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Phosphoenolpyruvate carboxykinase cytosolic and mitochondrial isoforms are expressed and active during hypoxia in the white shrimp *Litopenaeus vannamei*

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ABSTRACT

Hypoxic zones in marine environments are spreading around the world affecting the survival of many organisms. Marine animals have several strategies to respond to hypoxia, including the regulation of gluconeogenesis. Phosphoenolpyruvate carboxykinase (PEPCK) is a key regulatory enzyme of gluconeogenesis. The objective of this work was to study two isoforms of PEPCK, one mitochondrial (PEPKC-M) and one cytosolic (PEPCK-C), from the white shrimp *Litopenaeus vannamei* and the response to hypoxia. Both PEPCK isoforms are 72 kDa proteins and have 92% identity at the amino acid level. The mitochondrial isoform has a N-terminal signal peptide for mitochondrial import. Gene expression and enzymatic activity in subcellular fractions were detected in gills, hepatopancreas and muscle in normoxic and hypoxic conditions. Expression of PEPCK-C was higher than PEPCK-M in all the tissues and induced in response to hypoxia at 48 h in hepatopancreas, while the enzymatic activity of PEPCK-M was higher than PEPCK-C in gills and hepatopancreas, but not in muscle and also increased in response to hypoxia in hepatopancreas but decreased in gills and muscle. During limiting oxygen conditions, shrimp tissues obtain energy by inducing anaerobic glycolysis, and although gluconeogenesis implies energy investment, due to the need to maintain glucose homeostasis, these gluconeogenic enzymes are active with contrasting behaviors in the cytosol and mitochondrial cell compartments and appear to be up-regulated in hepatopancreas indicating this tissue pivotal role in gluconeogenesis during the response to hypoxia.

1. Introduction

Hypoxic sites in marine ecosystems are increasing around the world (Breitburg et al., 2018) due to global climate change and human activities, affecting the life of many marine organisms. Marine fauna are exposed to hypoxia due to tidal and wind effects in shallow water (Bell et al., 2003), eutrophication and stratification of the water column (Rabalais et al., 2002).

Several crustaceans can survive hypoxic conditions by diverse adaptations (McMahon, 2001), including the activation of anaerobic metabolism. The Pacific white shrimp, *L. vannamei* is native to the oriental coast of the Pacific Ocean and the major crustacean species used for aquaculture worldwide. In its natural habitat and in farming conditions, the white shrimp suffers fluctuations of oxygen levels (Parrilla-Taylor and Zenteno-Savín, 2011) and survives to low concentrations of dissolved oxygen or hypoxia (Rosas et al., 1999). In aquatic media, hypoxia is considered as the amount of dissolved oxygen (DO) in water lower than 2.8 mg L^{-1} (Diaz and Rosenberg, 1995), while 5–7 mg L^{-1} is referred as normoxia. As most animals, to obtain energy the shrimp uses aerobic metabolism with carbohydrates as glucose as the major fuel (Sánchez-Paz et al., 2007); however, when DO falls, the shrimp switches to anaerobic metabolism as indicated by the rapid increase of lactate in the hemolymph (Racotta et al., 2002; Soñanez-Organis et al., 2009). This results in depletion of glucose by the acceleration of glycolysis rate imposed by the need to generate ATP at substrate level phosphorylation, and because glucose reserves in the form of glycogen are very limited in this species (Sánchez-Paz et al., 2007). Therefore, to sustain glucose demand during hypoxia, glucose may be produced *de novo* from other metabolites such as lactate, pyruvate, glycerol and amino acids by gluconeogenesis. A key enzyme for this process is

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phosphoenolpyruvate carboxykinase (PEPCK; GTP, EC 4.1.1.32). PEPCK catalyzes the reversible formation of phosphoenolypyruvate (PEP) from oxaloacetate (OAA) and GTP. In eukaryotes two PEPCK isoforms are known, a mitochondrial (PEPCK-M) and a cytosolic (PEPCK-C) (Nordlie and Lardy, 1963).

Hepatopancreas appears to be the main site for gluconeogenesis in decapods crustaceans as shown by using labelled [^{14}C]-lactate and [^{14}C]-amino acids to follow glucose synthesis (Hervant et al., 1999; Vinagre and Da Silva, 2002), although some studies also implicated the gills (Chittó et al., 2001) and muscle (Schein et al., 2004) in this process. There are currently few detailed studies of gluconeogenesis and the enzymes involved in this process in crustaceans, and they have focused mostly on quantification of metabolites. In the crab *Chasmagnathus granulate*, metabolic adjustments in response to osmotic and anoxic stress occurs through gluconeogenesis (Oliveira and da Silva, 2000). However, there is no information about PEPCK and the effects of environmental stress in the shrimp *L. vannamei*. Herein, we identified a mitochondrial and a cytoplasmic isoform of PEPCK and investigated the effect of hypoxia in their expression and enzyme activity in the white shrimp.

2. Materials and methods

2.1. cDNA nucleotide and deduced amino acid sequences

The initial nucleotide sequences of the PEPCK isoforms cDNAs were obtained form available data. One PEPCK cDNA sequence was from GenBank (Accession no. AJ250829) and the other was included in a report of the white shrimp transcriptome (Ghaffari et al., 2014), but none of these were properly annotated. The deduced amino acid sequences were analyzed using "Expasy" http://web.expasy.org/translate/ and the signal peptide prediction for subcellular location was found using TargetP 1.1 (Emanuelsson et al., 2007) http://www.cbs.dtu.dk/services/TargetP/. Likewise, α-helices located in the N-terminal region were predicted using HELIQUEST http://heliquest.ipmc.cnrs.fr/ and ProtParam (http://ca.expasy.org/tools/protparam.html) was used to estimate the relative molecular weight and isoelectric point of both proteins.

2.2. Amino acid sequence alignment

PEPCK amino-acid sequences analyses were done with CLUSTAL Omega. PEPCK sequences used for the alignment were: *L. vannamei* GenBank Accession No. CAB85964; *Nephrops norvegicus* cytosolic isoform CAB65311, *Neohelice granulata* cytosolic isoform AAL78163; *H. sapiens* cytosolic isoform NP_002582; *Macrobrachium nipponense* mitochondrial isoform ALK82313; *Homo sapiens* mitochondrial isoform NP_004554; and finally, a PEPCK sequence from a report of the white shrimp transcriptome (Ghaffari et al., 2014).

