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The shrimp mitochondrial F_oF_1-ATPase inhibitory factor 1 (IF_1)

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Abstract The whiteleg shrimp species *Litopenaeus vannamei* is exposed to cyclic changes of the dissolved oxygen concentration of seawater and must neutralize the adverse effects of hypoxia by using ATP as energy source. In crustaceans, the mitochondrial F_OF_1-ATP synthase is pivotal to the homeostasis of ATP and function prevalently as a F_OF_1-ATPase. Hitherto, it is unknown whether these marine invertebrates are equipped with molecules able to control the F_OF_1-ATPase inhibiting the ATP consumption. In this study, we report two variants of the mitochondrial F_OF_1-ATPase Inhibitory Factor 1 (IF_1) ubiquitously expressed across tissues of the *Litopenaeus vannamei* transcriptome: the IF_1_Lv1 and the IF_1_Lv2. The IF_1_Lv1, with a full-length sequence of 550 bp, encodes a 104 aa long protein and its mRNA amounts are significantly affected by hypoxia and re-oxygenation. The IF_1_Lv2, with a sequence of 654 bp, encodes instead for a protein of 85 aa. Both proteins share a 69 % homology and contain a conserved minimal inhibitory sequence (IATP domain) along with a G-rich region on their N-terminus typical of the invertebrate. In light of this characterization IF_1 is here discussed as an adaptive mechanism evolved by this marine species to inhibit the F_OF_1-ATPase activity and avoid ATP dissipation to thrive in spite of the changes in oxygen tension.

Keywords IF_1, F_oF_1-ATPase · Hypoxia · Inhibitor · Transcriptional regulation · Shrimp

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Introduction

The principally acknowledged function of the mitochondria is the respiration given their ability to efficiently aid energy production via the synthesis of ATP, the final step of which is catalyzed by the F_OF_1-ATP synthase that is able to transform the kinetic energy of the electrochemical H^+ gradient to form the high-energy phosphate molecule ATP (Walker 1994; Boyer 1997). Mitochondria being the main site for ATP synthesis when O_2 is available are, at the same time, the primary consumers of oxygen within all aerobic cells (Solaini and Harris 2005).

Under O_2 conditions which are low for mitochondrial oxidation, the H^+ gradient across the mitochondrial inner membrane collapses, and the F_OF_1-ATPsynthase function is reversed, hydrolyzing ATP (acting as an F_OF_1-ATPase), in order to preserve the mitochondrial membrane potential ($\Delta\Psi_m$) in the attempt to avoid cell death caused by energy collapse (Campanella et al. 2008). Concomitantly, anaerobic glycolysis results in lactate accumulation, which is accompanied by a decrease of the intracellular pH and inhibition of F_OF_1-ATPase by the endogenous inhibitor IF_1 (Grover et al. 2004; Solaini and Harris 2005; Martinez-Cruz et al. 2012a).

The mammalian F_OF_1-ATPsynthase catalytic domain F_1 includes five extensively described subunits α, β, γ, δ, and ε and the inhibitor protein IF_1 (Campanella et al. 2009; Havlickova et al. 2010). Among these nuclear-encoded subunits, the first five subunits have been described in shrimp

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species (Martinez-Cruz et al. 2011; Martinez-Cruz et al. 2015) but there is no indication over the existence of IF_1 like proteins in crustaceans.

The F_OF_1-ATPase inhibitory factor 1 (IF_1), is an endogenous small and basic protein that binds to the catalytic F_1 domain of the F_OF_1-ATPase inhibiting the hydrolysis of ATP without affecting its synthesis (Campanella et al. 2008; Bason et al. 2011). This protein has been found in the mitochondria of yeasts, plants, nematodes and mammals. Bovine IF_1 consists of 84 amino acids (aa) and functions as a homodimer, which undergoes conformational changes under acidic conditions and subsequently binds to the ATPβ subunit of the F_1-ATPase domain inhibiting the hydrolysis of ATP as a mechanism to regulate the energy expenditure within the cell (Cabezon et al. 2003).

Currently, it is well known that hypoxia affects shrimp at physiological and molecular levels by disturbing metabolic pathways, promoting the reallocation of energy resources and reducing shrimp growth (Wei et al. 2008; Wang et al. 2009; Martinez-Cruz et al. 2012b). Various studies have examined the multiple effects of hypoxia on the survival, immune resistance, and genes expression in a number of crustacean species (Eads and Hand 2003; Burgents et al. 2005; Abe et al. 2007). Notably, up to date there are no reports on proteins inhibiting the mitochondrial F_OF_1 ATPsynthase activity in shrimp even though this could provide a more comprehensive understanding of the physiological adaptation of this species to environmental stress associated with hypoxia. With this study we aimed to characterize two cDNA sequences from the whiteleg shrimp (*Litopenaeus vannamei*) encoding for putative endogenous inhibitor IF_1, analyze their phylogenetic relationships and finally evaluate the effect of hypoxia in the expression of these IF_1 genes.

Materials and methods

Total RNA extraction and cDNA synthesis

Adult specimens of the shrimp *L. vannamei* were obtained from aquaculture facilities, and their organs were carefully dissected and maintained at −80 °C. Total RNA was isolated from various tissues including muscle, pleopods, gills, midgut gland and eyestalk, using the TRIzol® reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Total RNA integrity was analyzed in a 1 % agarose denaturing formaldehyde gel (Sambrook and Russell 2001). The total RNA concentration from all samples was determined by using a spectrophotometer (NanoDrop 1000, Wilmington, DE) at 260/280 nm.

Genomic DNA contamination of total RNA samples was eliminated using DNAse I (Roche, USA;1 U/μg RNA) following the manufacturer's instructions. 1 μg of each RNA sample was used for reverse transcription and cDNA was synthesized using the Quantitect Reverse Transcription System (QIAGEN, USA). The reactions were carried out in a total volume of 20 μL containing: 1 μL of RT primer mix, 4 μL of RT buffer 5X and 1 μL of Quantiscript reverse transcriptase. The reaction mixture was incubated 15 min at 42 °C in a shaker at low speed, and it was maintained 3 min at 95 °C. The cDNA was collected by centrifugation, and subsequently DEPC water was added.

