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# The cytochrome c oxidase and its mitochondrial function in the whiteleg shrimp *Litopenaeus vannamei* during hypoxia

L. R. Jimenez-Gutierrez · S. Uribe-Carvajal ·  
A. Sanchez-Paz · C. Chimeo · A. Muhlia-Almazan

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**Abstract** Cytochrome c oxidase (COX), which is located in the inner membrane of mitochondria, is a key constituent of the electron transport chain that catalyzes the reduction of oxygen. The Pacific whiteleg shrimp *Litopenaeus vannamei* is constantly exposed to hypoxic conditions, which affects both the central metabolism and the mitochondrial function. The purpose of this study was to isolate shrimp mitochondria, identify the COX complex and to evaluate the effect of hypoxia on the shrimp mitochondrial function and in the COX activity. A 190 kDa protein was identified as COX by immunodetection techniques. The effect of hypoxia was confirmed by an increase in the shrimp plasma L-lactate concentration. COX activity, mitochondrial oxygen uptake and protein content were reduced under hypoxic conditions, and gradually restored as hypoxia continued, this suggests an adaptive mitochondrial response and a highly effective COX enzyme. Both mitochondrial oxygen uptake and COX activity were completely inhibited by KCN and sodium azide, suggesting that COX is the unique oxidase in *L. vannamei* mitochondria.

**Keywords** COX activity · Hypoxia · Inhibition · Mitochondrial oxygen uptake · Shrimp

## Introduction

The synthesis of chemical energy and respiration in eukaryotic cells is carried out in the mitochondrion (Mayevsky and Rogatsky 2007). Cytochrome c oxidase (COX) is a multimeric complex which catalyzes the reduction of di-oxygen ( $O_2$ ) to water, being the terminal enzyme of the electron transport chain (Vijayasarathy et al. 2003; Dudkina et al. 2008; Fontanesi et al. 2008).

In the marine environment, the concentration of dissolved oxygen varies cyclically reaching levels considered as hypoxic, which affects the energy metabolism of marine invertebrates (Abele et al. 2007). Since oxygen is the final electron acceptor, the respiratory process of mitochondria is not completed during hypoxia, this promotes a failure on the oxidative phosphorylation process and the inhibition of ATP synthesis (Hochachka and Somero 2002).

In aerial breathers, the prolonged effect of hypoxia produces a failure in the activity of mitochondrial enzymes as COX, which responds to hypoxia by altering its subunits composition and reducing its activity (Prabu et al. 2006; Fukuda et al. 2007).

The Pacific whiteleg shrimp *Litopenaeus vannamei* is one of the most studied species around the world due its commercial importance (Paez-Osuna et al. 2003); however, scarce information concerning crustacean mitochondrial enzymes and their response to hypoxia is available.

In our knowledge only three studies have dealt with the response of COX to hypoxia in crustaceans, these include the quiescent embryos of *Artemia franciscana* (Hofmann and Hand 1990), the mud crab *Scylla serrata* (Paital and Chainy 2012), and the white shrimp *Litopenaeus vannamei* (Jimenez-Gutierrez et al. 2013), but many questions are open to

L. R. Jimenez-Gutierrez · C. Chimeo · A. Muhlia-Almazan (✉)  
Laboratory of Bioenergetics and Molecular Genetics,  
Centro de Investigacion en Alimentacion y Desarrollo, A.C.,  
Carretera a Ejido La Victoria Km 0.6, PO Box. 1735, Hermosillo,  
Sonora 83000, Mexico  
e-mail: amuhlia@ciad.mx

S. Uribe-Carvajal  
Department of Molecular Genetics, Instituto de Fisiologia Celular,  
Universidad Nacional Autonoma de Mexico, P.O. Box, 70-242,  
Mexico, D.F. 04510, Mexico

A. Sanchez-Paz  
Laboratorio de Referencia, Analisis y Diagnostico en  
Sanidad Acuicola, Centro de Investigaciones Biologicas  
del Noroeste (CIBNOR), S.C. (Campus Hermosillo),  
Calle Hermosa No. 101. Col. Los Angeles, Hermosillo,  
Sonora 83106, Mexico

understand COX function and responses in the crustaceans mitochondria.

