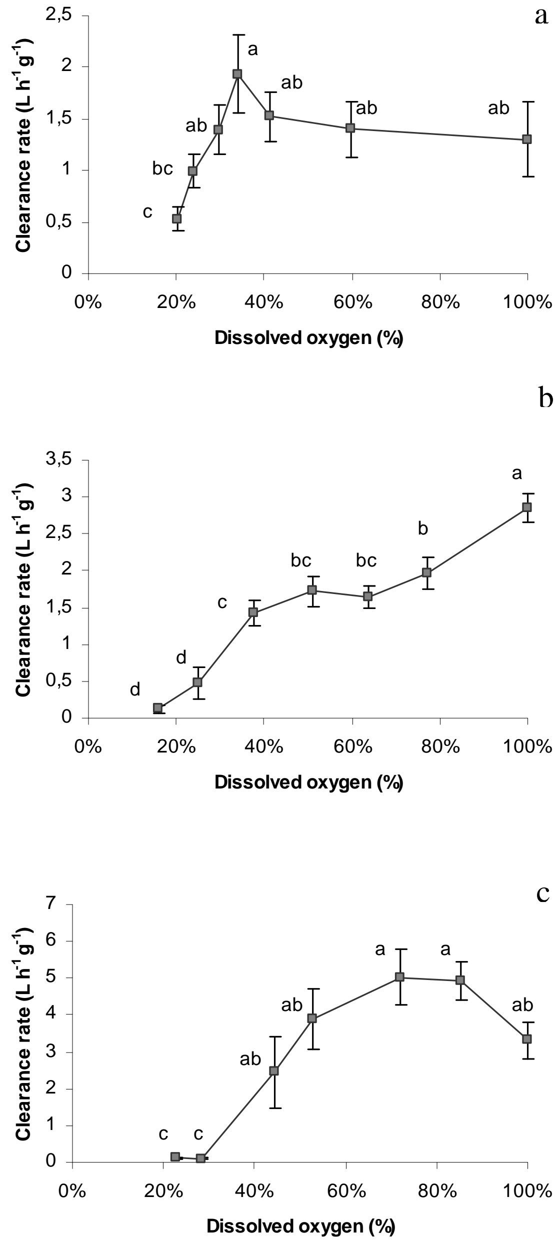
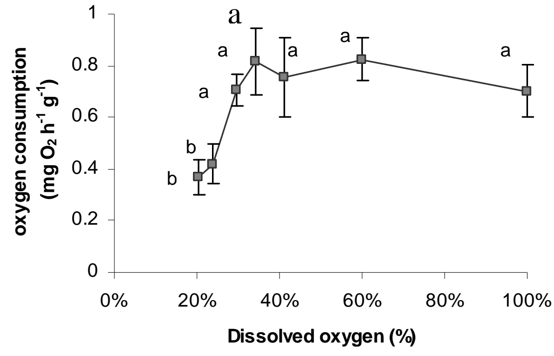
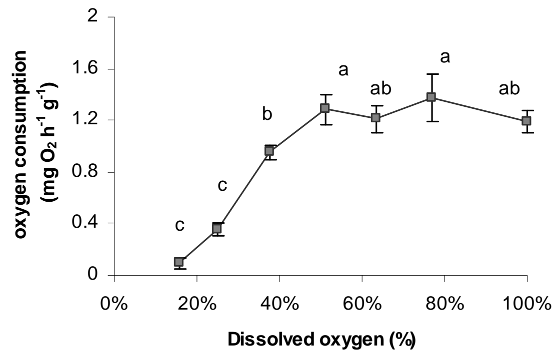


Figure 1: Effect of dissolved oxygen on clearance rate (CR) with the temperature (a) 15°C, (b) 20°C, (c) 25°C. (Means ± SE, n=7).



b



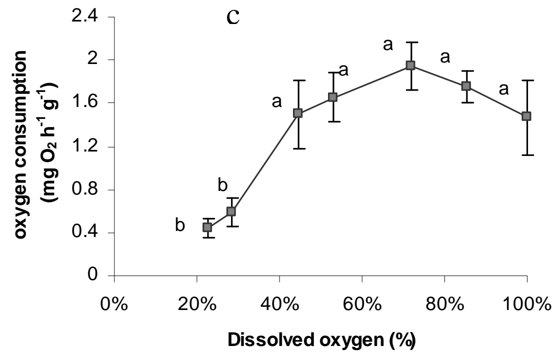


Figure 2: Effect of dissolved oxygen on oxygen consumption (OC) with the temperature (a) 15°C, (b) 20°C, (c) 25°C. (Means ± SE, n=7).