2.3. Phylogenetic analysis

PEPCK amino acid sequences from distinct taxa were obtained from the NCBI database (Table 2), and multiple alignment was performed with the ClustalW2 algorithm (Thompson et al., 1994). The phylogenetic tree was constructed using MEGA X (Kumar et al., 2018) and the Maximum Likelihood method, based on the Le Gascuel model (Le and Gascuel, 2008), with 1000 bootstrap replicates. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 0.5497)) and positions with < 90% site coverage were eliminated. The tree was rooted to a PEPCK sequence from *Corynebacterium glutamicum*.

2.4. Hypoxia bioassay

Briefly, for the hypoxia bioassay, juvenile intermolt shrimp *L. vannamei* (21.6 g ± 1.5) were acclimated in tanks with sea water constantly aerated and recirculated. Shrimps were exposed to hypoxia at 1.57 ± 0.2 mg DO per liter and normoxic controls were included. Hypoxia was produced by bubbling nitrogen gas as necessary and monitored with a portable oximeter (YSI model 55, Yellow Spring, OH, USA). After 3 h and 48 h of hypoxia, shrimps for each condition were collected, the tissues dissected and stored at −80 °C until used as reported by Cota-Ruiz et al., 2016.

2.5. RNA isolation and expression during hypoxia

To analyze gene expression, total RNA was extracted from hepatopancreas, gills and muscle using TRI Reagent (Sigma-Aldrich) from one group of shrimp subjected to hypoxia and a second one maintained in normoxia conditions as previously reported by Cota-Ruiz et al., 2016. The RNA was quantitated by A_{260nm} using a NanoDrop 2000c spectrophotometer (Thermo Scientific) and the integrity was confirmed by 1% agarose gel electrophoresis. To eliminate genomic DNA contamination, 13 μg of total RNA were incubated with RNase-free DNase I (Roche) at 37 °C for 20 min and verified by doing a q-PCR reaction with this RNA. Once verified for DNA decontamination, 500 ng of each total RNA were used for cDNA synthesis in duplicate with the Quantitect Reverse Transcription Kit (Qiagen) to have a final concentration of 25 ng/μL equivalents of the original total RNA in a volume of 20 μL.

PEPCK-M, PEPCK-C and ribosomal protein L8 (GenBank accession no. DQ316258.1) transcripts were quantified by RT-qPCR using a CFX-96 Real-Time PCR Detection System (Bio-Rad). Two cDNA reactions and then two qPCRs reactions for each shrimp sample were done (four data for each tissue per shrimp) in a 15 μL final volume. For PEPCK-M and ribosomal protein L8 (used for normalization) the reactions contained 7.5 μL of 2× SYBR Green qPCR Master Mix (Biotool), 5 μL of nuclease-free water, 0.75 μL of each primer (10 μM) and 1 μL of cDNA (25 ng of total RNA equivalents), and for PEPCK-C, the reaction contained 7.5 μL of 2× SYBR Green qPCR Master Mix, 5.75 μL of nuclease-free water, 0.375 μL of each primer (10 μM) and 1 μL of cDNA (derived from 25 ng of total RNA). For PEPCK-M the PCR conditions were as follows: 95 °C for 3 min, 40 cycles at: 95 °C for 30 s, 55 °C for 35 s and a 68 °C for 55 s, with a single fluorescence measurement at the extension step and a final melting curve program with 0.3 °C increments each 20 s from 60 °C to 116 °C. For PEPCK-C and L8, the PCR conditions were the same as PEPCK-M except that the annealing step was 57 °C for 25 s, and annealing temperature was 63 °C, respectively. Negative controls were included. The primers for both isoforms were designed using "Primer3" (Untergasser et al., 2012). The amplification of a 168 bp fragment for PEPCK-M was done with the primers PEPCK-M Fw (5′-AGCCAAAGAA CGTCCACATC-3′) and PEPCK-MRv (5′-CGATGAAGGTCTTGCTC TCC-3′), for PEPCK-C, the primers qPEPCK-C Fw (5′-GAAATTGCTCGC TCCGTAGC-3′) and qPEPCK-C Rv (5′-CGTGAACTTTGCTTGGCGAG-3′) generating a 157 bp fragment, and L8 was amplified using the primers L8F2 (5′-TAGGCAATGTCATCCCCATT-3′) and L8R2 (5′-TCCTGAAGGA AGCTTTACACG-3′) to amplify a 166 bp fragment (Trasviña-Arenas et al., 2013). Efficiency of amplification to each primer pair was determined running standard curves using ten-fold serial dilutions from 2.5×10^{1} to 2.5×10^{-6} of cDNA. In all cases, the amplification efficiency was 90–110%. Finally, the PEPCK-M and PEPCK-C relative expression to L8 was determined with the quantification cycle (Hervant et al., 1999) and calculated with the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.6. Subcellular fractionation and PEPCK activity

Samples of approximately 100–300 mg of hepatopancreas, gills and muscle of *L. vannamei* were homogenized in a 1:10 (w/v) buffer A (250 mM sucrose, 15 mM Tris-HCl, pH 8.0, 15 mM EDTA, 1 mM PMSF, 1 mM DTT). Samples of gills and muscle were homogenized with a sonifier Ultrasonic Branson 250 and hepatopancreas was homogenized

with a manual homogenizer Kontes to obtain a crude homogenate. The activity of the mitochondrial and cytosolic PEPCKs was measured in subcellular fractions. Mitochondrial and cytosolic fractions were obtained by centrifuging the crude homogenates at 1000 ×*g* for 30 min at 4 °C to separate the debris, the supernatant was collected and centrifuged at 1200 ×*g* for 10 min at 4 °C, finally, the supernatant was collected and centrifuged at 20,000 ×*g* for 20 min at 4 °C; the supernatant was collected as the cytosolic fraction and the resulting pellet was the mitochondrial fraction. The pellet was then resuspended in 100 µL of buffer B (125 mM sucrose, 15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1% sodium deoxycholate) to break the mitochondrial membrane and release the mitochondrial enzymes. Both fractions were then diluted 1:10 in buffer A, except the fractions from hepatopancreas that were diluted 1:20 in buffer A.

