



# Alternative mitochondrial respiratory chains from two crustaceans: *Artemia franciscana* nauplii and the white shrimp, *Litopenaeus vannamei*

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## Abstract

Mitochondrial ATP is synthesized by coupling between the electron transport chain and complex V. In contrast, physiological uncoupling of these processes allows mitochondria to consume oxygen at high rates without ATP synthesis. Such uncoupling mechanisms prevent reactive oxygen species overproduction. One of these mechanisms are the alternative redox enzymes from the mitochondrial respiratory chain, which may help cells to maintain homeostasis under stress independently of ATP synthesis. To date, no reports have been published on alternative redox enzymes in crustaceans mitochondria. Specific inhibitors were used to identify alternative redox enzymes in mitochondria isolated from *Artemia franciscana* nauplii, and the white shrimp, *Litopenaeus vannamei*. We report the presence of two alternative redox enzymes in the respiratory chain of *A. franciscana* nauplii, whose isolated mitochondria used glycerol-3-phosphate as a substrate, suggesting the existence of a glycerol-3-phosphate dehydrogenase. In addition, cyanide and octyl-gallate were necessary to fully inhibit this species' mitochondrial oxygen consumption, suggesting an alternative oxidase is present. The in-gel activity analysis confirmed that additional mitochondrial redox proteins exist in *A. franciscana*. A mitochondrial glycerol-3-phosphate dehydrogenase oxidase was identified by protein sequencing as part of a branched respiratory chain, and an alternative oxidase was also identified in this species by western blot. These results indicate different adaptive mechanisms from artemia to face environmental challenges related to the changing levels of oxygen concentration in seawater through their life cycles. No alternative redox enzymes were found in shrimp mitochondria, further efforts will determine the existence of an uncoupling mechanism such as uncoupling proteins.

**Keywords** Alternative enzymes · Artemia · Mitochondria · Shrimp · Branched respiratory chain

## Introduction

The mitochondrial electron transport chain (ETC) comprises four respiratory complexes embedded into the mitochondrial internal membrane (MIM), which carry electrons to reduce oxygen. Multimeric complexes I (NADH dehydrogenase), III (cytochrome *c* reductase), and IV (cytochrome *c* oxidase) function as proton pumps from the mitochondrial matrix to the intermembrane space to produce a proton gradient that complex V, ATP synthetase, uses to produce ATP (Nicholls and Ferguson 2003).

Under non-phosphorylating conditions, when ADP is low, the ETC is slowed by a high proton transmembrane gradient. Thus semiquinone concentrations are increased, free radicals accumulate and unspecifically react with oxygen to form ROS (Baradaran et al. 2013).

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To avoid overproducing ROS in the absence of ATP synthesis, mitochondria reduce the proton gradient, thus accelerating oxygen consumption. The presence of non-pumping alternative redox enzymes in the ETC is one of the uncoupling mechanisms described in mitochondria from yeast species such as *Yarrowia lipolytica* and *Debaryomyces hansenii* (Guerrero-Castillo et al. 2011; Cabrera-Orefice et al. 2014).

Mitochondrial alternative enzymes, including NADH-dehydrogenase type 2 (NDH2), glycerol-phosphate-dehydrogenase (mitGPDH), and alternative oxidase (AOX), are in the MIM, do not pump protons, and may be part of a branched respiratory mitochondrial chain (Umbach and Siedow 2000; Kadenbach 2003; Rasmusson et al. 2004).

To date, alternative oxidases are confirmed to exist in some invertebrates such as the nematode, *Pratylenchus vulnus*, and the mollusks *Crassostrea gigas*, *Mercenaria mercenaria* and *Aplysia californica* (McDonald et al. 2009). However, crustaceans mitochondrial proteins and their functions have been scarcely studied, and to date, no reports exist in alternative enzymes in the mitochondria of these species, which may help, in some extent, to explain how these species adapt to low environmental oxygen levels.

Among crustaceans, branchiopods and decapods can survive in extreme marine environments including high salinity levels, low oxygen concentrations, and continuously changing water temperatures (Abatzopoulos et al. 2002; Puente 2009). *Artemia franciscana* may survive under anoxia for years as cysts by strongly downregulating their metabolism (Abatzopoulos et al. 2002), and decreasing in ATP turnover (Clegg 1997; Patil 2012). The white shrimp, *Litopenaeus vannamei*, is exposed to extreme diurnal variations in the dissolved oxygen concentrations in seawater, and can survive at very low oxygen levels as 0.2 mg/L (Perez-Rostro et al. 2004).

This work determined whether alternative dehydrogenases and oxidases exist in both *A. franciscana* nauplii and *L. vannamei* mitochondria, by evaluating the in-gel activity of the mitochondrial enzymes that participate in the ETC, and evaluated the oxygen consumption of mitochondrial fractions of both species in the presence of different respiratory substrates and specific inhibitors. The results may explain the ability of these species to adapt to changes in the environmental oxygen concentrations.

## Materials and methods

### Artemia cysts hatching and shrimp sampling

Dehydrated and encysted gastrulae of *A. franciscana* were obtained from Brine Shrimp Direct, Inc. (Utah, USA). Three grams of the cysts were placed into a 1.5-L conical plastic container of seawater at 25 °C with constant light and aeration

for 24 h. After hatching, *A. franciscana* nauplii were separated from cyst shells by a siphon and filtered.

Fifteen juvenile *L. vannamei* shrimp were obtained from aquaculture facilities and tested for pathogens. After acclimatization to laboratory conditions (28 °C, 34 ppt of salinity, and 6 mg O<sub>2</sub>/L), organisms were placed in ice and their pleopods were dissected.