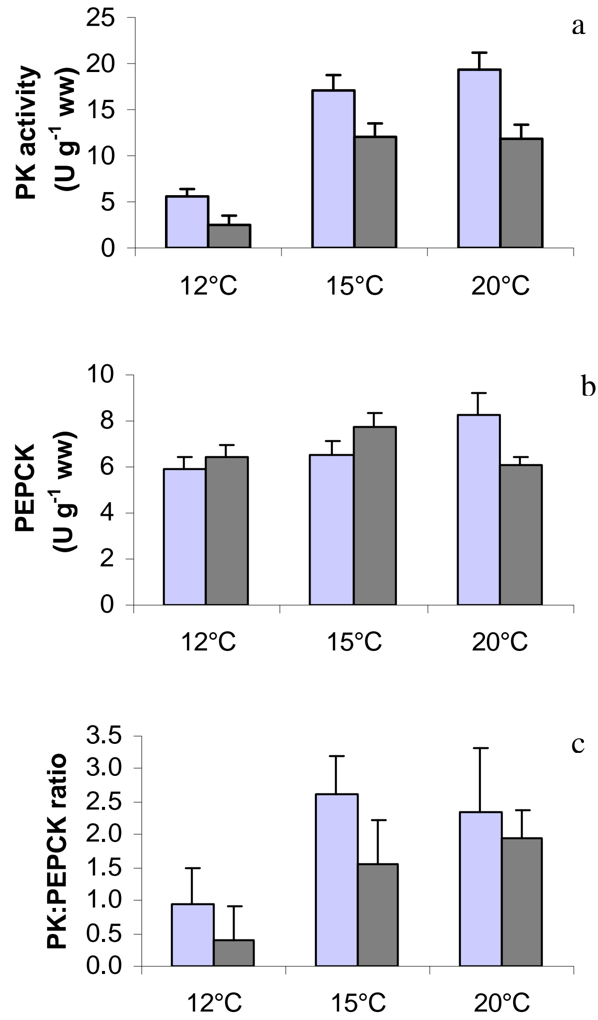


Figure 3: Effect of temperature and hypoxia on (a) PK activity, (b) PEPCK activity, (c) PK/PEPCK ratio. Normoxia (grey) and hypoxia (dark) (Means ± SE, n=12).

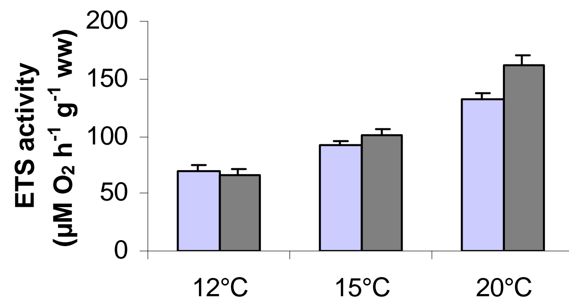


Figure 4: Effect of temperature and hypoxia on ETS activity. Normoxia (grey) and hypoxia (dark) (Means ± SE, n=12).

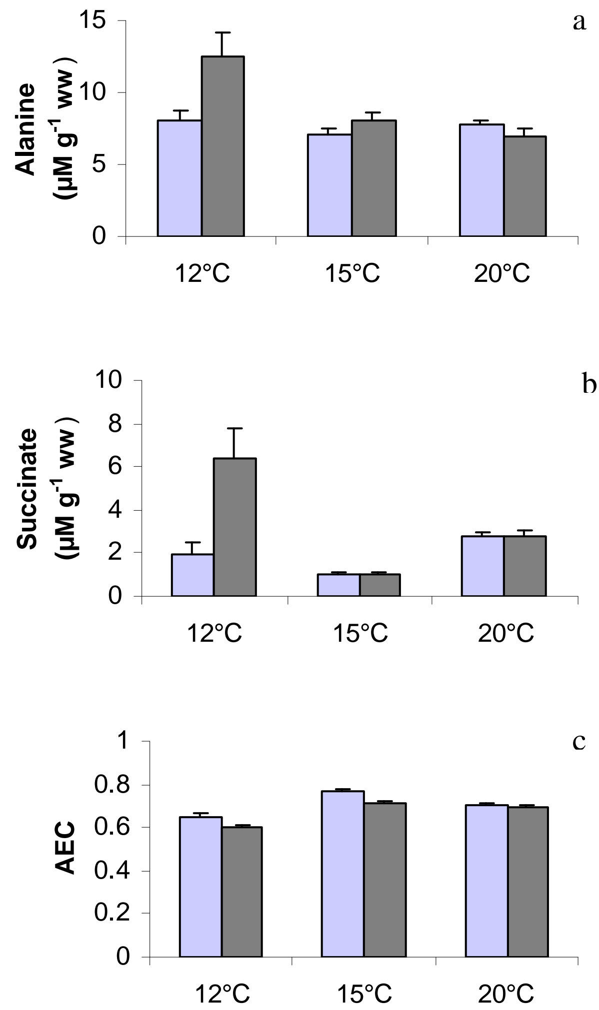


Figure 5: Effect of temperature and hypoxia on (a) alanine (b) succinate content and (c) AEC. Normoxia (grey) and hypoxia (dark) (Means ± SE, n=12).