The aim of this study was to isolate mitochondrial proteins from abdominal muscle of shrimp. Immunodetection was used to identify the COX complex. The effect of hypoxia was evaluated in the L-lactate concentrations of plasma, the mitochondrial oxygen uptake of shrimp muscle, the mitochondrial content of protein and the response of COX activity was evaluated under normoxia and hypoxia.

## Materials and methods

### Hypoxia assay

Adult shrimp of the species *L. vannamei* ( $7.2 \pm 1.5$  g each) were obtained from aquaculture facilities and maintained at CIBNOR, Hermosillo, Sonora. A total of 84 organisms were randomly distributed in three 60 L plastic tanks filled with marine water. Shrimp were acclimatized for 8 days at controlled conditions as 28–30 °C, 35 ppt salinity, and a dissolved oxygen concentration of 6 mg/L (187.6 μM O<sub>2</sub>). Marine water was daily exchanged (50 %) and shrimp were fed daily with commercial pelletized food (35 % protein).

After acclimation shrimp were kept at normoxia (6 mg/L or 187.6 μM O<sub>2</sub>) as controls and shrimp were sampled at this condition from each tank. The air stones were removed from tanks and nitrogen gas was bubbled to induce hypoxia. Tanks were covered with a plastic sheet each, to prevent atmospheric oxygen contact. Oxygen concentration in water was carefully and continuously monitored during the assay in each tank using a digital submersible oximeter.

During the hypoxic phase 7 shrimp were sampled from each tank at: 2.0 mg/L (62.5 μM O<sub>2</sub>), 1.0 mg/L (31.26 μM O<sub>2</sub>) and after increasing oxygen at 2.0 mg/L. The oxygen content was gradually reduced until it reached 2.0 mg/L, and then shrimp were maintained at this condition and sampled after 6 h. Afterwards, oxygen was reduced to 1.0 mg/L and shrimp were sampled after 6 h at this condition and finally the nitrogen gas was replaced by air stones and the oxygen concentration was gradually raised to 2.0 mg/L (62.5 μM O<sub>2</sub>), and 6 h later shrimp were sampled. The hypoxia bioassay endured 18 h since shrimp were sampled at normoxia.

All sampled shrimp were individually weighed, and the hemolymph was extracted from the base of the third pereiopod with an insulin syringe with a 28 gauge needle preloaded with 500 μL of shrimp anticoagulant solution (450 mM NaCl, 10 mM KCl, 10 mM EDTA, 10 mM HEPES at pH 7.3; Vargas-Albores et al. 1993). Hemolymph samples were centrifuged at 5,724×g for 10 min at 4 °C, and plasma (supernatant) was separated from hemocytes and stored at –20 °C until use, then each abdominal muscle was excised, submerged in liquid nitrogen and immediately used for mitochondria isolation.

### L-lactate concentration in shrimp plasma

To elicit the effect of hypoxia in experimental shrimp, L-lactate concentration was measured in plasma samples using the L-lactate Standard kit (Randox, UK), following the manufacturer instructions. Measurements were performed in a microplate spectrophotometer FLUOstar Omega (BMG LABTECH, Germany), the reaction including: 200 μL of reactive solution and 10 μL of shrimp plasma. All samples were analyzed in triplicate following the increase in absorbance at 550 nm. L-lactate concentration was determined in shrimp samples at normoxia and hypoxia.

### Mitochondria isolation from shrimp muscle

Individual shrimp samples from the same normoxia/hypoxia condition were pooled to obtain 100 g of muscle tissue, and 8 volumes of cold isolation buffer A (300 mM sucrose, 150 mM KCl, 20 mM HEPES, 1 mM EGTA, pH 7.5) were added. Subsequent procedures were carried out at 4 °C. The tissue was homogenized in a blender at high speed for 1 min and the pH was adjusted to 7.5 immediately. The neutralized and minced muscle was placed in a double layer cheesecloth. Mitochondria were obtained from the collected solution by differential centrifugation as mentioned in Martinez-Cruz et al. (2012) with some modifications: the mitochondrial pellet was resuspended in 0.5 mL of buffer B (300 mM sucrose, 150 mM KCl, 20 mM HEPES and 0.025 mM EGTA, pH 7.5). The soluble protein concentration was measured at 280 nm using a Nanodrop spectrophotometer (Thermo scientific, USA).