### Intact mitochondria isolation

Mitochondria were isolated by differential centrifugation as described by Jimenez-Gutierrez et al. (2014) with some modifications. *A. franciscana* nauplii (2 g wet weight) or dissected shrimp pleopods (5 g wet weight) were placed in ice-cold extraction buffer 1 (0.125 M sucrose, 0.375 M sorbitol, 1 mM EGTA, 150 mM KCl, 0.5% [w/v] fatty acid-free BSA, and 20 mM K-HEPES, pH 7.5). Samples were homogenized using a Dounce homogenizer. The homogenate was centrifuged for 10 s at 3024  $\times$  g at 4 °C, and the supernatant was removed and centrifuged at 17,418  $\times$  g for 15 min at 4 °C. The supernatant was discarded and pellets were suspended in two volumes of ice-cold extraction buffer 2 (0.125 M sucrose, 0.375 M sorbitol, 0.025 mM EGTA, 150 mM KCl, 0.5% [w/v] fatty acid-free BSA, and 20 mM K-HEPES, pH 7.5). A third centrifugation step was performed at 1089  $\times$  g for 5 min at 4 °C; the supernatant was removed, and a last centrifugation step was performed at 17,418  $\times$  g for 15 min at 4 °C.

Mitochondrial pellets were suspended in 300  $\mu$ L of ice-cold extraction buffer 2. Soluble mitochondrial protein content was measured using the Bradford method (Bradford 1976). Isolated mitochondrial fractions were used immediately to measure oxygen consumption, separate enzymes by electrophoresis, and evaluate their in-gel activities.

### Measurement of the mitochondrial oxygen consumption rate

The mitochondrial oxygen consumption rate from artemia and shrimp was evaluated to determine whether alternative dehydrogenases and oxidases were present. Mitochondrial oxygen consumption was determined by an oximeter coupled to a Clark-type oxygen electrode (Strathkelvin instruments model 782, Scotland). Respiration buffer included 0.125 M sucrose, 0.375 M sorbitol, 150 mM KCl and 20 mM K-HEPES (pH 7.5), 1 mM MgCl<sub>2</sub> and 10 mM Tris-phosphate (Pi).

Mitochondrial coupling was evaluated using 10 mM succinate as the respiratory substrate, 0.1 mM ADP to promote phosphorylation (state III), and 0.6  $\mu$ M CCCP (carbonyl cyanide m-chlorophenyl hydrazone) as an uncoupler.

To determine the presence of NDH2 or mitGPDH, 1 mM NADH or 10 mM glycerol-3-phosphate (G3P) were used as respiratory substrates, oxygen consumption was accelerated with 0.6  $\mu$ M CCCP, and inhibitors, including 50  $\mu$ M rotenone

and 5  $\mu\text{M}$  antimycin A, were added to identify alternative enzymes (Cabrera-Orefice et al. 2014). The AOX presence in mitochondria was determined using 10 mM succinate as the respiratory substrate, 0.6  $\mu\text{M}$  CCCP, 50  $\mu\text{M}$  octyl-gallate (OG), and 100  $\mu\text{M}$  KCN as inhibitors (Guerrero-Castillo et al. 2009). All assays included 1 mg protein/mL in a final volume of 200  $\mu\text{L}$ , and were performed in triplicate.

### Mitochondrial enzymes separation by hrCN-PAGE, and in-gel activity determination

High-resolution clear native polyacrylamide gels (hrCN-PAGE) were performed as described by Wittig et al. (2007) to identify NADH-dehydrogenase, succinate-dehydrogenase, glycerol-phosphate dehydrogenase, cytochrome *c* oxidase (COX), and ATP synthetase complexes in the isolated artemia and shrimp mitochondria. The isolated artemia, shrimp and bovine (as controls) mitochondria were centrifuged twice at  $17,418 \times g$  for 10 min at 4 °C. The first pellet was suspended in 100  $\mu\text{L}$  of wash buffer (250 mM sorbitol and 25 mM imidazole, pH 7.0), and a second pellet was suspended in 50  $\mu\text{L}$  of no-salt buffer (0.25 M sucrose, 10 mM trizma base, pH 7.0), solubilized with 1 g of dodecyl-D-maltoside (LM) or digitonin per gram of protein, and vortexed for 1 h at 4 °C. After solubilization mitochondria were centrifuged at  $24,675.5 \times g$  for 1 h at 4 °C, and the soluble protein concentration of the supernatant was determined by the Bradford method (Bradford 1976).

A total of 250  $\mu\text{g}$  of mitochondrial protein were resolved in a 4–12% polyacrylamide gradient gel. The cathode buffer 1 contained 50 mM tricine, 7.5 mM imidazole, 0.01% LM, 0.05% sodium deoxycholate, and anode buffer 2 contained 25 mM imidazole (pH 7.0) (Wittig et al. 2007).

Once the mitochondrial proteins were separated in the hrCN-PAGE, the in-gel NADH dehydrogenase activity was detected by incubating the gel in a solution containing 0.5 mg of nitro-blue tetrazolium bromide (NBT) per mL, 10 mM Tris (pH 7.0), and 1 mM NADH. The in-gel glycerol phosphate dehydrogenase (mitGPDH) activity was determined using 0.5 mg NBT, 10 mM Tris (pH 7.0), and 5 mM glycerol-3-phosphate (pH 7.0), or 5 mM succinate (pH 7.4) to determine succinate dehydrogenase activity (Zerbetto et al. 1997).