PEPCK activity was measured in duplicates from four individual samples in the carboxylation direction by following the oxidation of NADH (modified from Wiese et al., 1991). This assay is based on coupled reactions where the OAA produced by PEPCK was used as substrate by malate dehydrogenase to produce malate. The oxidation of NADH by malate dehydrogenase was measured at 340 nm at 25 °C in a microplate using a Multiskan GO (Thermo scientific) spectrophotometer every 15 s for up to 6 min (in agitation). The absorbance change ratio in the linear range and the NADH absorbance coefficient value of 6.22×10^{-3} L mol^{-1} cm^{-1} were used to calculate the enzyme activity. One unit of enzyme activity is defined as the amount of enzyme resulting in the production of 1 µmol of product per min at 25 °C.

The reaction mixtures contained 100 mM HEPES-KOH buffer, pH 7.4, 90 mM $KHCO_3$, 100 mM KCl, 6 mM $MnCl_2$, 4 mM PEP, 2 mM GDP, 0.15 mM NADH, 5 mM DTT and 6 U/mL of malate dehydrogenase in a final volume of 153 µL (including 3 µL of sample). Protein concentration was determined by the Bradford method (Bradford, 1976) to calculate the specific enzymatic activity of each sample. To confirm appropriate subcellular fractionation, spectrophotometric activities of lactate dehydrogenase (Worthington, 1988) and cytochrome *c* oxidase (Cytocrome c Oxidase Assay Kit, Sigma-Aldrich) were determined as cytoplasmic and mitochondrial markers, respectively.

2.7. Statistics

Statistical analyses were done using the NCSS 11 software 2017 package (NCSS LLC, Kaysville, Utah, USA). The data for RT-qPCR and enzymatic activity were tested for normality distribution (Kolmogorov-Smirnoff test) and analyzed by two-way ANOVA (hypoxia X time). Fisher LSD test were used to find differences between means at a significant level of $p < 0.05$.

3. Results

3.1. PEPCK cDNAs and deduced amino acid sequences

The cDNA nucleotide sequence of PEPCK (GenBank accession no. AJ250829) from hepatopancreas (Fig. 1) is 2186 bp. This sequence presents the start and stop codon at positions 169 and 2104, respectively. The open reading frame (ORF) is 1935 bp and codes for a protein of 645 amino acids. The calculated molecular mass was 71.86 kDa with a predicted pI of 7.23, and this agrees well with the molecular mass of mammalian and avian PEPCKs (Beale et al., 1985; Cook et al., 1986). A mitochondrial signal peptide was found in the N-terminal, where secondary structure predictions revealed an α-helix of 31 amino acids that form an amphipathic helix which corresponds to the hydrophobic face (Val, Val, Phe, Leu, Ala, Ala) and polar residues between the two opposite faces of the α-helix, typical of mitochondrial targeting peptides, hence, this sequence corresponds to the mitochondrial isoform (PEPCK-M).

The sequence from a report of the white shrimp transcriptome is 2242 bp in length (Fig. 2). It has the start and stop codon at positions 76 and 2038 respectively. The ORF is 1962 bp encoding a 654-amino acid protein. The calculated molecular mass was 72.55 kDa with a predicted pI of 7.22. Secondary structure predictions did not reveal any targeting peptide; therefore, we conclude that this corresponds to the cytosolic isoform (PEPCK-C). None of these two sequences had been reported properly and the analysis clearly reveals and distinguishes the mitochondrial and cytosolic isoforms. The nucleotides sequences of the PCR fragments used for expression analysis were identical to the previously obtained sequences.

3.2. Amino acid sequence alignment

Sequence analysis of the deduced PEPCK revealed high homology between both isoforms from *L. vannamei* (92%). The predicted sequence of PEPCK-C from *L. vannamei* shows high identity to PEPCK-Cs from other organisms, such as the Norway lobster *N. norvegicus* 82%, the burrowing crab *N. granulata* 78% and 67% with *H. sapiens*; and for PEPCK-M from *L. vannamei* with others PEPCK-M showed the highest identity with the river prawn *M. nipponense* 86% and 66% with *H. sapiens*.

From the alignment of different PEPCK amino acid sequences (Fig. 3), we found well conserved regions and fully conserved residues among the different taxa; residues involved in metal binding: Asp 109, Asp 112, Glu 117 (*L. vannamei* PEPCK-C) and Asp 101, Asp 104, Glu 109 (*L. vannamei* PEPCK-M); catalytic residues: Lys 272, His 293, Thr 321, Asp 340, Asp 341 (*L. vannamei* PEPCK-C) and Lis 264, His 284, Thr 311, Asp 330, Asp 331 (*L. vannamei* PEPCK-M); and the hyper-reactive cysteine 318 and 308 (Holyoak et al., 2006) *L.vannamei* PEPCK-C and PEPCK-M respectively.

3.3. Phylogenetic analysis

The resulted tree (Fig. 4) constructed from 20 amino acid sequences of PEPCK shows clearly three groups, the first one corresponding to invertebrates (including both *L. vannamei* PEPCKs) regardless of the isoform and includes the decapods in one cluster with high bootstrap values (99%), with crustaceans forming a single cluster but the bootstrap value is low (~40%), probably due to the few sequences available and used in this analysis. The other two clusters correspond to vertebrates and are formed by a mitochondrial and a cytosolic branch with a bootstrap value of 89%, consistent with Tsang et al., 2008, that indicates that PEPCK is a well-conserved protein that plays a fundamental role in many life forms. While these last two branches reflected a more conventional taxonomy separating the mitochondrial from the cytosolic isoform, this cannot be reflected in the crustacean cluster where both isoforms are included. However, since there are no more sequences available of crustacean's PEPCKs, this result must be interpreted with caution. Also, as the identity between PEPCK-M and PEPCK-C from *L. vannamei* is very high (92%), this is likely to be the same for other crustaceans, hence the clustering of these sequences by taxon and not by isoform.