### Mitochondrial oxygen uptake measurements

Freshly-isolated mitochondria from shrimp at normoxia/hypoxia were used to measure oxygen uptake by means of an oximeter coupled to a Clark-type electrode (Strathkelvin instruments model 782, Scotland). Shrimp mitochondria were incubated in a solution containing: 5 μL of 0.5 M MgCl<sub>2</sub>, 10 μL of 0.5 M KPO<sub>4</sub>, 20 μL of 0.5 M isocitrate as respiratory substrate and 10 μL of 0.5 M ADP at 25 °C, mitochondria were added in a final concentration of 300 μg of protein. At the end of the experiment 10 μL of 0.5 M KCN (potassium cyanide) were added to inhibit COX activity, and respiration buffer (220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4) to reach a final volume reaction of 200 μL. All samples were analyzed in triplicate.

### COX activity and inhibition assays

COX activity was measured in each mitochondrial isolate from shrimp at normoxia/hypoxia conditions using a CARY 50 BIO spectrophotometer at 550 nm and the cytochrome c

oxidase assay kit CYTOCOX1 (Sigma) following manufacturer conditions with some changes: volumes were adjusted to a final reaction volume of 1.5 mL and 300 µg of mitochondrial protein were added. Measurements were performed in triplicates at 25 °C. The inhibition assays were carried out by adding 15 µL of 200 µM KCN, then reactions were incubated 30 min at room temperature; afterwards, activity reduction was determined in the spectrophotometer as described above.

#### Statistical analysis

Obtained data were analyzed for normality and homoscedasticity using the NCSS 2007 software. Statistical significance in data was evaluated by one way-ANOVA at  $p < 0.05$ ; finally, significant differences among normoxia/hypoxia conditions were evaluated by a Tukey-Kramer multiple-comparison test at  $p < 0.05$ .

#### Mitochondrial protein separation by blue native electrophoresis (BN-PAGE)

To separate mitochondrial proteins, identify COX complex, and evaluate COX activity in polyacrylamide gels up to 150 µg of shrimp muscle protein from each normoxia/hypoxia condition were solubilized in a n-dodecyl-β-maltoside solution (DM; 4 mg/mg mitochondrial protein), and solubilization buffer (750 mM aminocaproic acid, 50 mM Bis-Tris, pH 7.0) to reach a final volume of 30 µL.

Samples were incubated on ice during 30 min with periodical agitation and centrifuged at 23,447×*g* for 55 min at 4 °C. The supernatant was recovered and coomassie blue was added to a final concentration of 0.1 % (w/v) and finally loaded on to a 4–12 % polyacrylamide gradient gel. BN-PAGE was performed according to Schägger and von Jagow (1995) method, with modifications. Briefly, two cathode buffers were used: cathode buffer 1 (50 mM tricine, 15 mM Bis-Tris, 0.02 % coomassie blue), cathode buffer 2 (50 mM tricine, 15 mM Bis-Tris, 0.002 % coomassie blue), and anode buffer (50 mM Bis-Tris pH 7.0).

Electrophoresis was run at 13 mAmp for each gel with cathode buffer 1, then after 20 min this buffer was replaced by cathode buffer 2, and kept until the end. Gels were stained with silver nitrate and the protein content of each band was calculated relative to the control by densitometry using the Image™ Lab 2.0. (Bio-Rad, USA).

#### Immunoblotting and COX I detection

Shrimp mitochondrial proteins previously separated by BN-gel were transferred into a PVDF membrane using a Transblot SD semi-dry transfer cell (Bio-Rad) and transfer buffer (390 mM glycine, 48 mM Tris and 2 % ethanol at pH 7.2). Proteins were transferred at 15 V for 25 min, then the membrane was blocked

for 2 min with 2 % Tween-20 in 50 mM Tris-HCl, 150 mM NaCl, 15 mM Na<sub>3</sub>N<sub>3</sub> pH 7.2. Afterwards, the blot was incubated in a shaker for 1 h at room temperature with purified rabbit anti-human COX I polyclonal antibody (DAKO, Denmark) diluted 1:500 (v/v) in TBS buffer (500 mM Tris, 150 mM NaCl, pH 7.2), then incubated at 4 °C overnight.