The in-gel cytochrome C oxidase activity was detected by incubating the hrCN-PAGE in a mixture of 50 mM phosphate buffer (pH 7.4), 10 mg of reduced cytochrome C dissolved in 1 mL of 50 mM phosphate buffer, and 1 mg/mL of diamino-benzidine (Wittig and Schägger 2007). The in-gel ATPase activity was measured by incubating the hrCN-PAGE in incubating-buffer (270 mM glycine and 35 mM Tris, pH 8.4) for 60 min, and then in activity buffer (270 mM glycine, 35 mM Tris, pH 8.4, 6 mM ATP, 14 mM  $\text{MgSO}_4$  and 0.2%  $\text{Pb}(\text{NO}_3)_2$ ) for 24 h (Wittig et al. 2007). All gels were

stirred constantly at room temperature until the color reaction developed.

### In-gel digestion and tandem mass spectrometry analysis (LC-MS/MS)

Artemia mitochondrial proteins were separated by hrCN-PAGE as previously described (Wittig et al. 2007), and stained with 0.2% Coomassie brilliant blue. Specific protein bands with GPDH activity were excised from the preparative gels (Fig. 8). Before the in-gel digestion, protein bands were destained and reduced with 10 mM DTT in 25 mM ammonium bicarbonate followed by protein alkylation with 55 mM iodoacetamide. Proteins were digested overnight at 37 °C with sequencing grade trypsin (Promega, Madison, WI, USA). Nanoscale LC of tryptic peptide separation was performed using a nanoACQUITY UPLC System (Waters, Milford, MA, USA), and LC-MS/MS analysis was performed by a SYNAPT HDMS Q-TOF (Waters) as previously reported (Joaquin-Ramos et al. 2014) with the brief modification that accurate mass data were collected in an alternating Data Dependent Acquisition mode (DDA). In low energy mode, data were collected at a constant collision energy of 3 eV. In elevated-energy mode, the collision energy was increased from 15 to 45 eV for 3 s of integration.

To identify proteins by a homology database search, the MS/MS spectra datasets were used to generate PKL extension files using the Protein Lynx Global Server v2.4 (PLGS, Waters). Proteins were then identified using PKL files and the PEAKS Studio v8.0 (Bioinformatics Solutions Inc. Ontario CAN). Searches were conducted against an in-house database containing the Branchiopoda subset of the nrNCBI protein database (600,199 sequences, January 2017), plus 1085 protein sequences corresponding to mitochondrial GPDHs from the NCBI and the contaminants collection from the Max Plank Institute of Biochemistry (247 sequences). Trypsin was used as the specific protease, and one missed cleavage was allowed. Carbamidomethyl cysteine was set as a fixed modification, and methionine oxidation was specified as a variable modification. The precursor and fragment ion mass tolerances were set at 20 ppm and 0.1 Da, respectively. Significant protein scores (>20) indicated successful identifications with the identity or extensive homology having a false discovery rate < 0.1%.

### Mitochondrial proteins separation by SDS-PAGE and AOX immunodetection

Mitochondrial isolates from artemia nauplii (*A. franciscana*), Pacific oyster (*Crassostrea gigas*), mango (*Mangifera indica*) and huitlacoche (*Ustilago maydis*) were solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate supplemented

with proteases inhibitors; 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM PMSF and protease inhibitor cocktail (Santa Cruz Biotechnology)).

Samples were lysed in a multi-Vortex V-32 (Biosan, Riga, Latvia) for 30 min at 4 °C, and centrifuged at 15,160  $\times$  g for 10 min. Supernatant was recovered and the soluble protein content was determined. Solubilized proteins (100  $\mu$ g) were diluted in a 4X buffer (500 mM Tris [pH 6.8], 10% glycerol, 10% SDS, 0.05%  $\beta$ -mercapto-ethanol, and 0.01% bromophenol blue). Samples were boiled for 5 min and then loaded and separated by SDS-PAGE on 10% (*w/v*) polyacrylamide gels. Proteins were transferred to PVDF membranes using 25 mM potassium phosphate, 25 mM sodium phosphate, 12 mM Tris, 192 mM glycine, and 20% methanol, pH 7.0 (Towbin et al. 1979).

The transferred proteins were blocked with 5% BSA in TTBS buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6) for 1 h at 4 °C, and then incubated with a *Sauromatum guttatum* (voodoo lily) monoclonal anti-AOX antibody (diluted 1:100; Elthon et al. 1989) overnight in agitation at 4 °C. The membrane was washed with TBS-T and incubated for 1 h at 37 °C with horseradish peroxidase-conjugated secondary antibody. Once the membrane was washed, the bands were developed by chemiluminescence using an ECL kit (Amersham Biosciences, GE, Healthcare).