3.4. Both isoforms of PEPCK are expressed in normoxia and hypoxia

Expression of PEPCK in normoxia (as control) and hypoxia (3 h and 48 h) were evaluated in hepatopancreas, gills and muscle (Fig. 5, panels A, B and C). The transcripts of both isoforms of PEPCK were detected in the three analyzed tissues, indicating gluconeogenic capacity. In all the cases, expression of the cytosolic form was higher than the mitochondrial one. The higher levels of PEPCK transcripts were found in gills at 48 h in normoxia from the cytosolic isoform. Interestingly, there was a change in the expression of PEPCK-M in gills at 48 h in the normoxic condition, with 11-fold higher value than the hypoxia corresponding group and it is also higher than the normoxic group at 3 h. In hepatopancreas, both isoforms had a significant difference due to hypoxia but only after 48 h compared to the normoxic control, with 3.6 and 2.5-fold

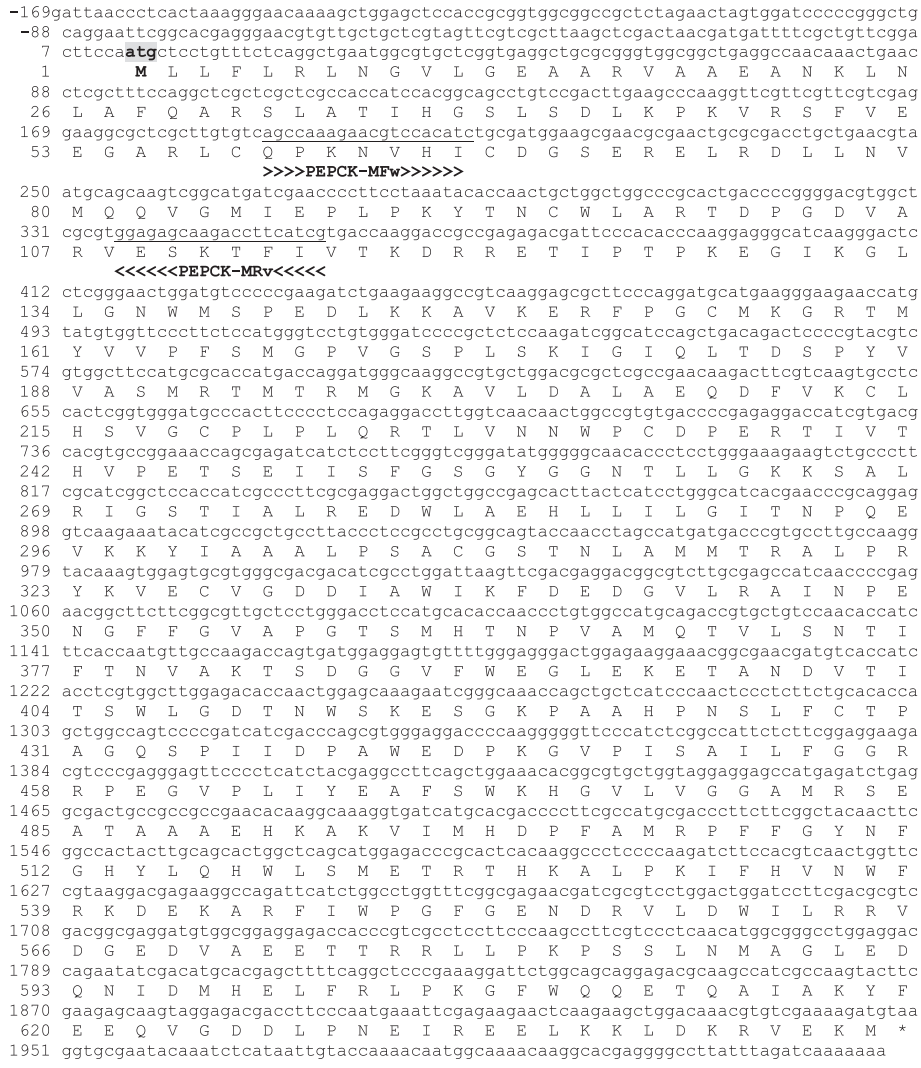

Fig. 1. Nucleotide and deduced amino acid sequence of *L. vannamei* PEPCK-M. The start methionine is shown in bold and the stop codon by an asterisk. The primer positions for qPCR are shown with (> > > > >) for forward and (< < < < <) for reverse and underlined.

higher for PEPCK-C and PEPCK-M respectively. Last, the transcripts of PEPCK-C in muscle had the lower expression levels and showed a decrease of 6-fold after 3 h of hypoxia compared to its corresponding control group, with opposite results compared to the expression in gills.

3.5. PEPCK enzymatic activity is regulated in long-term hypoxia in hepatopancreas

To evaluate the effect of hypoxia on PEPCK activity, we measured the enzymatic activity in mitochondrial and cytosolic subcellular fractions from tissues from hypoxic and normoxic shrimp. Activity of PEPCK was detected in hepatopancreas, gills and muscle in all treatments here evaluated (Fig. 6), with higher activity of the mitochondrial isoform compared to the cytosolic one in gills and hepatopancreas but not in muscle. The highest activity for the cytosolic fraction was detected in muscle at 3 h of normoxia, while for the mitochondrial fraction, it was detected in hepatopancreas at 48 h of hypoxia. Interestingly, only in hepatopancreas there was higher activity of PEPCK-C and PEPCK-M (2.8 and 11-fold respectively) due to hypoxia but only at 48 h compared to its time-pair normoxic control. In all the cases, comparisons of each isoform were done with the respective control time in normoxic, and it is interesting to note that there are changes in expression and enzyme activity even in normoxic conditions as reported previously for FBP in the same shrimp species (Cota-Ruiz et al., 2016).

To ensure no cross-contamination between subcellular fractions, cytochrome *c* oxidase activity (Table 1) was determined in cytosolic and mitochondrial fractions of the three tissues, and it was only detected in the mitochondrial fraction, hence, no mitochondrial contamination was detected. Also, lactate dehydrogenase (LDH) activity (Table 1) was used as cytosolic marker and it was detected mainly in the cytosolic fraction, therefore, no major cytosolic contamination occurred.

4. Discussion

4.1. PEPCK isoforms

To ensure proper targeting and import, most of the mitochondrial proteins (70%) synthesized in the cytosol as “preproteins” contain cleavable targeting signals. These signals are found in the N-terminal of the newly synthesized proteins, recognized by receptor proteins localized in the organellar outer membranes (Vogtle et al., 2009; Zybailov et al., 2008), and after translocation to the organelle are excised to produce mature proteins. In this study, we analyzed two isoforms of PEPCK (mitochondrial and cytosolic) by the presence (or not) of a cleavable targeting peptide. We found high identity among all the