The blot was washed three times for 5 min each with wash buffer (TBS buffer, 15 mM sodium azide, and 0.05 % Tween-20) and then incubated with goat anti-rabbit IgG/HRP (DAKO, Denmark) peroxidase conjugated secondary antibody diluted 1:2000 (v/v) in TBS buffer for 1 h at room temperature. The blot was washed three times for 5 min each with wash buffer without sodium azide, and then washed with distilled water two times for 5 min each. To develop the peroxidase reaction 10 mg of diaminobenzidine were dissolved in TBS buffer with 12 µL of H<sub>2</sub>O<sub>2</sub> freshly prepared. The membrane was incubated by shaking 30 min until the signal was developed, and the reaction was stopped with distilled water.

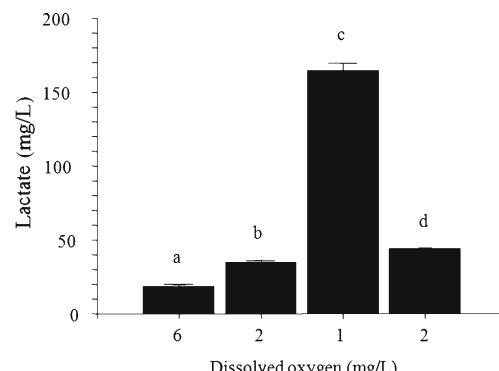
#### In-gel activity of COX

A BN-PAGE was performed as mentioned before, immediately the in-gel activity of COX was determined according to Wittig et al. (2007) with some modifications: the gel was incubated at room temperature in activity buffer (50 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.0) with 20 mg diaminobenzidine and 10 mg of cytochrome c, to a final volume of 50 mL. Incubation lasted 30 min or until the signal was developed. The reaction was stopped with distilled water.

## Results

#### L-lactate concentration in shrimp plasma

Figure 1 shows the profile of L-lactate concentration in plasma of shrimp exposed to normoxic and hypoxic conditions. Lactate concentration in shrimp plasma was significantly



**Fig. 1** Plasma lactate concentration in the white shrimp *L. vannamei* exposed to normoxia and hypoxia. Different letters show significant difference at  $p < 0.05$ . Bars represent means  $\pm$  SE

lower in normoxia than that detected in shrimp exposed to progressive hypoxia ( $p < 0.05$ ). The lactate concentration slightly increased at 2 mg/L (62.5  $\mu\text{M O}_2$ ), it increased abruptly at 1 mg/L (31.26  $\mu\text{M O}_2$ ), reaching levels 9-fold higher than those observed in organisms at normoxia. At the end of the assay, when dissolved oxygen concentration increased from 1 to 2 mg/L (31.26 to 62.5  $\mu\text{M O}_2$ ), the lactate concentration decreased but did not reach the same levels as those measured in shrimp under normoxic conditions.

#### Mitochondria isolation from shrimp muscle

The total recovery of mitochondrial protein showed sharp variations among normoxia and hypoxia groups (Table 1). The amount of extracted mitochondrial proteins (mg) from 100 g of shrimp muscle at the early hypoxia condition (2.0 mg/L or 62.5  $\mu\text{M O}_2$ ) was drastically reduced in a 51 % when compared to the protein recovered from shrimp under normoxia; then values gradually increased as hypoxia continued and, after reoxygenation to 2.0 mg/L, the protein recovery reached 10.39 mg, a higher value than that observed at normoxia.

#### Mitochondrial oxygen uptake measurements

Figure 2 shows that significant differences were observed on the mitochondrial oxygen uptake among normoxia/hypoxia groups ( $p < 0.05$ ). The maximum rate of oxygen consumption by mitochondria was detected in shrimp at normoxia (11.33 nmolO<sub>2</sub>/min/mg protein,  $p < 0.05$ ). When the concentration of dissolved oxygen decreased to 2 and 1 mg/L (62.5 and 31.26  $\mu\text{M O}_2$ ), the mitochondrial oxygen uptake was reduced in 92 and 82 %, when compared to mitochondria from shrimp at normoxia; whereas, these values increased in shrimp mitochondria at reoxygenated 2 mg/L to less than the rate observed during normoxia ( $p < 0.05$ ). Finally, the addition of KCN completely inhibited the oxygen uptake in all analyzed groups (Fig. 2).