IF_1 mRNA sequencing

The synthesized cDNA was used as a template to obtain the IF_1 full-length sequence. The bovine IF_1 sequence (GenBank accession number: NM_175816) was used to found other species transcripts encoding IF_1 proteins on the GenBank database. A multiple alignment analysis was performed including the mRNA sequences of IF_1 from animal species to design oligonucleotides (Table 1).

The PCR amplification of two cDNA fragments encoding shrimp IF_1 transcripts was carried out as follows: 2.5 μL of 10X Top Taq PCR buffer, 2.5 μL of 10X coral dye, 5 μL of Q solution, 0.5 μL of 200 μM dNTPs mix, 0.125 μL of Top Taq DNA polymerase (Qiagen™, USA), 0.5 μL of each 20 μM forward and reverse oligonucleotides, cDNA as template (150 ng RNA equivalents), and water to a total volume of 25 μL. PCR conditions were: 95 °C for 3 min (1 cycle), 95 °C for 45 s, 50 °C for 1 min, 72 °C for 2 min (35 cycles) and a final extension of 72 °C for 10 min. PCR products were analyzed on a 1 % agarose gel and stained with SYBR Safe (Invitrogen, Carlsbad, CA).

In order to sequence the untranslated regions (UTRs) of both IF1 transcripts, the oligonucleotides SMART IV and CDS III/3 from the SMART™ cDNA Library Construction Kit (Clontech, USA), and the First Strand cDNA synthesis kit were used to synthesize cDNA as described in (Jimenez-Gutierrez et al. 2013). PCR products were purified using the Nucleospin Extract II purification kit (Macherey-Nagel, Germany) according to the manufacturer's instructions, and sequenced at the Laboratory of Molecular and Systematic Evolution Facility at the University of Arizona (Tucson, AZ, USA).

Results were analyzed using BLAST algorithms (http://blast.ncbi.nlm.nih.gov/; Altschul et al. 1990), and the multiple alignment analysis and the percentage of identity among sequences were performed using the Clustal W algorithm (Thompson et al. 1994). Protein sequences were translated, and the predicted molecular weight and isoelectric point were obtained using the EXPASY translate tool (http://expasy.org/).

IF_1 mRNA expression in shrimp tissues

In order to determine whether any or both IF_1 transcripts are expressed in different shrimp tissues/organs, the cDNAs from

Table 1 Specific oligonucleotides used for PCR amplification of shrimp IF_1_Lv1, IF_1_Lv2 and the reference gene L8 from the whiteleg shrimp *L. vannamei*

Gene	Oligonucleotide Name	Sequence (5′-3′)	cDNA position (nts)
IF_1	IF1Fw3	GAAAGGGCGAGCCAGGTGAT	59–87
IF_1	IF1Fw4	CTTCAGGAAATTGGAGCAAG	177–196
IF_1	IF1Fw5	CGAGTTCCACAAGGAGCAAG	237–256
IF_1	IF1Fw6	AATTCATTCATCGTATC	−20–0
IF_1*	IF1Fw7	GAAATTTGGGGATATCCATCTG	326–347
IF_1	IF1Rv3	CCTATAGGGGTTTAAAGTCGTAGG	342–319
IF_1	IF1Rv4	CCGAGTGTTTCTACGGTAG	279–261
IF_1	IF1Rv5	GCTCAAGGTGTTCCTCGTTC	256–237
IF_{1*}	IF1Rv6	GTTCAATCATTTCATCGTCAC	542–521
$IF_1$2*	IF2Fw4	GGAAGAAGAGCAGATGAAGA	189–208
$IF_1$2*	IF2Rv3	TGGTTCTTGCTCCATTCTTG	306–288
L8*	L8Fw4	CAGAACGTCATGGATACC	144–162
L8*	L8Rv4	CCGCAGTAGACAAACTG	313–296

*Oligonucleotides used during qRT-PCR

previously isolated RNAs were synthesized as described above, and used as template in the PCR amplification of each IF_1 individual mRNA. The midgut gland (MG), gills (G), pleopods (Pl), eyestalk (ES) and muscle (M) samples were evaluated, and PCR reactions and conditions were identical to those mentioned above. The PCR products were loaded in a 1.5 % agarose gel and each IF_1_Lv1, IF_1_Lv2, and the reference gene L8.

IF_1 protein model

The 3D models of the IF_1_Lv1 protein (residues 34–104) in its monomer and dimer states, were generated using homology modeling procedures and the coordinates of the IF_1 protein from bovine mitochondria (Cabezon et al. 2001; Gordon-Smith et al. 2001; Gledhill et al. 2007; PDB codes: 2V7Q-J, 1HF9 and 1GMJ) as templates. Model coordinates were built using the SWISS-MODEL server (Guex et al. 1999; Schwede et al. 2003) available at http://swissmodel.expasy.org/, and their structural quality was checked using the analysis programs provided by the same server (Anolea/Gromos). Global model quality estimation scores (QMEAN4; Benkert et al. 2011) are: QMEANscore4: 0.718; Z-Score: -0.394, that are within the range of those accepted for homology-based structure models. To optimize geometries, models were energy minimized using the GROMOS 43B1 force field implemented in DeepView (Guex and Peitsch 1997), using 500 steps of steepest descent minimization followed by 500 steps of conjugate-gradient minimization.

Phylogenetic analysis of IF_1

A multiple alignment analysis including the IF_1 amino acid sequences from reported taxa and the shrimp IF_1_Lv1 was performed for comparison using Clustal X v1.81 software (Thompson et al. 1994). A total of 25 sequences were aligned including 20 from invertebrates, 4 from vertebrates and 1 from yeast.