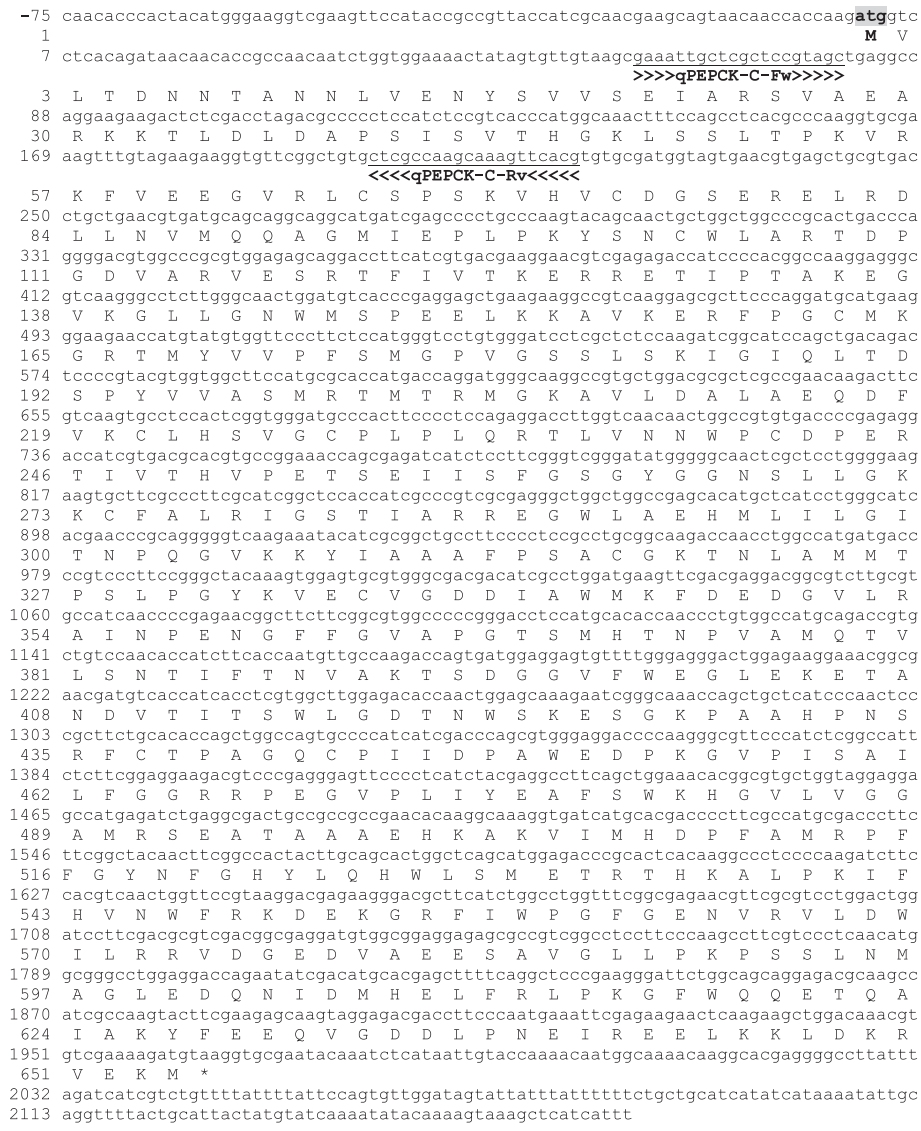

Fig. 2. Nucleotide and deduced amino acid sequence of *L. vannamei* PEPCK-C. The start methionine is shown in bold and the stop codon by an asterisk. The primer positions for qPCR are shown with (> > > > >) for forward and (< < < < <) for reverse and underlined.

deduced proteins herein studied; therefore, the evolutionary preservation of this enzyme was evident. Particularly, the "hyper-reactive" cysteine residue is completely conserved in all PEPCKs and it is known that the modification of this residue inactivates this enzyme (Lewis et al., 1989). The presence of this residue in the active site suggest two roles: one would be the regulation through the steric inhibition of nucleotide binding and the other as a mechanism for stimulation of nucleotide product release; this is due to the mobility of this cysteine which once in contact with Mn, stimulates the product release (Holyoak et al., 2006).

4.2. PEPCK expression is regulated in a tissue-specific manner in response to hypoxia

In hypoxic conditions, the white shrimp rely on several mechanisms for its survival, including the shift from aerobic to anaerobic glycolysis (Soñanez-Organis et al., 2009), indicated by lactate accumulation in plasma (Racotta et al., 2002; Soñanez-Organis et al., 2010) and the up-regulation of glycolytic enzymes such as PFK (Cota-Ruiz et al., 2016), HK and LDH (Soñanez-Organis et al., 2012). In hepatopancreas, expression of both PEPCKs was slightly induced at 48 h of hypoxia (Fig. 5), thus, the gluconeogenic pathway is active during hypoxia although this implies energy use in conditions where ATP synthesis in mitochondria is halted by the limitation of oxygen. Similar results under hypoxia were found for FBP expression in hepatopancreas of *L. vannamei* (Cota-Ruiz et al., 2015), PEPCK of *Palaemonetes pugio* hepatopancreas (Brown-Peterson et al., 2008) and glucose-6-phosphatase in liver of fish *Gillichthys mirabilis* (Gracey et al., 2001), all involved in the production of glucose *de novo*. An increment in the glucose levels in plasma due to hypoxia (Soñanez-Organis et al., 2009) altogether with the induction of two glucose transporters, GLUT1 and GLUT2 in hepatopancreas in hypoxic conditions (Martinez-Quintana et al., 2015; Martinez-Quintana et al., 2014) suggest a mobilization of glucose from hepatopancreas to other tissues through hemolymph. In contrast, the expression in muscle drops from 3 h of hypoxia for PEPCK-C, and no other changes were found for the rest of treatments or the mitochondrial isoform, this can be related with down-regulation of HIF-1α (a transcription factor) in muscle in hypoxic conditions (Soñanez-Organis et al., 2009) and that the transcriptional activation of PEPCK is HIF-1-dependent (Choi et al., 2005); similar results were found in gills where no significant changes on the transcript levels of PEPCK-C (Fig. 5) appear at any time here measured. This suggest that under hypoxic