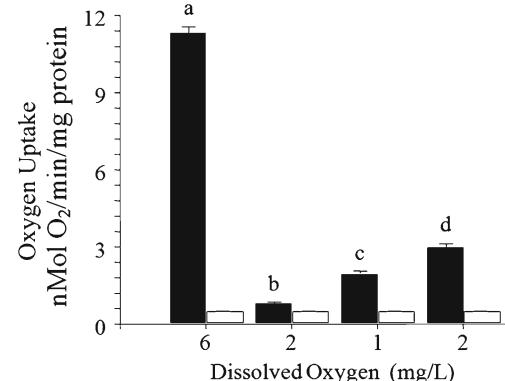
#### COX activity and inhibition assays

The COX activity of shrimp mitochondria at normoxia (16  $\mu\text{Mol/min/mg protein}$ ; Fig. 3) was significantly higher

**Table 1** Recovery of mitochondrial protein from 100 g of shrimp muscle at normoxia and hypoxia

Dissolved oxygen concentration (mg/L)	Protein concentration ( $\mu\text{g}/\mu\text{L}$ )	Total volume ( $\mu\text{L}$ )	Total protein (mg)
6	20.316	440	8.939
2	10.680	408	4.357
1	12.866	470	6.047
2 <sup>a</sup>	20.583	505	10.394

<sup>a</sup> Reoxygenated

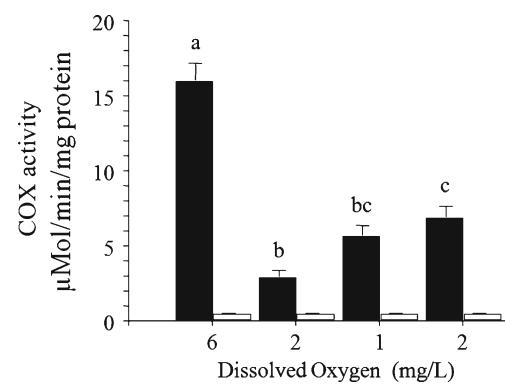


**Fig. 2** Mitochondrial oxygen uptake and inhibition by KCN at different dissolved oxygen concentrations. Dark columns show mitochondrial oxygen uptake, white columns show mitochondrial oxygen uptake inhibition by KCN. The oxygen uptake was measured in phosphorylating state (III) induced with ADP. Oxygen uptake in the presence of KCN was measured after a steady state was reached. Mitochondria (300  $\mu\text{g}$  protein) were added to a final volume of 200  $\mu\text{L}$ . Data from three independent experiments are expressed as means  $\pm$  SE. Different letters show statistical differences ( $p < 0.05$ )

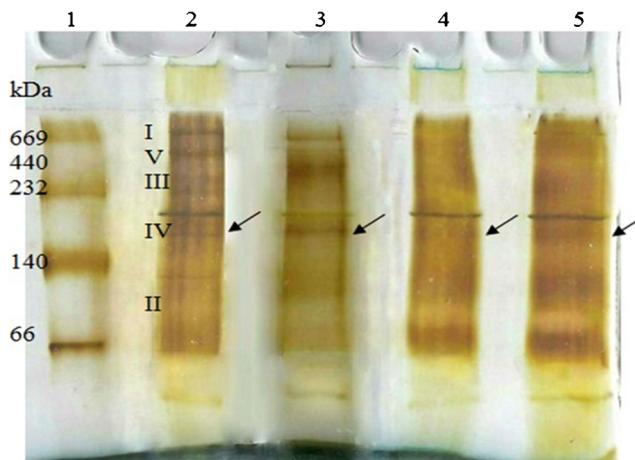
than that measured in organisms under hypoxic conditions ( $p > 0.05$ ). The COX activity decreased 81 % during hypoxia at 2 mg/L (62.5  $\mu\text{M O}_2$ ), and 64.5 % at 1 mg/L (31.26  $\mu\text{M O}_2$ ) when compared to mitochondria from shrimp at normoxia. However, when water was reoxygenated to 2 mg/L, the COX activity slightly increased being a 69 % of the activity detected at normoxia. The COX activity was completely inhibited (100 %) by KCN in all shrimp mitochondrial extracts (Fig. 3).