The alignment was used to construct a phylogenetic tree based on the Jones model of substitution (Jones et al. 1992). The tree was constructed using the neighbor-joining algorithm (Saitou and Nei 1987), the bootstrap method was applied for assessing confidence analysis of the clades based on 1000 replicates in MEGA 4 software (Hillis and Bull 1993; Tamura et al. 2007).

Hypoxia bioassay and IF_1_Lv1 mRNA quantification

Adult specimens of the whiteleg shrimp *L. vannamei*, weighing 30 ± 1 g each, were obtained from aquaculture facilities. Shrimp were randomly distributed in six 1000-L tanks filled with 300 L of seawater, and maintained for 8 days under controlled conditions (28 °C, 35 ppt salinity, and 6 mg O_2/L) for acclimatization. Shrimp from each tank (n = 30) were fed twice daily with commercial pelletized food (35 % protein). Daily water exchange averaged 70 % of the total volume.

During the hypoxia assay, after acclimatization, shrimp from the 6 tanks were not fed for 24 h. Three tanks were kept under normoxic conditions (6 mg/L), and the air stones from 3 tanks were removed to induce hypoxia by covering each tank with a plastic sheet and the air supply was gradually replaced by bubbling nitrogen gas into the water (Martinez-Cruz et al. 2012a). As oxygen concentration decreased, it was continuously monitored and carefully controlled during the experiment with a digital submersible oximeter.

Three shrimp were collected from each tank at normoxia (6.0 mg/L) as controls, and at the same time,

3 shrimp were collected from each of the 3 tanks at hypoxia. The first shrimp samples were taken after 6 h at 2.0 mg/L, then oxygen was reduced to 1.5 mg/L, maintained in this condition for 6 h and then shrimp were collected. The 3 experimental tanks at hypoxia were reoxygenated and, once the oxygen concentration in water reached 2.0 mg/L, shrimp were maintained 6 h and collected; finally shrimp were collected after 6 h at 7.0 mg/L. The hypoxia bioassay lasted 24 h, and the abdominal muscle of shrimp was dissected and individually stored in liquid nitrogen until use.

The cDNAs of shrimp samples from the normoxia-hypoxia-reoxygenation assay were synthesized as described above, and used to perform a qRT-PCR analysis in triplicates from each cDNA sample. Relative amounts of IF_1_Lv1 mRNA were evaluated using a real-time PCR analysis and SYBR green I as fluorescent dye. Specific oligonucleotides were designed with Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) for the IF_1_Lv1 cDNA sequence (Table 1). The ribosomal protein L8 was included in the analysis as a reference gene (GenBank accession number: DQ316258).

Real-time PCR reaction mixtures were prepared in a 20 μL final volume as follows: 10 μL of 2× iQ SYBRGreen super mix (Bio-Rad, USA), 0.7 μL of 5 μM each of forward and reverse oligonucleotides and cDNA (equivalent to 50 ng total RNA) in a StepOne™ Real-Time thermal cycler (Applied Biosystems, USA). Amplification was performed as follows: 1 cycle at 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 53 °C (IF_1_Lv1) or 61 °C (L8) for 1 min and 72 °C for 35 s. The specificity of the amplified products was confirmed by a melting curve analysis raising the temperature from 60 to 95 °C with an increase of 0.3 °C every 15 s. Each amplification plate included no-template controls for each pair of oligonucleotides.

PCR efficiency (E) was determined by the formula $E = 10^{(-1/slope)}$ through a dynamic-range evaluation for each gene, and efficiency results were compared between them to be equal. The $2^{-(\Delta\Delta CT)}$ method reported by Livak and Schmittgen (2001) was used to calculate mRNA changes in the expression of *IF_1-Lv1* gene relative to the reference gene *L8* following the formula $2^{-(\Delta\Delta CT)} = ((\text{mean } CT_1^{IF} - \text{mean CTL8})\text{hypoxia} - (\text{mean } CT_1^{IF} - \text{CT L8})\text{normoxia}$.

The statistical analyses were performed using NCSS, 2007 software. Z-test was used to determine the normal distribution of data, and Levene's test confirmed variances homogeneity. One-way ANOVA was performed to test the significance of hypoxia effect. Fisher's multiple comparisons test was used to determine differences between means. Statistical significance differences were considered at $p < 0.05$ (Zar 1984).

Results

IF_1_Lv1 and IF_1_Lv2 cDNA sequences

Two different nucleotide sequences in the transcriptome of the whiteleg shrimp were identified as putative endogenous F_OF_1-ATPase inhibitor factor IF_1. The full-length cDNA sequence of IF_1_Lv1 (GenBank accession number: KF306266; Fig. 1a) consists of 548 bp, a start codon (ATG) at position 1, and a stop codon (TAA) at position 313. The coding region of 315 bp includes on its 5'- end a 63 bp signal peptide (21 residues), and a 3'-UTR of 233 bp that contains a polyadenylation signal TAAATA at position 502, and a polyA tail at position 548.

The IF_1_Lv2 cDNA partial sequence is 654 bp long and includes a 258 bp coding sequence with a start codon at 1, and the stop codon TAG is found at position 256. The signal peptide sequence is identical to the IF_1_Lv1 transcript (63 pb), and the partial 3'- UTR consists of a 396 bp sequence including a polyadenylation signal TAAATA at position 606, no polyA tail was found (GenBank accession number: KR819267; Fig. 1b).

Both IF_1 transcripts share an identity percentage of 78 %, and IF_1_Lv2 includes an insert of 106 bp at position 191 of IF_1_Lv1, this insertion produces two proteins 69 % identical (Figs. 1 and 3).