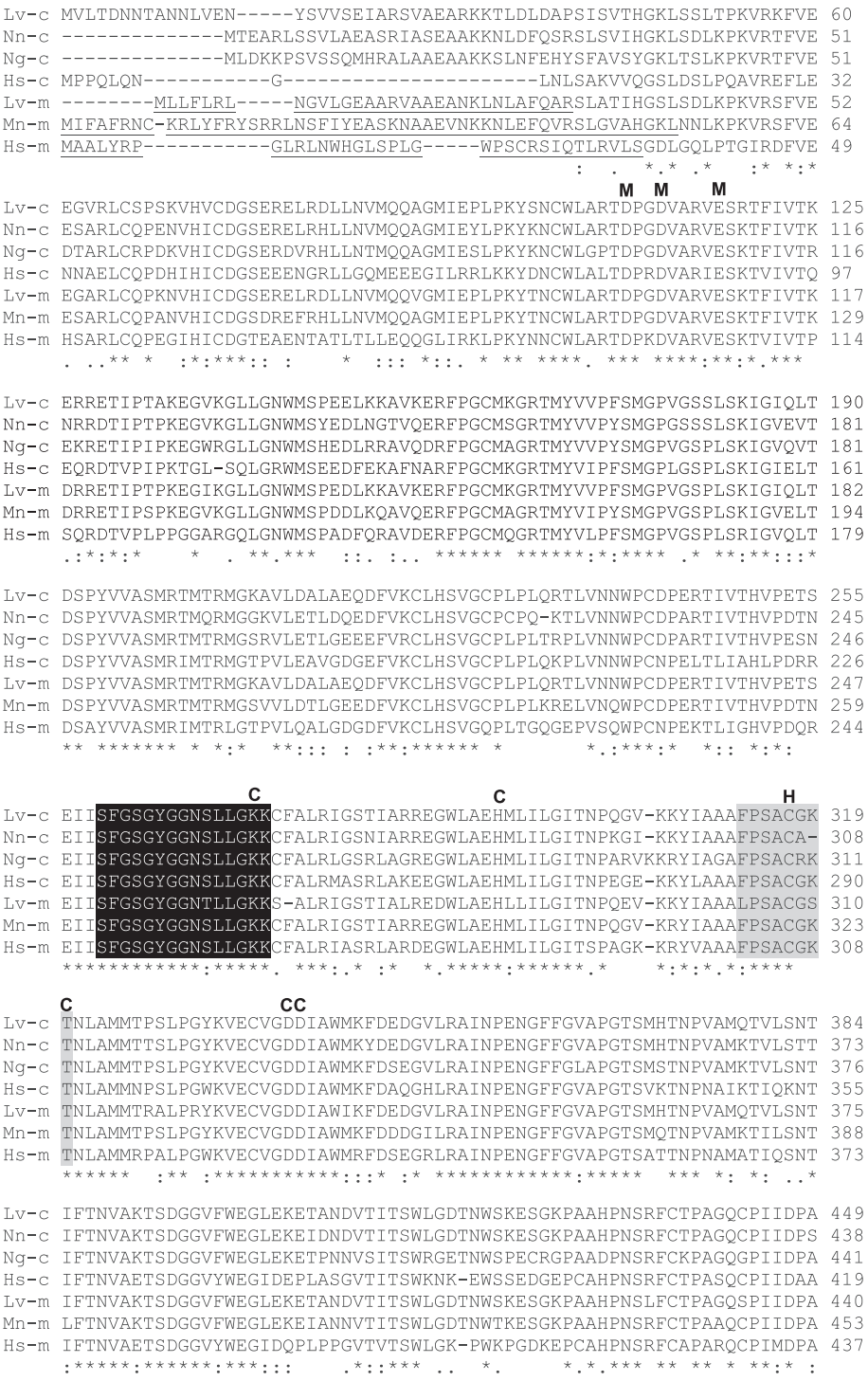

Fig. 3. Amino acid sequence comparisons among selected GTP-PEPCKs. Lv-c (*L. vannamei*, cytosolic), Nn-c (*N. norvegicus*, cytosolic), Ng-c (*N. granulata*, cytosolic), Hs-c (*H. sapiens*, cytosolic), Lv-m (*L. vannamei*, mitochondrial), Mn-m (*M. nipponense*, mitochondrial), Hs-m (*H. sapiens*, mitochondrial). Mitochondrial targeting peptides are underlined. Letters above the sequences indicates residues involved in metal binding (M); catalytic residues (C); hyper-reactive cysteine (H). Residues highlighted by black shading background represent PEPCK specific domain; gray shading background represent kinase site. Symbols below the sequences mean: (*), identical residues; (:), conservative substitutions; and (.), semi-conservative substitutions.

conditions there is no induction of glucose synthesis on muscle and gills, and the same behavior was reported for FBP, where in gills and muscle of *L. vannamei*, its expression was lower during hypoxia (Cota-Ruiz et al., 2015).

4.3. Hepatopancreas PEPCK activity: Its role for glucose homeostasis in hypoxia

Contrasting patterns occurred in the changes of enzyme activity; there is higher PEPCK-M than PEPCK-C activity in gills and hepatopancreas, but lower PEPCK-M than PEPCK-C in muscle, but the activity in muscle in any case is higher but with smaller changes compared to

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Lv-c WEDPKGVPISAILFGGRRPEGVPLIYEAFSWKHGVLVGGAMRSEATAAAEHKAKVIMHDPFAMRP 514
Nn-c WEDPKGVPISAILFGGRRPQGVPLVYEAFNWKHGVMVGGSMRSEATAAAEHKGKVIMQNPFAMTP 503
Ng-c WEDPKGVPISAILFAGR-PPGLPLVYEAYDWKHGVMVGAAMRSEATAAAEHKAQVIMHDPFAMRP 505
Hs-c WESPEGVPIEGIIFGGRRPAGVPLVYEALSWQHGVFVGAAMRSEATAAAEHKGKIIMHDPFAMRP 484
Lv-m WEDPKGVPISAILFGGRRPEGVPLIYEAFSWKHGVLVGGAMRSEATAAAEHKAKVIMHDPFAMRP 505
Mn-m WEDPKGVPISAILFGGRRPQGVPLVYEAFDWKHGVLIGGAMRSEATAAAEHKGKVIMHDPFAMRP 518
Hs-m WEAPEGVPIDAIIFGGRRPKGVPLVYEAFNWRHGVFVGSAMRSESTAAAEHKGKIIMHDPFAMRP 502
     ** *:****..*:*.** * *:**:*** .*:***::*.:****:*******.::**::**** *