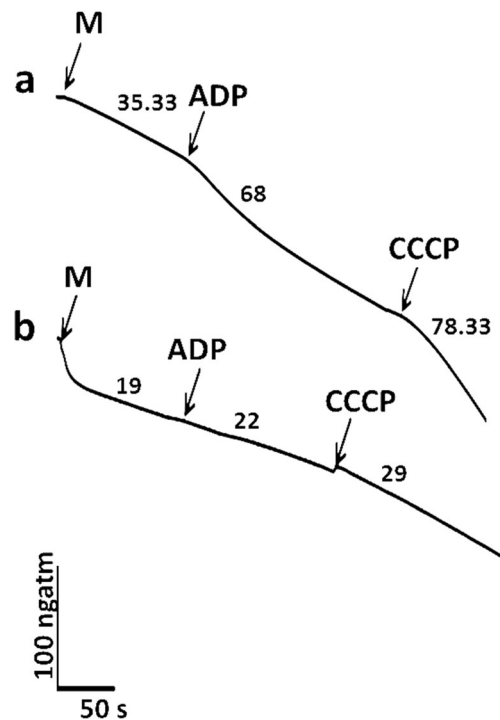
## Results

### Mitochondrial oxygen consumption and detection of alternative enzymes NDH2, mitGPDH, and AOX

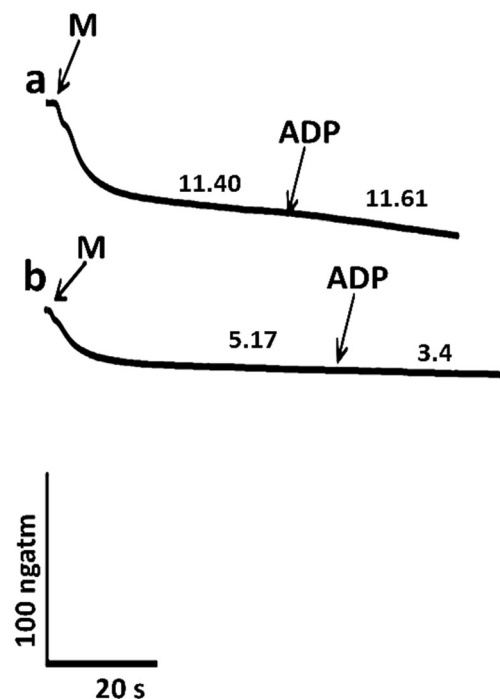
The oxygen consumption rate from isolated mitochondria was measured using specific substrates and inhibitors of classic or alternative enzymes that may be part of a putative branched respiratory chain in *A. franciscana* and *L. vannamei* mitochondria.

Figure 1 shows the oxygen consumption rate of both, artemia and shrimp mitochondria at states IV, III, and uncoupled (Fig. 1 traces a, b). Succinate was used as a respiratory substrate, and adding ADP or CCCP (as an uncoupler) increased oxygen consumption, which confirmed that isolated mitochondria from both crustaceans were coupled. The mitochondrial respiratory control (RC) was calculated as the ratio of states III/IV. RC values of 1.92 and 1.15 were obtained from artemia and shrimp, respectively.

In the presence of NADH as a substrate (Fig. 2 traces a, b), the mitochondrial oxygen consumption in both crustaceans was lower than that observed in Fig. 1, and resulted from the remnant respiratory substrates in the mitochondrial matrix. No significant differences were detected after ADP addition. These results suggest the absence of an external NDH2 that



**Fig. 1** Oxygen consumption rate of intact mitochondria from *Artemia franciscana* (trace a) and *Litopenaeus vannamei* (trace b). Succinate was used as a respiratory substrate. Numbers indicate the rate of oxygen consumption in ngatm O/mg protein/ min. Mitochondrial protein (M), at 1 mg/mL was used in each experiment. Mean values from triplicates are shown



**Fig. 2** Alternative NADH dehydrogenase determination in isolated mitochondria from *Artemia franciscana* (trace a) and *Litopenaeus vannamei* (trace b). Numbers indicate the rate of oxygen consumption rate in ngatm O/mg protein/min. Mitochondrial protein (M) was used in each experiment. Mean values from triplicates are shown

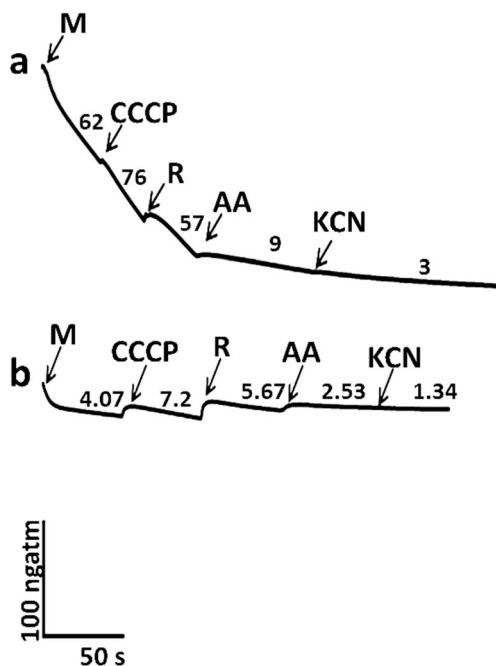


would oxidize external NADH and accelerate the rate of oxygen consumption.

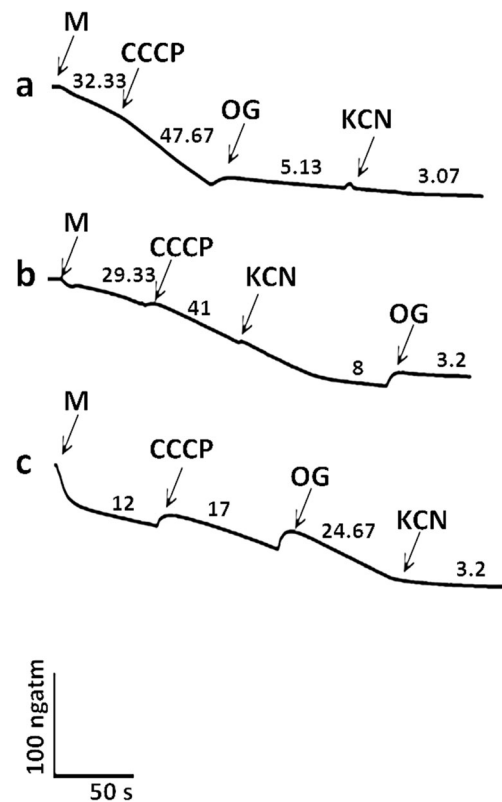
A mitGPDH was detected using glycerol-3-phosphate (G3P) as a respiratory substrate. *Artemia* mitochondria actively consumed oxygen both, before and after CCCP was added. Rotenone was added to inhibit complex I, thus ensuring that oxygen consumption depended on a mitGPDH. After antimycin A addition, mitochondrial oxygen uptake significantly decreased (Fig. 3 trace a). These results suggest that mitGPDH is part of an alternative respiratory chain that donates electrons to complex III.