Both transcripts were found to be expressed in all tested shrimp tissues, but differences were detected mainly at pleopods in which IF_1_Lv2 was observed to be highly and repeatedly more expressed than IF_1_Lv1 (Fig. 2). The nucleotides sequence of IF_1_Lv1 shares higher identities with that of other IF_1 proteins of different animal species than IF_1_Lv2. The IF_1_Lv1 transcript shares 52 % identity with that of the crayfish *Procambarus clarkii* (GenBank accession number: HQ414578), which is the unique IF_1 nucleotides sequence reported for crustaceans. Shrimp IF_1_Lv1 also shares identities with insects such as the bee *Apis florea* (30 %, GenBank accession number: XM_003695878), and the red beetle *Tribolium castaneum* (34 %, GenBank accession number: XM_966457). When compared to vertebrates, shrimp IF_1_Lv1 showed lower identities, 29 % with the bovine IF_1 (GenBank accession number: NM_175816), and with the human IF_1 transcript variant 1 (GenBank accession number: NM016311).

IF_1_Lv1 and IF_1_Lv2 deduced proteins

The deduced shrimp protein IF_1_Lv1 comprises 104 aa residues including a signal peptide (21 residues) and a mature protein of 83 residues (22–104). This protein has a predicted molecular mass of 9.24 kDa and a pI of 8.93. A conserved domain IATP also known as the "minimal inhibitory sequence" was detected in the shrimp sequence at positions

Fig. 1 **a** Shrimp IF_1_Lv1 cDNA and deduced amino acid sequences (GenBank accession number: KF306266). **b** Shrimp IF_1_Lv2 partial cDNA and deduced amino acid sequences. Start and stop codons in an open frame. Double underlined nucleotides indicate the poly A signal, and poly A tail. Underlined residues indicate the signal peptide and black shaded letters show the IATP domain (minimal inhibitory sequence). Residues in an open frame indicate the flanking regions implicated in stabilizing the IF_1_Lv and F_OF_1-ATPase complex

a

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-35  GTGGAGCGGCCTTCAAGAATTCATTCATCGTCATCATGGCTCTGCGACAGACTTCTCTTTTGCGCCTTCGCTCACCCCTG
  1                                        M  A  L  R  Q  T  S  L  L  R  L  R  S  P  L

 46  GCTGTGTGTGTGCGAAGCATGGGAAAGGGCGAGCCAGGTGATGGGGCTGGCCGTGGTGGAGGAAGTGGTGGCACAATCAGG
 16  A  V  C  V  R  S  M  G  K  G  E  P  G  D  G  A  G  R  G  G  G  S  G  G  T  I  R

127  GATGCTGGTGGTGCCTTCGGCAAGCGTGAGGCAGCACAAGAAGAACAGTACTTCAGGAAATTGGAGCAAGAACAGTTGAAA
 43  D  A  G  G  A  F  G  K  R  E  A  A  Q  E  E  Q  Y  F  R  K  L  E  Q  E  Q  L  K

208  AAGTTAAAAAAGGGCCTTCAAAAGGAAATCGAGTTCCACAAGGAGCAAGTGGAGGCTCACAAAGATGCCATCAAAAGGCAC
 70  K  L  K  K  G  L  Q  K  E  I  E  F  H  K  E  Q  V  E  A  H  K  D  A  I  K  R  H

289  GAGGAACGTCTGAATGAGCTGAAGTAAAGAGGATGCTGAAATTTGGGGATATCCATCTGTATTGCAACTTGCATCATTCTG
 97  E  E  R  L  N  E  L  K  *

370  GGAAGAAGTAATTACAAGGAGAAAAAAATGCATCTATTTCAGTTTTTGTTTTTTACTGATTCAAAAAAGATGCATTATTTC
451  ATGGTAAAAAAAATATATATACATATATATATATATATAATTGGTATGATGTAAATATAATGGCTTTGATTATCAAGTTAG

532  TAAAGTAGCAGTGCTTTAAAAAAAAAAAAAAAAAAAAAAA
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b

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-237  AAGCAACCGGAAATAGTCATCATATTTCTACATCTATTTGAATTCACTATCCCCCCTCAAAAAAAGTCAGATATGGATAGT
-156  CAAAACTTTTACCCGCTTTCTAAATGATCCAAGGTATTATCACTACAAAACCAAAAAAGGAAGGTAGGCTTCGAACATTAC
 -75  CCTTCCCCCACTAGGTTGGCAATCCTGAGATAAAGAAGGCGTGGAGCGGCCTTCAAGAATTCATTCATCGTCATCATGGCT
   1                                                                          M  A

   7  CTGCGACAGACTTCTCTTTTGCGCCTTCGCTCACCCCTGGCTGTGTGTGTGCGAAGCATGGGAAAGGGCGAGCCAGGTGAT
   3  L  R  Q  T  S  L  L  R  L  R  S  P  L  A  V  C  V  R  S  M  G  K  G  E  P  G  D

  88  GGGGCTGGCCGTGGTGGAGGAAGTGGTGGCACAATCAGGGATGCTGGTGGTGCCTTCGGCAAGCGTGAGGCAGCACAAGAA
  30  G  A  G  R  G  G  G  S  G  G  T  I  R  D  A  G  G  A  F  G  K  R  E  A  A  Q  E

 169  GAACAGTACTTCAGGAAATTGGAAGAAGAGCAGATGAAGAAGCTGAAGGATTTACAGTCCAGTCAAGGGAAGGAGAAGAAA
  57  E  Q  Y  F  R  K  L  E  E  E  Q  M  K  K  L  K  D  L  Q  S  S  Q  G  K  E  K  K

 250  CCAAAATAGTGATAGCTTCAAGATTGAATGTTTTCAATCAAGAATGGAGCAAGAACAGTTGAAAAAGTTAAAAAAGGGCCT
  84  P  K  *

 331  TCAAAAGGAAATCGAGTTCCACAGGGAGCAAGTGGAGGCTCACAAAGATGCCATCAAAAGGCACGAGGAACGTCTGAATGA
 412  GCTGAAGTAAAGAGGATGCTGAAATTTGGGGATATCCATCTGTATTGCAACTTGCATCATTCTGGGAAGAAGTAATTACAA
 493  GGAGAAAAAAAATGCATCTATTTCAGTTTTTGTTTTTTACTGATTCAAAAAAGATGCATTATTTCATGGTAAAAAAAATATA
 574  TATACATATATATATATATAATTGGTATGATGTAAATATAATGGCTTTGATTATCAAGTTAGTAAAGTAGCAGTGCCTAAC
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40 to 73 (Figs. 1a and 3). The shrimp IF_1_Lv1 protein shares a higher percentage of identity with other marine invertebrates as the crayfish *P. clarkii* deduced protein (70 %), the IF_1_Lv2 (69 %), and the purple sea urchin *Strongylocentrotus purpuratus* (55 %) (Fig. 3).