Lv-c FFGYNFGHYLQHWLSMETRTHKALPKIFHVNWFRKDEKGRFIWPGFGENVRVLDWILRRVDGEDV 579
Nn-c FFGYNFGQYLQHWLSMETRTDKALPKIFHVNWFRKVKKGRFIWPGFGDNVRVLDWILKRVDGEDV 568
Ng-c FFGYNFGHYLQHWLSMEGRTTKQMPKIFHINWFRKNEKGRFKGPGFGENVRVLDWILRRVEGQDV 570
Hs-c FFGYNFGKYLAHWLSMAQHPAAKLPKIFHVNWFRKDKEGKFLWPGFGENSRVLEWMFNRIDGKAS 549
Lv-m FFGYNFGHYLQHWLSMETRTHKALPKIFHVNWFRKDEKARFIWPGFGENDRVLDWILRRVDGEDV 570
Mn-m FFGYNFGHYLQHWLSMENRTDKPLPKIFHVNWFRKDEKGRFIWPGF------------------- 564
Hs-m FFGYNFGHYLEHWLSMEGRKGAQLPRIFHVNWFRRDEAGHFLWPGFGENARVLDWICRRLEGEDS 567
     *******:** *****  :    :*:***:****: : .:*  ***

Lv-c AEESAVGLLPKPSSLNMAGLEDQNI-DMHELFRLPKGFWQQETQAIAKYFEEQVGDDLPNEIREE 643
Nn-c AEESGRRLLPKDPPSTMAGLEHENV-DMDELFSLPKELWEQEVRDSTKYFAEQVGDDLPNEVREQ 632
Ng-c AEESAIACLPSPDPSTLEGLEDHAHRTPEEAFSLPKDFWSQEIREIRKYFDEQVGSELPNEVRAR 635
Hs-c TKLTPIGYIPKEDALNLKGLGHI---NMMELFSISKEFWEKEVEDIEKYLEDQVNADLPCEIERE 611
Lv-m AEETTRRLLPKPSSLNMAGLEDQNI-DMHELFRLPKGFWQQETQAIAKYFEEQVGDDLPNEIREE 634
Mn-m ------------------------------------------------------------
Hs-m ARETPIGLVPKEGALDLSGLRAI---DTTQLFSLPKDFWEQEVRDIRSYLTEQVNQDLPKEVLAE 629
Lv-c LKKLDKRVEKM 654
Nn-c LKILEKRIEKM 643
Ng-c LKKLEKRVEKM 646
Hs-c ILALKQRISQM 622
Lv-m LKKLDKRVEKM 645
Mn-m -----------
Hs-m LEALERRVHKM 640
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Fig. 3. (*continued*)

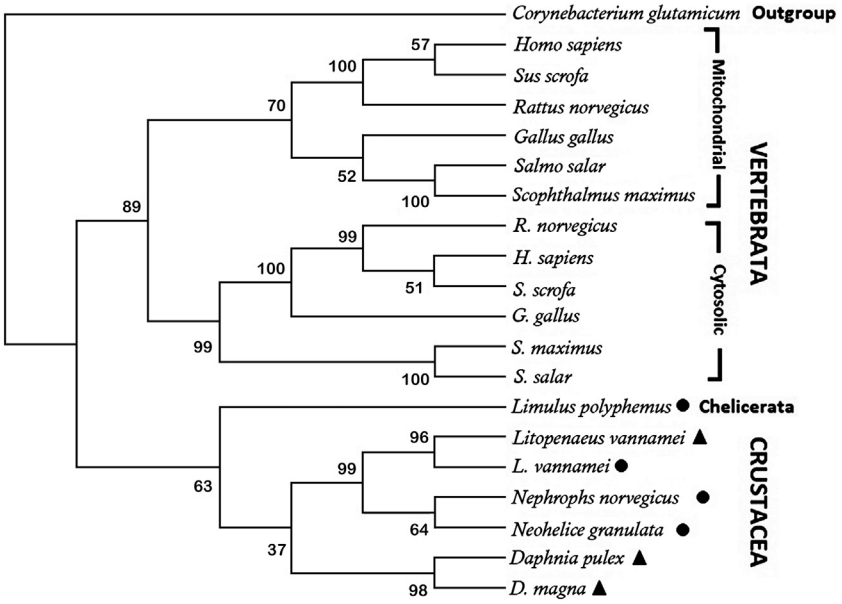

Fig. 4. Phylogenetic tree of PEPCK sequences. Twenty amino acid sequences were included in the analysis and there were 617 positions in the final dataset. The tree was inferred by the Maximum Likelihood method with the LG + G model. The numbers indicate the bootstrap percentage supporting each branch calculated from 1000 replicates. The GenBank accession numbers are listed in Table 2. Circle and a triangle after the sequence indicates cytosolic and mitochondrial isoform respectively.

gills and hepatopancreas. Also, there are differences in normoxic conditions in the different times. This may be due to the fed status of the animals, since feeding is suspended when the hypoxia treatment starts, but all the animals were maintained in the same conditions, the only change was the amount of oxygen. In hepatopancreas we found that the activity of both PEPCK isoforms did not decrease after 48 h of exposure to hypoxia, which is in contrast due to the significant decrease in both of these isoforms observed between 3 and 48 h in normoxia, and we in fact observed a significant increase in the activity of PEPCK-M in hypoxia. Additionally, reports in *L. vannamei* found a rise in glucose concentrations at 24 h and 48 h of hypoxia in hepatopancreas (Martinez-Quintana et al., 2016) and in hemolymph (Soñanez-Organis et al., 2010). These findings propose that hepatopancreas could supply the demand of glucose to other tissues because of the acceleration of the rate of anaerobic glycolysis. Also, our results are somewhat in agreement with a report in rat hepatocytes, where they found the induction of glucose endogenous production and its release due to hypoxia (Choi et al., 2005). In summary, herein we report the presence of two isoforms of *L. vannamei* PEPCK, mitochondrial and cytosolic. Although many authors have reported evidence for unique ways that animals use to survive to oxygen-deprived environments, the information about the molecular and biochemical mechanisms of response to this stress in marine crustacean species is still scarce. Both PEPCK isoforms transcripts were detected in all conditions here studied, indicating gluconeogenic capacity in hepatopancreas, gills and muscle. Also, we report that the expression of PEPCK is higher in hepatopancreas in response to