#### Mitochondrial protein separation by blue native electrophoresis (BN-PAGE)

Figure 4 shows the shrimp characterized mitochondrial proteins from different treatments including complex I: NADH dehydrogenase, complex V: ATP synthase, complex III: cytochrome c reductase, complex IV: cytochrome c oxidase, and



**Fig. 3** COX activity and inhibition by KCN at different dissolved oxygen concentrations. Dark columns show COX activity, white columns show COX inhibition by KCN. Different letters show statistical differences ( $p < 0.05$ ). Bars represent means  $\pm$  SE



**Fig. 4** BN-PAGE native gel corresponding to mitochondrial complexes isolated from shrimp at different dissolved oxygen concentrations. *Lane 1*: Molecular weight marker. *Lane 2*: Normoxia (6 mg/L). *Lane 3*: Hypoxia (2 mg/L). *Lane 4*: Hypoxia (1 mg/L), *Lane 5*: Hypoxia (2 mg/L reoxygenated). Roman numbers indicate mitochondrial complexes: I-NADH dehydrogenase, II- Succinate dehydrogenase, III- Cytochrome c reductase, IV- Cytochrome c oxidase and V- ATP synthase. Arrows indicate the COX monomer

complex II: succinate dehydrogenase. The COX monomer of *L. vannamei* showed a molecular weight of ~190 kDa.

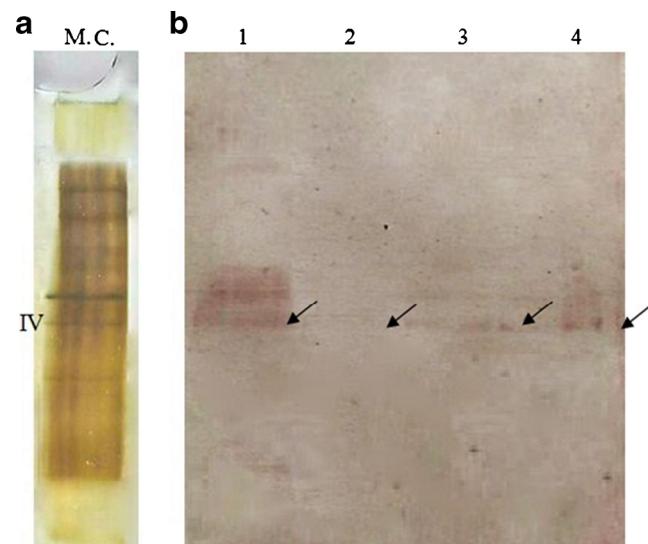
The protein band of COX monomers in mitochondrial extracts from each treatment varied in intensity, and the percentage of protein content from each band relative to the control (being normoxia 100 %) was: 89.58 % for hypoxia at 2 mg/L, 93.53 % for hypoxia at 1 mg/L and 96.58 % for hypoxia at 2 mg/L reoxygenated.

#### Immunoblotting and COX I detection

The shrimp mitochondrial subunit COX I was identified in a BN-PAGE by using a commercially available human polyclonal antibody, which specifically recognized shrimp COX I among mitochondrial proteins of shrimp at normoxia and hypoxia (Fig. 5). In this assay it was necessary to increase the amount of protein (250 µg) of the mitochondrial proteins of shrimp at each condition to detect COX I. Figure 5 shows the COX monomer (190 kDa) but the presence of various detected proteins suggests the possibility of COX interacting with other mitochondrial proteins.

#### In-gel activity of COX

The protein bands exhibiting COX activity in the mitochondrial extracts of shrimp at normoxia and hypoxia are shown in Fig. 6. The COX monomer of each treatment is observed as an intense band; however, additional activity bands were detected in each treatment due to the enzyme interactions with mitochondrial proteins, as other OXPHOS proteins. As observed in Fig. 6 the highest COX activity was detected in the