In contrast, the shrimp mitochondrial oxygen consumption rate was low when G3P was the substrate (Fig. 3 trace b). Adding CCCP promoted a slight increase inhibited by rotenone and antimycin A. These results suggest that while *artemia* mitochondria exhibited high mitGPDH activity, this enzyme was not present in shrimp mitochondria.

The oxygen consumption by *artemia* mitochondria was inhibited by octyl-gallate (OG), and then by KCN (Fig. 4 traces a, b). The results suggest an AOX presence since adding OG significantly decreased oxygen uptake (Fig. 4 trace a). At this point, electrons may be redirected to COX to maintain the ETC; then, after KCN addition, oxygen consumption is stopped. When KCN was added first, it partially inhibited oxygen consumption, but OG addition fully inhibited it (Fig. 4 trace b).



**Fig. 3** Mitochondrial glycerol phosphate-dehydrogenase (mitGPDH) determination in *Artemia franciscana* (trace a) and *Litopenaeus vannamei* (trace b). Numbers indicate the rate of oxygen consumption in ngatm O/mg protein/min. Mitochondrial protein (M) at 1 mg/mL, rotenone (R), antimycin (AA), and cyanide (KCN) were used in each experiment. Mean values from triplicates are shown

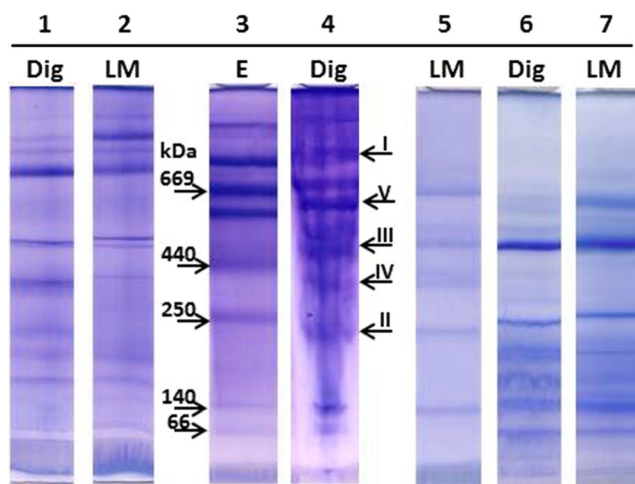


**Fig. 4** Alternative oxidase (AOX) determination in the mitochondria from *Artemia franciscana* (trace a, b) and *Litopenaeus vannamei* (trace c) mitochondria. Succinate was used as a respiratory substrate. Numbers indicate the rate of oxygen consumption in ngatm O/mg protein/min. Mitochondrial protein (M) at 1 mg/mL, octyl-gallate (OG), and cyanide (KCN) were used in each experiment. Mean values from triplicates are shown

Shrimp mitochondria were uncoupled after CCCP addition (Fig. 4 trace c); however, no inhibition resulted from adding OG, and KCN completely inhibited oxygen consumption, consistent with previous results for this species mitochondria (Jimenez-Gutierrez et al. 2014), suggesting an absence of AOX.

### hrCN-PAGE and mitochondrial enzymes in-gel activities

To confirm alternative mitochondrial redox enzymes presence in each species, their in-gel activities were determined. Figure 5 shows the mitochondrial proteins in a hrCN-PAGE from shrimp, bovine, and *artemia*. Figure 6a and b show the in-gel NADH dehydrogenase (NDH) activity in *artemia* and shrimp mitochondria, respectively. At least, two activity bands were detected in both species: the  $\approx 1000$  kDa band indicates the NADH-dehydrogenase complex and one or two additional proteins with molecular masses approximately 60–80 kDa. These additional bands with dehydrogenase activity were confirmed by protein sequencing methods as dehydrogenases from the Krebs



**Fig. 5** Isolated mitochondrial proteins from *Litopenaeus vannamei* (lanes 1–2), bovine (lanes 4–5), *Artemia franciscana* (lanes 6–7). I–V mitochondrial complexes. E: Amersham High Molecular Weight (lane 3). DG: protein solubilized with digitonin. LM: protein solubilized with dodecyl-D-maltoside

cycle such as malate dehydrogenase and succinate dehydrogenase, indicating the absence of any type 2 NADH-dehydrogenase in both species mitochondria.

Figure 6c and d show the in-gel activity of mitGPDH in both, artemia and shrimp mitochondria, respectively. A single band was detected in bovine fractions with a molecular mass around 161.4 kDa, as it has been previously reported (Zimin et al. 2009). A single band detected in the artemia mitochondria suggests that mitGPDH is present in this species' respiratory chain. No bands with GPDH activity were detected in the shrimp mitochondria.