Lower identities were detected when IF_1_Lv1 was compared to insects (*Tribolium castaneum* 50 %, and *Apis florea* 45 %) and vertebrates as the human isoform-1 (38 %), and bovine (36 %).

The IF_1_Lv2 deduced protein is shorter than IF_1_Lv1 (Fig. 3). However, it includes an identical signal peptide and the IATP domain, which characterizes these inhibitory proteins. Differences between both deduced proteins are mainly observed in the C-terminus, and the identity percentages of

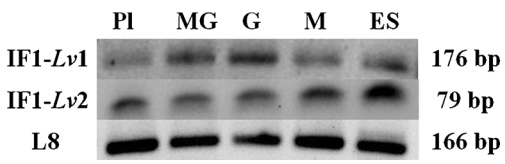

Fig. 2 IF_1-Lv transcripts expressed on various tissues of the white shrimp *Litopenaeus vannamei*. (Pl) pleopods, (MG) midgut gland, (G) gills, (M) muscle, and (ES) eye stalk

IF_1_Lv2 with those of other species are lower when compared with IF_1_Lv1 (Fig. 3).

Since shrimp IF_1 predicted proteins IF_1_Lv1 and IF_1_Lv2 seem to contain many of the previous reported elements of a functional inhibitory protein, their potential ability to inhibit shrimp F_OF_1 ATPase was determined through a comparative analysis with the bovine model, both in their monomeric and dimeric forms (Figs 4a, b, c).

In agreement with the bovine IF_1 model (Cabezon et al. 2000a), both shrimp proteins share the conserved elements that allow formation of the dimer and confer pH-sensitivity to the protein (Fig. 4c). Some glutamic acid residues, which are located in highly conserved regions in IF_1 proteins of various species, including bovine (E25 and E30), and both shrimp proteins (E52 and E56; Fig. 3), may confer the ability of pH sensing (Ando and Ichikawa 2008). Many of these residues may also participate on the conformational changes of the protein under oxidative stress conditions, as hypoxia, when the mitochondrial matrix achieves mildly acidic pH (Cabezon et al. 2003).

Shrimp IF_1 proteins also share some features resembling the bovine N-terminal inhibitory sequence 1–45 (27–71

Fig. 3 IF_1 Protein multiple alignment. *IF_1_Lv1 and IF_1_Lv2*: *Litopenaeus vannamei*. *Bterrestris*: *Bombus terrestris*, *Aflorea*: *Apis florea*, *Tcastaneum*: *Tribolium castaneum*, *Pclarkii*: *Procambarus clarkii*, *Spurpuratus*: *Strongylocentrotus purpuratus*, *Celegans*: *Caenorhabditis elegans*, *Btaurus*: *Bos taurus*, *Hsapiens1*: *Homo sapiens* isoform 1, *Rnorvegicus*: *Rattus norvegicus*, *Mmusculus*: *Mus musculus*. Framed region indicates the IATP domain, black-shaded residues indicate essential sites for inhibitory capacity, and gray shaded residues in indicate the interaction sites among IF_1 monomers and, in the human and bovine sequences, the three histidine residues conferring pH-sensitivity to IF_1

shrimp; Figs. 4a, b) that may inhibit the mitochondrial ATPase on its monomeric form in agreement with the recent findings of Gledhill et al. (2007); Bason et al. (2011), and Bason et al. (2014). These include: 1) A high content of charged residues, 19 in bovine and 22 in shrimp sequences; 2) scarcity of bulky residues as L, F, V, Y, I and W, which in the bovine sequence are 8 and 10 in the shrimp sequence, and 3) a fold index analysis that indicates a predicted disorder score of −0.16 for the bovine sequence, and −0.19 for the shrimp sequence.

Phylogenetic relationships of IF_1_Lv1

The phylogenetic analysis of crustacean IF_1_Lv1 was performed using a total of 25 protein sequences of several other