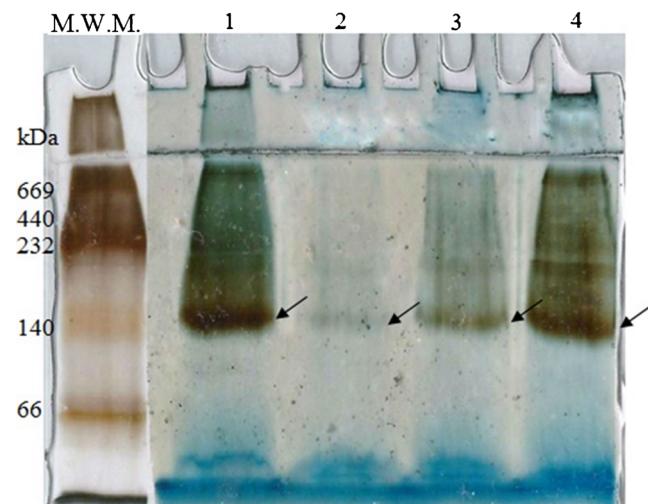


**Fig. 5** COX I subunit immunodetection in the mitochondrial fractions of shrimp at normoxia and hypoxia. *M.C.* mitochondrial complexes silver stained. Roman numbers represent the COX monomer. *Lane 1*: Normoxia (6 mg/L). *Lane 2*: Hypoxia (2 mg/L). *Lane 3*: Hypoxia (1 mg/L), *Lane 4*: Hypoxia (2 mg/L reoxygenated). Arrows indicate the COX monomer

mitochondrial extract of shrimp at normoxia, whereas at hypoxia 2 mg/L (62.5 µM) showed the lowest activity.

#### Discussion

A central idea supports the need for better understanding marine crustaceans and their mitochondrial systems: Shrimp are able to survive at very low oxygen concentrations during



**Fig. 6** COX activity in the muscle mitochondria of the shrimp *L. vannamei* during normoxia and hypoxia. *MWM* molecular weight marker, silver stained. *Lane 1*: Normoxia (6 mg/L). *Lane 2*: Hypoxia (2 mg/L). *Lane 3*: Hypoxia (1 mg/L), *Lane 4*: Hypoxia (2 mg/L reoxygenated) coomassie stained. Arrows indicate the COX monomer specific activity

prolonged time, and their mitochondrial machinery is quite different to other animals, and seems to be highly effective on facing this condition (Martinez-Cruz et al. 2012; Jimenez-Gutierrez et al. 2013). Moreover, the lack of studies on these species has caused a gap in the knowledge about the diverse mechanisms of adaptation that allow organisms, from unicellular to the more complex models as vertebrates, to surpass environmental conditions as hypoxia.

Shrimp physiologically responded to hypoxia by increasing the concentration of L-lactate in plasma, indicating a shift to the anaerobic pathway due to the lack of oxygen, a phenomenon that has been reported in several other animal species, including crustaceans (Dabos et al. 2011; Martinez-Cruz et al. 2012).

In mammals, besides the metabolic rate reduction and the cellular response to hypoxia as lactate production, also observed in marine invertebrates (Racotta et al. 2002; Re and Diaz 2011), the mitochondrial function is known to fail, and the oxygen uptake and OXPHOS activity are reduced in the rat skeletal muscle (Chen et al. 2012), these responses are known to rapidly occur in some specialized tissues as detected in rat arterial cells as reported by Buckler and Turner (2013).

The values of mitochondrial oxygen uptake from shrimp in this study are consistent with previous reports in other invertebrates as the polychaetes *A. marina* (7.8 nmolO<sub>2</sub>/min/mg protein; Hildebrandt and Grieshaber 2008) and *Nereis pelagica* (6 nmolO<sub>2</sub>/min/mg protein), and the bivalve *Arctica islandica* (7 nmolO<sub>2</sub>/min/mg protein; Keller et al. 2004; Abele et al. 2007), but are much lower than the mitochondrial oxygen uptake of bovine heart (179 nmolO<sub>2</sub>/min/mg protein; Abele et al. 2007) and the murine monocyte macrophages (36 nmolO<sub>2</sub>/min/mg protein; Vijayasarathy et al. 2003), since invertebrates are oxyconformer organisms, and their mitochondrial oxygen uptake is much lower than that measured in vertebrates.

In this study a significant reduction in the mitochondrial oxygen uptake, the mitochondrial protein content of shrimp muscle, and the COX activity of shrimp mitochondria were detected after 6 h of hypoxia at 2.0 mg/L (62.5 μM). As hypoxia continued (12 h) and the oxygen concentration of water was reduced to 1 mg/L (31.26 μM) such variables increased, and after the oxygen concentration of water increased to 2 mg/L (18 h) increasing values were detected in the three variables. These gradually increased values detected after 12 h of exposure to hypoxia (Table 1; Figs. 2 and 3) suggest a response of shrimp mitochondria to hypoxia as an attempt to compensate and restore their functions.