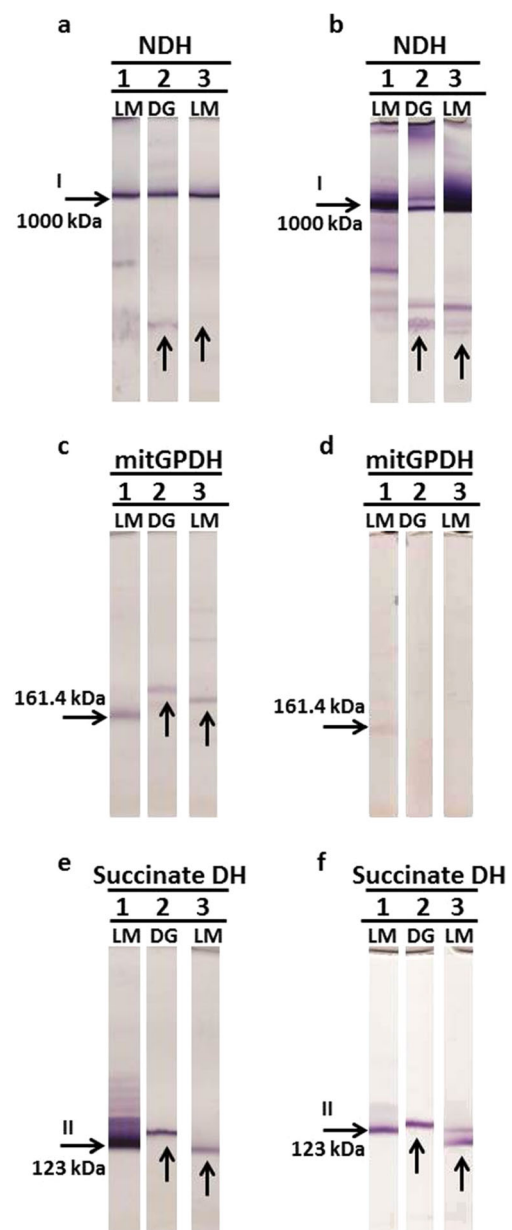
Succinate dehydrogenase activity is shown in fig. 6e and f for artemia and shrimp, respectively. A single band was detected in both gels with a molecular mass of approximately 123 kDa for the bovine enzyme, as well as *A. franciscana* and *L. vannamei*.

Figure 7a and b show the in-gel activity of complex IV, or COX, and a single 410 kDa band confirmed bovine COX activity (Wittig and Schagger 2007; Wittig et al. 2010). Figure 7a shows no other proteins with oxidase activity in the mitochondria of *A. franciscana*, since AOX is not detected by this method. No additional bands with oxidase activity were detected in the shrimp mitochondria (Fig. 7b).

The in-gel ATP synthase activity revealed a single band of an approximately 1194 kDa active dimer from the bovine mitochondria. A single band was observed in both, *A. franciscana* and *L. vannamei* mitochondria (Fig. 7c and d).

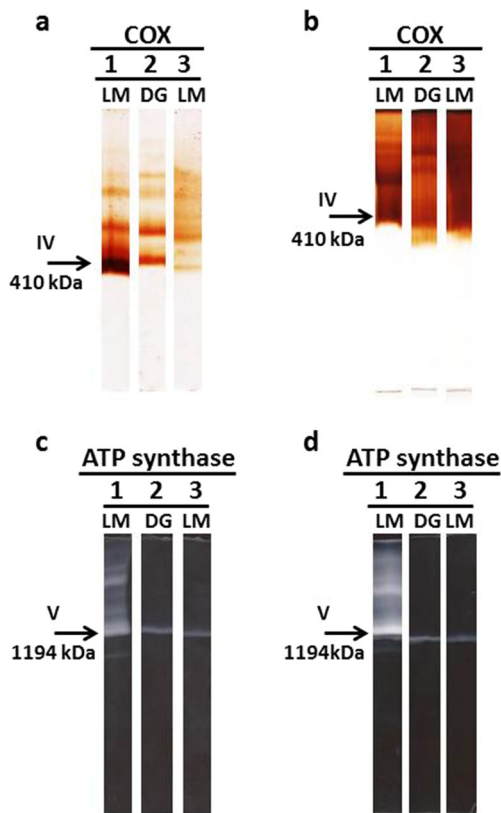
## Database search and protein identification

To confirm artemia mitGPDH existence and identity, the results from comparing the PKL extension files from LC-MS/MS analysis of X band (Fig. 8), against the in-house nrNCBI protein database are shown in Table 1. The X band analysis



**Fig. 6** In-gel activities of mitochondrial complexes I, II, and mitGPDH from bovine, *Artemia franciscana* and *Litopenaeus vannamei*. In-gel NADH-dehydrogenase activity. From: **a** bovine (lane 1), and artemia (lanes 2–3); **b** bovine (lane 1), and shrimp (lanes 2–3). In-gel mitGPDH activity from: **c** bovine (lane 1), and artemia (lanes 2–3); **d** bovine (lane 1), and shrimp (lanes 2–3). In-gel succinate-DH activity from: **e** bovine (line 1), and artemia (lanes 2–3); **f** bovine (line 1), and shrimp (lanes 2–3). DG: protein solubilized with digitonin. LM: protein solubilized with dodecyl-D-maltoside

showed 3 peptides that shared high identity with the alternative protein glycerol-phosphate-dehydrogenase from organisms including the octopus *Octopus bimaculoides*, the marsupial *Monodelphis domestica*, and the acari *Metaseiulus occidentalis*, obtaining scores above 20 ppm. These results confirmed mitGPDH as part of a branched respiratory chain in *A. franciscana*.



**Fig. 7** In-gel activities of mitochondrial complexes IV and V from bovine, *Artemia franciscana* and *Litopenaeus vannamei*. In-gel cytochrome oxidase activity from: **a** bovine (lane 1), and artemia (lanes 2–3); **b** bovine (lane 1), and shrimp (lanes 2–3). In-gel ATPase activity from: **c** bovine (lane 1), and artemia (lanes 2–3); **d** bovine (lane 1), and shrimp (lanes 2–3). DG: protein solubilized with digitonin. LM: protein solubilized with dodecyl-D-maltoside