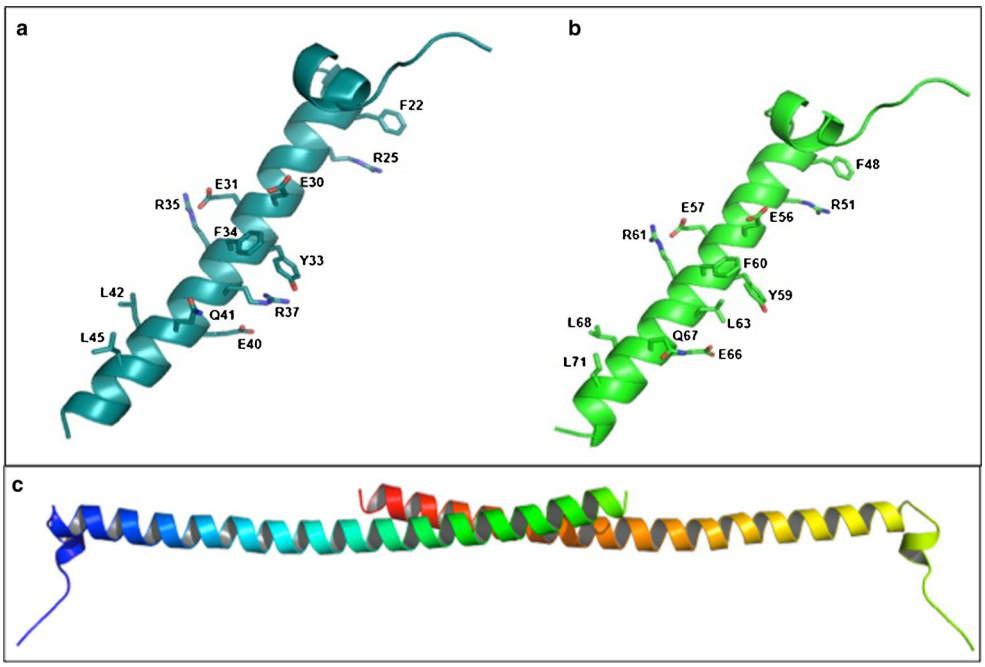

Fig. 4 **a** The molecular model of the bovine IF_1 taken from Gledhill et al. (2007). Copyright (2007) National Academy of Sciences, U.S.A. **b** The predicted molecular model IF_1_Lv1 monomer from *L. vannamei*. **c** The predicted structure of the IF_1 shrimp dimer. Atoms are presented in a ribbon side-view diagram, and the theoretical interactions between them are showed in the COOH-overlapping ends

taxa. Analysis included: two crustaceans, the whiteleg shrimp *L. vannamei* and the deduced protein of the red swamp crayfish *Procambarus clarkii.* Figure 5 shows the resulting yeast-rooted tree which is comprised by 3 main clades: Clade A, defined by the yeast IF_1 protein sequence; clade B, which includes the sequences of vertebrates; and finally the invertebrates sequences from Arthropoda and Cnidaria are grouped into clade C. The IF_1 sequences from insects form a well-resolved sub-clade, and both crustacean proteins are grouped together, close to the sequences of Cnidarians (all of them marine invertebrates) and clearly separated from vertebrate proteins.

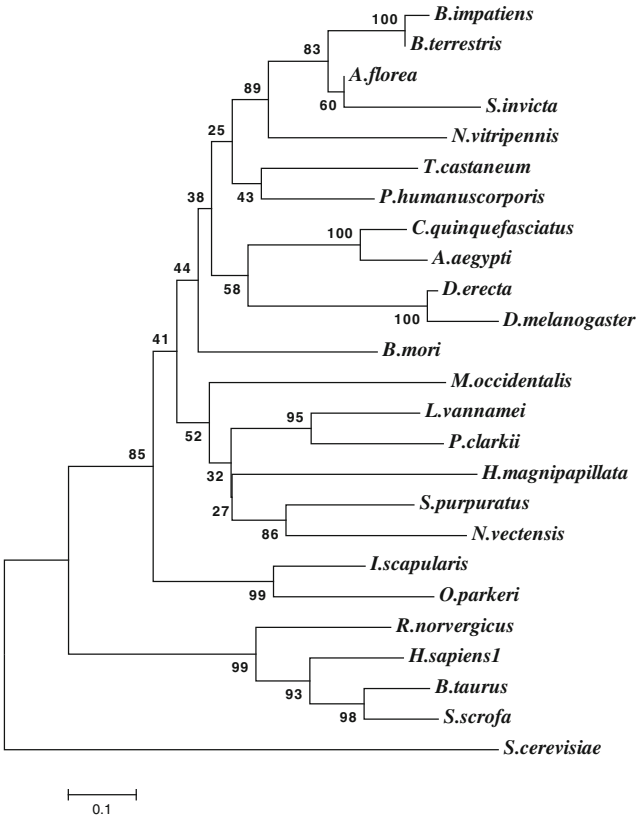

Fig. 5 Shrimp IF_1 phylogenetic tree topology using neighbor-joining method with pairwise deletion from amino acid sequences. Numbers above/below the nodes indicate nonparametric bootstrap values (1000 replicates). (*Culex quinquefasciatus* GenBank accession number: XP0018421173; *Aedes aegypti* GenBank accession number: XP001648854; *Drosophila erecta* GenBank accession number: XP001976392; *D. melanogaster* GenBank accession number: AAL48937; *Tribolium castaneum* GenBank accession number: XP971550; *Pediculus humanus corporis* GenBank accession number: XP002433204; *Nasonia vitripennis* GenBank accession number: XP001602785; *Bombus impatiens* GenBank accession number: XP003487447; *Bombus terrestris* GenBank accession number: XP003401492; *Apis florea* GenBank accession number: XP003695926; *Solenopsis invicta* GenBank accession number: EFZ13965; *Bombyx mori* GenBank accession number: NP001091821; *Metaseiulus occidentalis* GenBank accession number: XP 003745769; *Litopenaeus vannamei* GenBank accession number: KF306266; *Procambarus clarkii* GenBank accession number: AEB54654; *Hydra magnipapillata* GenBank accession number: XP002164073; *Strogylocentrotus purpuratus* GenBank accession number: XP 785704; *Nematostella vectensis* GenBank accession number: XP001625547; *Ixodes scapularis* GenBank accession number: XP002399280; *Ornithodoros parkeri* GenBank accession number: ABR23451; *Rattus norvegicus* GenBank accession number: AAA41360; *Homo sapiens* transcript-1 GenBank accession number: NP057395; *Bos taurus* GenBank accession number: NP787010; *Sus scrofa* GenBank accession number: NP001090955; *Saccharomyces cerevisiae* GenBank accession number: NP0101100)

According to the cross-species identity percentages and phylogenies, these results indicate that the IF_1 protein from invertebrates shares more than the conserved regions previously described, including a long G-rich sequence in the N-terminus side of the IATP domain that distinguishes invertebrates as insects and crustaceans from the mammals sequences (Fig. 5).

Shrimp IF_1_Lv1 mRNA levels

The shrimp IF_1_Lv1 steady state mRNA levels at normoxia, hypoxia and re-oxygenation were evaluated by real-time PCR (qRT-PCR). Each of the melting curves generated a single PCR product for each gene in all samples, and PCR efficiencies of IF_1 and L8 were 104 and 106 %, respectively.

Figure 5 shows the statistical differences detected among different conditions relative to the L8 ribosomal protein ($p < 0.05$). The IF_1 mRNA amounts found at normoxia were 12, 97 and 94 % higher than those observed during hypoxia at 2.0, 1.5 and 2.0 mg/L, and 46.6 % higher than levels at re-oxygenation.