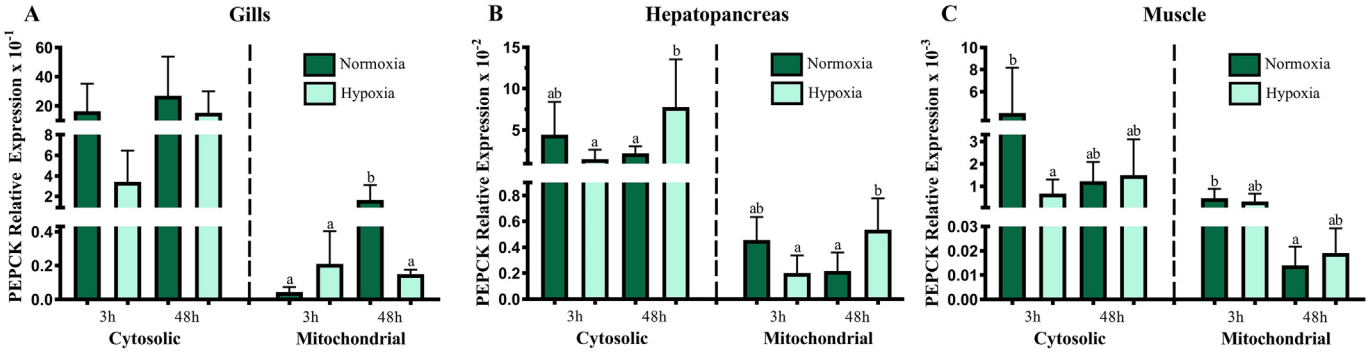

Fig. 5. Effect of hypoxia in PEPCK gene expression in shrimp tissues. A) Gills, B) Hepatopancreas and C) Muscle. The expression levels were normalized to the ribosomal protein L8 transcript as $2^{-\Delta Ct}$. Data are presented as means (n = 4–5) and bars represent the standard deviation. Different letters indicate significant differences ($p < 0.05$; Fisher's LSD Multiple Comparison Test).

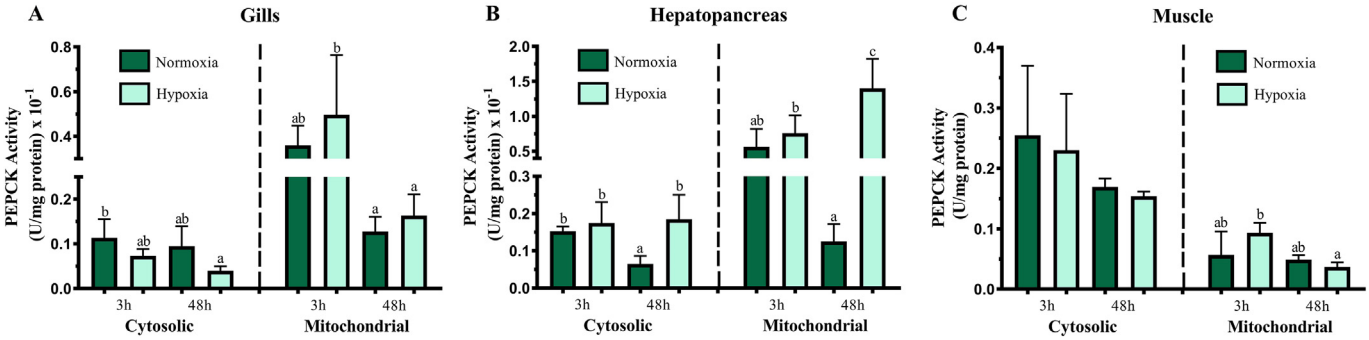

Fig. 6. Effect of hypoxia in PEPCK activity in shrimp tissues. A) Gills, B) Hepatopancreas and C) Muscle. Data are presented as means (n = 3–4). Different letters indicate significant differences ($p < 0.05$; Fisher's LSD Multiple Comparison Test). Bars represent the standard deviation.

Table 1
Cytochrome c oxidase and LDH activities in mitochondrial and cytosolic subcellular fractions.

Tissue	Subcellular fraction	Cytochrome *c* oxidase activity (mU/mg protein)	LDH activity (mU/mg protein)
Hepatopancreas	Mitochondrial	56.03	1.08
	Cytosolic	0	42.95
Gills	Mitochondrial	30.40	0
	Cytosolic	0	29.81
Muscle	Mitochondrial	8.83	126.9
	Cytosolic	0	837.9

hypoxia. Similarly, PEPCK activity of both isoforms was higher in hepatopancreas in hypoxia compared to its corresponding normoxic control. Therefore, hepatopancreas appears to be the main tissue where gluconeogenesis is induced due to hypoxia, probably exporting glucose through hemolymph to other tissues to sustain the glucose demand in the hypoxic condition. Nonetheless, further studies about characterization of the gene and promoter regions for each isoform, along with purification and kinetic studies of both PEPCKs are necessary for deeper knowledge of the mechanisms in which biosynthesis pathways such as gluconeogenesis are regulated during hypoxia in different tissues in crustaceans.

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Table 2
PEPCK amino acid sequences used for phylogenetic analysis.

Scientific name	Taxonomy	Subcellular location	GenBank accession no.
Litopenaeus vannamei	Crustacea	Mitochondrial	CAB85964.1
L. vannamei[a]	Crustacea	Cytosolic	
Nephrophs norvegicus	Crustacea	Cytosolic	CAB65311.1
Neohelice granulata	Crustacea	Cytosolic	AAL78163.1
Daphnia pulex	Crustacea	Mitochondrial	EFX80236.1
D. magna	Crustacea	Mitochondrial	KZS05421.1
Limulus polyphemus	Chelicerata	Cytosolic	XP_013788949.1
Homo sapiens	Vertebrata	Mitochondrial	NP_004554.3
H. sapiens	Vertebrata	Cytosolic	NP_002582.3
Rattus norvegicus	Vertebrata	Mitochondrial	NP_001101847.2
R. norvegicus	Vertebrata	Cytosolic	NP_942075.1
Sus scrofa	Vertebrata	Mitochondrial	XP_020953977.1
S. scrofa	Vertebrata	Cytosolic	NP_001116630.1
Gallus gallus	Vertebrata	Mitochondrial	NP_990801.1
G. gallus	Vertebrata	Cytosolic	NP_990802.1
Scophthalmus maximus	Vertebrata	Mitochondrial	AGJ83084.1
S. maximus	Vertebrata	Cytosolic	AGJ83085.1
Salmo salar	Vertebrata	Mitochondrial	NP_001117114.1
S. salar	Vertebrata	Cytosolic	ACI34142.1
Corynebacterium glutamicum	Actinobacteria	N/A	WP_096458918.1

[a] Obtained from a white shrimp transcriptome report (Ghaffari et al., 2014).

Author contributions

GYP and CARR conceived, designed and analyzed the experiments. CARR, APU, KCR, LLC and EMVS performed and analyzed the experiments. CARR and GYP drafted the MS and all the authors critically revised and approved the final MS to be published.

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