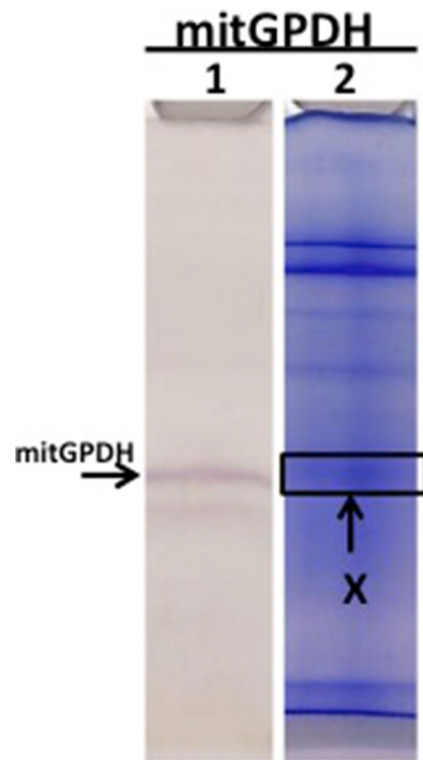
### Mitochondrial AOX confirmation by western blot

AOX identity was confirmed by Western blot analysis on the isolated mitochondria from artemia. Figure 9 shows a single band of approximately 25 and 37 kDa, which confirmed that AOX was present in the *A. franciscana* nauplii. No AOX was detected in the isolated mitochondrial proteins from shrimp (data not shown).

Positive controls included AOX from oyster, mango and huitlacoche mitochondria, and each of these enzymes were previously detected and confirmed (Considine et al. 2001; McDonald et al. 2009; Cardenas-Monroy et al. 2017).

### Discussion

*Artemia franciscana* and *Litopenaeus vannamei* are two crustaceans with the ability to survive in adverse environmental conditions, including widely variable dissolved oxygen concentrations (Abatzopoulos et al. 2002; Puente 2009). *Artemia* successfully faces anoxia by significantly reducing ATP turnover



**Fig. 8** X band from MS-MS analysis of artemia mitochondria loaded on a hrCN-PAGE (4–12% gradient). In-gel activity of mitGPDH (lane 1) and hrCN-PAGE stained with coomassie brilliant blue (lane 2). Black rectangle indicates the excised band corresponding to GPDH (band X)

(Patil 2012). Conversely, the white shrimp *L. vannamei*, has been identified as a hypoxia-tolerant species that can adapt to continuous oxygen changes in seawater throughout its life cycle. In culture ponds or shallow estuaries, seawater oxygen levels may vary diurnally from normoxia during the day (8 mg/L) to hypoxia at night (1 mg/L) (Greccay and Stierhoff 2002; Puente 2009).

Mitochondria, as organelles that use oxygen to produce energy, is closely related to a species' ability to face oxidative stress and maintain cellular homeostasis. The physiological mitochondrial uncoupling ability is an important response to control the oxidative stress using different mechanisms to prevent mitochondrial ROS production such as alternative dehydrogenases and oxidases (Nicholls and Ferguson 2003).

Respiratory control of isolated mitochondria by both crustaceans was lower than in mammalian mitochondria, such as the bovine heart, which yielded a RC of approximately 7. Previous studies have reported similar values in mitochondria isolated from *L. vannamei* by using the same respiratory substrate (Chimeo et al. submitted), and the coupled state of these isolates was confirmed by the increased oxygen consumption velocity in the mitochondria when ADP was added. In addition, these RC values are similar those of the polychaete *Nereis pelagica*, and the bivalve *Arctica islandica* with RCs of 1.9 and 2.5, respectively (Tschischka et al. 2000). These

**Table 1** Identification of *Artemia franciscana* GPDH by LC-MS/MS and homology database search

Band	Protein	Organism	Accession number <sup>a</sup>	Theor. MW/pI <sup>e</sup>	Score <sup>f</sup>	PM <sup>g</sup> /SC <sup>h</sup>	Peptide sequences
X	Glycerol-3-phosphate dehydrogenase, mitochondrial-like isoform X2	<i>Octopus bimaculoides</i>	XP_014772456.1	61.0/7.15	39.26	1/1%	K.ALELFPMLK.R Oxidation M
	Glycerol-3-phosphate dehydrogenase, mitochondrial-like isoform X1	<i>Monodelphis domestica</i>	XP_007494252.1	80.9/6.65	55.91	1/2%	R.LVQDYGLESEVAQ.H
	PREDICTED: Glycerol-3-phosphate dehydrogenase, mitochondrial	<i>Metaseiulus occidentalis</i>	XP_003739777.1	79.3/7.23	36.60	1/2%	R.LHEEFYIEAEVR.Y

<sup>a</sup> Accession numbers according to NCBI protein database; <sup>b</sup> Theoretical molecular weight and pI; <sup>f</sup> PEAKS Score reported after searching against a database containing the Brachiopoda subset of the nrNCBI protein database (600,199 sequences, January 2017), plus 1085 protein sequences corresponding to mitochondrial GPDHs obtained from the NCBI and the Contaminants collection from The Max Plank Institute of Biochemistry (247 sequences). Scores >20 indicate a protein False Discovery Rate <0.1%. <sup>g</sup> Peptides matched; <sup>h</sup> Sequence coverage

values may be due to the low concentration of molecular oxygen in the marine seawater compared with that in the atmosphere; thus aquatic organisms and their respiratory rates are thought to be adapted to this condition.

In this study, oxygen consumption data suggested that various alternative enzymes exist including dehydrogenases and oxidases. The in-gel activities of respiratory complexes I, II, IV and V, in the mitochondria isolated from *A. franciscana* nauplii and shrimp under normoxic conditions were compared with bovine mitochondrial enzymes as standards. More than one protein band with dehydrogenase activity and alternative oxidase immunodetection were detected in the artemia nauplii mitochondria, suggesting the existence of AOX, and mitGPDH as part of an alternative respiratory chain in the *A. franciscana* nauplii mitochondria. No AOX- or GPDH-like enzymes were detected in the shrimp mitochondria.