After 12 h of hypoxia (at 1.5 mg/L) the IF_1 mRNA transcript expression decreased abruptly, reaching a minimum until the system was re-oxygenated, when the amount of this transcript rose to a maximum (88.7 %). However, it is important to note that the initial amount of IF_1 mRNA was not reached again once the oxygen levels returned to the original conditions (Fig. 6).

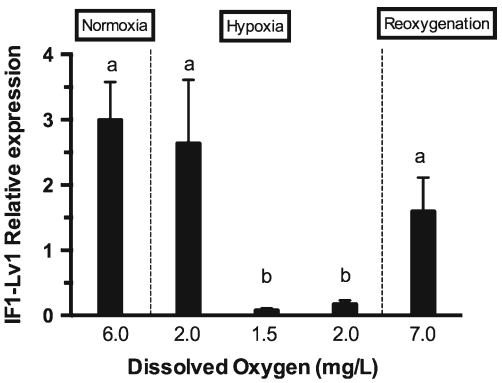

Fig. 6 IF_1-Lv1 mRNA changes relative to the reference gene L8 in the muscle of shrimp during normoxia, hypoxia and re-oxygenation. Data represent mean ± standard error (representative experiment $n = 12$). Different letters indicate statistical significant differences ($p < 0.05$)

Discussion

In eukaryotes, the mitochondrial F_OF_1-ATPsynthase may function as an F_OF_1-ATPase in hypoxia/ischemia events hydrolyzing ATP and translocating H^+ from the matrix to the intermembrane space to restore the lost mitochondrial membrane potential ($\Delta\Psi_m$). In this reverted function, the protein IF_1 plays an essential role inhibiting the F_OF_1-ATPase and preserving intracellular ATP levels (for reviews see Campanella et al. 2009; Faccenda and Campanella 2012).

In the animal kingdom, the model of the bovine IF_1 is the most studied and therefore used by us to establish comparisons with the envisaged shrimp IF_1. Here we have indeed identified two shrimp transcripts (IF_1_Lv1 and IF_1_Lv2) in *L. vannamei* both expressed in all tested tissues/organs, which resemble sequences mimicking the inhibitory domains to interact with the FoF_1-ATPase.

The IF_1_Lv2 encodes for a protein shorter than the IF_1_Lv1 with which still shares the main basic elements: i) mitochondrial import sequence, and ii) inhibitory sequence for the ATPase; however, its short C-terminal region (residues 70–85) lacks identity with the bovine sequence, and probably, the ability of forming the antiparallel α-helical coiled-coil of the IF_1 homodimer (Gordon-Smith et al. 2001; Cabezon et al. 2000b).

The existence of pseudogenes or transcripts encoding for IF_1 proteins has been detected in species such as rats (Samuel et al. 1995) and the nematode *Caenorhabditis elegans* (Ichikawa et al. 2006) implying that IF_1-like proteins localized outside mitochondria may equally regulate cellular FoF_1 ATPases (Ichikawa et al. 2006). Future studies will confirm whether both these shrimp transcripts are differentially expressed in a condition-specific manner, their gene origin as mRNA variants of the same gene, or different genes encoding each transcript form and their ability to inhibit the mitochondrial ATPase.

Although the in vitro inhibitory ability of both shrimp proteins remains to be confirmed (Chimeo et al. *in preparation*), the inclusion of the IATP domain (contained in the six residues F48, R51, E52, A53, E56 and Y59) which is involved in the inhibition of FoF_1-ATPase activity of the bovine model (Van Raaij et al. 1996; Gordon-Smith et al. 2001; Ichikawa and Ogura 2003) represents a strong indication of this.

IF_1 proteins may form oligomers and dimers through pH-sensitive conformational changes given their specific pH-sensitive residues (Cabezon et al. 2001) and notably both the shrimp IF_1 proteins lack the three residues at positions H48, H49 and H55 proposed to confer such activity at high pH (Cabezon et al. 2000a). Notably, also the yeast IF_1 lacks these residues whilst the yeast E21 -found in both shrimp sequences at E52- is also known to be important for the pH- sensitivity (Ichikawa et al. 2001) suggesting this to be the mechanism via which the change in conformation may occur in this species' proteins. In 2003, Ichikawa and Ogura suggested that the inhibitory mechanism is similar for the IF_1 proteins in the various species, but the mammalian one may require unique residues not necessarily retrievable in precedent steps of the evolution. This would also account for a plausible evolutionary adaptation in the mammals with the need just of a single IF_1 gene whilst in crustaceans as well as zebrafish (Shah et al. 2012) two are instead needed.

Even though the suggested inhibitory mechanism in the bovine species is mediated by the dimeric IF_1, where the C-terminal regions of the protein interact with each other and are involved in the FoF_1-ATPase dimer stabilization (Cabezon et al. 2000a, 2000b), recent published data suggest that IF_1 monomers may suffice to inhibit the ATPase (Gledhill et al. 2007). Furthermore, through a deep structural analysis, Bason et al. (2014) suggested that the initially disordered structure of IF_1 N-terminal region passes through various states interacting with the $\alpha_E\beta_E$, $\alpha_{TP}\beta_{TP}$, and $\alpha_{DP}\beta_{DP}$ catalytic interfaces of ATPase until it reaches its final inhibitory extended form, which includes several interaction sites with subunits ATPα, ATPβ, and ATPγ from the F_1 domain of ATPase (Bason et al. 2014).