Previous studies have demonstrated that COX is a key regulator of the oxidative phosphorylation system, being part of a complex mechanism which is known to be controlled at different levels, including transcriptional factors as HIF-1, NRF2 and Sp1 controlling both, the genes expression of mitochondrial and nuclear subunits which are part of the

whole complex, and the mitochondrial and cellular activities (Grossman and Lomax 1997; Ongwijitwat and Wong-Riley 2005; Hüttemann et al. 2012; Buckler and Turner 2013).

In a recent study, Jimenez-Gutierrez et al. (2013) suggested that nuclear COX subunits of shrimp are transcriptionally regulated by NRF2 and Sp1 transcription factors, and that hypoxia significantly affected the mRNA levels of three nuclear encoded subunits of the COX complex from *L. vannamei*. A coordinated response to hypoxia among the mitochondrial and nuclear encoded COX subunits was detected, the mRNA levels decreased at the beginning of the hypoxia exposure at 2 mg/L (62.5 μM O<sub>2</sub>), and increased at 1.5 mg/L (46.90 μM O<sub>2</sub>; Jimenez-Gutierrez et al. 2013), which is in agreement with the results obtained in the present study.

The integrated results of both studies suggest a hypoxia-induced reduction in the mRNA and protein levels of COX subunits probably controlled at the transcriptional level, via factors (NRF2 and Sp1) during the first 6 h of exposure; as hypoxia continues at 1–1.5 mg/L (12 h of exposure) and at 2.0 mg/L (18 h of exposure), the mRNA and protein synthesis are partially restored and potentially associated to the increase in COX activity, the recovery of mitochondrial oxygen uptake, and the decrease in lactate levels observed in this study. This last suggestion is in agreement with the work of Liu et al. (2002), in which it is suggested that animals exposed to hypoxia may show gradual acclimatization resulting in the recovery of the mitochondrial function under long term hypoxia.

In 2006, Papandreou et al. reported that the decreased mitochondrial oxygen uptake under hypoxic conditions is a response mediated by the HIF-1 transcription factor in vertebrates; however, the role of HIF-1 in the regulation of the transcription of the shrimp COX genes should be analyzed and confirmed.

The complete inhibition of shrimp mitochondrial oxygen uptake and COX activity by KCN was repeatedly observed at different concentrations and various incubation times (data not shown); the results obtained may suggest that under the current experimental conditions, there is no evidence of the existence of an alternative oxidase (AOX) in shrimp mitochondria, which supports the hypotheses of McDonald et al. (2009) and Jimenez-Gutierrez et al. (2013).

The AOX presence has been previously reported in other species as the yeasts *Yarrowia lipolytica* (Guerrero-Castillo et al. 2009) and *Candida krusei* (Costa-de-Oliveira et al. 2012), the polychaete *A. marina* (Hildebrandt and Grieshaber 2008), the urochordate *Ciona intestinalis* (Dassa et al. 2009) and some plant species (Pierron et al. 2012). In this way, the absence of an AOX in crustaceans is consistent with the lack of reports for any arthropod AOX (McDonald et al. 2009).

Scarce information has been published about COX activity in invertebrates. In agreement with the results in this study,

Yang et al. (2010) reported the decreasing COX activity of the cotton bollworm *Helicoverpa armigera* during diapause, a physiological state of low metabolism. Studies in the brine shrimp *Artemia franciscana* have shown that biosynthesis in embryos during pre-emergence development is directly controlled via pH changes, and the COX synthesis is inhibited under anaerobic dormancy (Hofmann and Hand 1990).

Taken together, these results indicate a shrimp adaptive response to hypoxia where the mitochondrial machinery plays a key role by reducing the oxygen uptake and the protein synthesis. Shrimp mitochondria have a highly effective COX enzyme that lacks the ability of differentially-expressing subunits isoforms under hypoxia as observed in mammals (Jimenez-Gutierrez et al. 2013); furthermore, the lack of an AOX, that provides other invertebrate species the ability to anaerobically produce energy (Philip and Abele 2007), may implicate that COX is the unique oxidase in the respiratory chain of shrimp mitochondria. Further studies about the enzyme kinetics and oxygen affinity at normoxia/hypoxia should confirm the efficiency of the enzyme and its unique central role in the shrimp mitochondria.

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