A mitGPDH was confirmed in artemia via protein sequence analysis. This enzyme is tentatively located on the external side of the MIM, uses glycerol-3-phosphate from the cytosol as substrate, and reduces ubiquinone to the ubiquinol that enters the respiratory chain. The transferred electrons may then reach complexes III/IV or AOX as previously observed by Guerrero-Castillo et al. (2012) in yeast. Adding antimycin A partially inhibits oxygen consumption, but no sensitivity to rotenone or

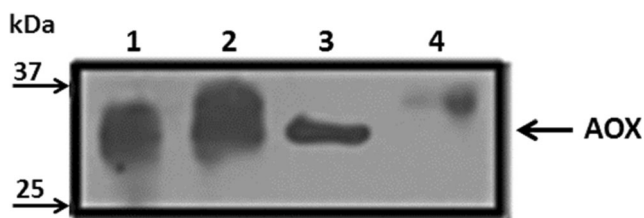
succinate, suggested that artemia mitGPDH reduces ubiquinone to ubiquinol, allowing electrons to reach complex IV from complex III or AOX from the ubiquinol. This explains why adding antimycin does not fully inhibits mitochondrial respiration (Fig. 3 trace a).

The fact that mitGPDH is present in recently hatched artemia nauplii may be associated with the cyst's energetic metabolism since it is based primarily on trehalose use, which is the cyst's first energy source. The two glucose molecules produced by trehalose hydrolysis are used during glycolysis, the Krebs cycle, and the ETC to produce ATP via oxidative phosphorylation. When environmental conditions are favorable, large amounts of glycerol accumulate inside the cyst, generating high osmotic pressure for hatching (Clegg 1964). Consequently, the large stores of trehalose and glycerol-3-phosphate produced from the dihydroxyacetone via glucose oxidation are used as respiratory substrates, increasing the ETC, and the mitochondrial oxygen consumption.

Glycerol-3-phosphate is used by mitGPDH to reduce FAD from feeding the ETC without pumping protons into the intermembrane space; thus, the proton gradient decreases, oxygen uptake is maintained, and ROS production remains steady.

Some species' mitochondria include an external NADH2 to support the ETC and accelerate oxygen uptake (Nicholls and Ferguson 2003). No evidence of an external NDH2 in either the artemia or shrimp mitochondria was observed in this study since no changes were detected in the oxygen consumption of both species with NADH as a respiratory substrate. We suggest that no alternative dehydrogenases as NDH2 exist in crustaceans, as those observed in the branched mitochondrial chains of the fungus *Neurospora crassa* (Duarte et al. 2003) and in the yeast, *Saccharomyces cerevisiae* (Marres et al. 1991).

Previous studies have reported the existence of a NDH2 and mitGPDH in organisms such as the yeast, *D. hanseni*.



**Fig. 9** AOX immunodetection from *Artemia franciscana* nauplii mitochondria (lane 1), *Crassostrea gigas* mitochondria (lane 2), *Mangifera indica* mitochondria (lane 3) and *Ustilago maydis* mitochondria (lane 4)



These enzymes are only expressed in the stationary phase of cell growth to maintain the ETC activity, thus decreasing the proton gradient in the intermembrane space since these enzymes do not pump protons through the MIM. This allows uncoupled of ATP synthesis and decreased ROS production (Cabrera-Orefice et al. 2014).

AOX existence was suggested by the decreased oxygen uptake in artemia mitochondria after octyl-gallate (OG) addition, since it specifically inhibits the enzyme (Hoefnagel et al. 1995). These results regarding oxygen consumption and AOX immunodetection indicate that artemia nauplii express an AOX as part of a branched respiratory chain that maintains the electrons flow and the cellular redox potential. The enzyme was also confirmed to be active at the same time as COX, but it remains to be studied whether this mitochondrial uncoupling mechanism is expressed at any specific life-cycle stage or specific environmental conditions as anoxia, hypoxia or reoxygenation.

To date, AOXs are known to participate in maintaining intracellular oxygen pressure when ADP is low or when intracellular oxygen levels rise due to the oxygen saturation in the classical respiratory chain, decreasing the risk of free radicals production and cell damage by oxidative stress (Korshunov et al. 1997; Tschischka et al. 2000).

In artemia, high amounts of energy are required after the cysts hatch, and the organisms face increased oxygen concentrations. At this early stage, embryos must maintain their recently-activated cellular functions, to grow, swim and molt (Abatzopoulos et al. 2002); thus, mitGPDH and both oxidases, COX and AOX, are active. These enzymes may maintain ATP levels and control the oxygen molecule concentration to avoid excessive ROS production as suggested in other invertebrate species (Abele et al. 2007).

Although previous reports of mitochondrial alternative enzymes have confirmed that AOX exists in marine mollusks and other invertebrates (McDonald et al. 2009), to our knowledge, these results confirm for the first time, that alternative enzymes exist in crustaceans mitochondria. A mitGPDH and AOX in artemia nauplii mitochondria, both as part of a branched respiratory chain, may represent an adaptive trait used by artemia nauplii to survive the high oxygen concentrations that these organisms face after cysts eclosion. Future studies will provide additional information about these proteins specific function and the adaptive mechanisms these species have developed along their life cycle.

Since no alternative enzymes were found in shrimp mitochondria as it occurs in artemia, further efforts will be needed to determine the existence of other mitochondrial uncoupling mechanisms. To date, no evidence exists of a permeability transition response in artemia and shrimp (Menze et al. 2005; Rodriguez-Armenta, in preparation); however, recently two uncoupling proteins (UCP4 and UCP5) have been confirmed in *L. vannamei* mitochondria (Mendez-Romero, in

preparation), suggesting these proteins are the only confirmed uncoupling mechanism in shrimp mitochondria.

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