Accordingly, the IF_1 ability to inhibit ATPase activity may not only depend on the pH-sensibility to complete a conformational change, but also in the inhibitor structure to bind the F_1 catalytic domains. The results obtained in the current study show that the shrimp IF_1_Lv1 and IF_1_Lv2 sequences include the bovine residues 31–49 (shrimp 57–75), that are the conserved sites according to Bason et al. (2014), which may therefore establish polar interactions in the $\alpha_E\beta_E$ catalytic site mediating enzyme inhibition. These are sites at shrimp residues IF_1-E57 with R455 β_E (hydrogen bond), IF_1-Y59 with K448 β_E, and IF_1-Q67 with D497 β_E (also reported by Gledhill et al. 2007). Additional interacting hydrophobic residues Y33, F34, L42 and L45 of the bovine protein were also found in the shrimp IF_1 as Y59, F60, L68 and L71 binding the β_{TP}, and a salt bridge between IF_1-R51 and ATPγ E258 (Bason et al. 2014). Furthermore, some other residues reported in the bovine model interaction IF_1 with the F_1-ATPase subunits ATPα, ATPβ and ATPγ (Cabezon et al. 2003; Ichikawa et al. 2005; Gledhill et al. 2007; Bason et al. 2011; Bason et al. 2014) were also found in the previously reported shrimp F_1-ATPase subunits including: in shrimp ATPβ (GenBank accession number: GQ848644.1; Martinez-Cruz et al. 2011) residues β_{TP}D433 (bovine D386), β_{DP}M440 (bovine M393), and β_EE501 (bovine E454); in shrimp ATPγ (GenBank accession number: HM036579; Martinez-Cruz et al. 2015) residues I36 (bovine I16), and N35 (bovine N15); in shrimp ATPα (GenBank accession number: GQ848643; Martinez-Cruz et al. 2015) residues E393 (bovine E353), E395 (bovine E355), Q436 (bovine Q396), E439 (bovine E399), and F443 (bovine F403).

The presence of the above-mentioned conserved residues suggests the ability of the N-terminal region of the shrimp IF_1 protein to inhibit the F_1-ATPase monomer even though this remains to be confirmed.

Among the differences detected between the IF_1 analyzed sequences, is the glycine-rich region (8 residues) close to the N-terminus of the shrimp IATP domain to emerge (Figs. 3 and 4b), since it is observed in marine invertebrates as the sea urchin and crustaceans, likewise in the insects and the nematode *C. elegans* (Ichikawa et al. 2006). This conserved G-rich region has been reported in the mammalian model as the flanking sequence of the IATP domain in stabilizing the complex IF_1-F_1 (Cabezon et al. 2000a). Although this region is dispensable for the inhibitory activity of IF_1 according to Van Raaij et al. (1996) findings, we suggest that it is the one capable to confer higher flexibility to the IF_1 protein and enhance the interaction within the F_1-ATPase domain in these species.

Although recent findings imply that the mitochondrial proteome results from species-specific subunits addition to the ATP synthase core (Grey 2015), it has not been yet established whether these endogenous nucleus-encoded ATPase inhibitors appear along the evolution of the animal kingdom. In the same way, previous studies have also indicated that these are old proteins sharing common ancestors with yeast paralogous proteins such as the 9 and 15 K stabilizing factors: both mitochondrial proteins with no inhibitory activity but identical sequences to the IF_1 (Matsubara et al. 1983).

The phylogenetic information obtained in this study reveals a close relation among the IF_1 protein sequences of Crustaceans and Insects, which is not surprising since both subphyla are known to be sister groups sharing a common ancestor, the Tetraconata (Hexapoda + Crustacea) (Andrew 2011).

According to the identity percentages shared among species and to the phylogenetic analysis, our findings indicate that in spite of the large taxonomic distances between vertebrates, invertebrates and yeast, IF_1 is a protein that has been conserved along evolution: a finding which is per se indicative of a central role(s) in the regulation of mitochondrial and cellular function.

As previously shown by Campanella et al. (2008), IF_1 expression level varies among rodent tissues, and this may determine cell death in response to oxygen deprivation. All tested shrimp tissues express both IF_1 transcripts, and the differences observed may also in this animal species account for alternative need for energy requirement and condition.

Here we found that hypoxia condition promoted significant changes in the mRNA levels of IF_1 in the shrimp muscle including a sharp decrease at 1.5 and 2.0 mg/L, and a 8.8-fold increase after reoxygenation at 7.0 mg/L. These results suggest a transcriptional response to the lack of oxygen, which is in agreement with previous reports that have found changes in the mRNA levels of mitochondrial and nuclear genes by the effect of hypoxia on crustacean species as the grass shrimp *Palaemonetes pugio* (Brown-Peterson et al. 2008), and the whiteleg shrimp *L. vannamei* (Muhlia-Almazan et al. 2008; Jimenez-Gutierrez et al. 2013).

On the other hand, reduced transcript levels of IF_1 suggest that the mRNAs may be degraded during hypoxia, probably as a consequence of the pH reduction (Garrido et al. 2006), or by the action of cytoplasmic nucleases (Guhaniyogi and Brewer 2001). After hypoxia, when the system was re-oxygenated, the increased amount of IF_1_Lv1 mRNA suggests that the transcriptional/translational mitochondrial machinery re-starts functioning as the oxygen level increases. At this point pH increases, the IF_1 protein is released from the F_1-ATPase and the ATPase activity gradually decreases as previously detected in shrimp mitochondria by Martinez-Cruz et al. (2012a), and the synthesis of ATP is expected to reactivate (Solaini and Harris 2005).

Martinez-Cruz et al. (2012a) demonstrated that the white shrimp mitochondrial ATPase activity increased at low oxygen level (2.0 mg/L), reduces at prolonged hypoxia (13 h) as well as at re-oxygenation (7.0 mg/L) speculating on the existence in crustaceans of an ATPase inhibitor element.

Our results confirm this by describing the presence of IF_1-like transcripts that encode for a protein regulated at the transcriptional level (Fig. 5) and possibly at translational or post-translational level (Campanella et al. 2008) to control ATP consumption and preserve energy to fulfill demands until oxygen levels are restored (Fujikawa et al. 2012). The characterization of an IF_1 dependent protective mechanism of the vital bio-energy equilibrium in *Litopenaeus vannamei* may be of tangible value to design comparative based studies aimed at obtaining protective approaches in low oxygen conditions for mammals.

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