Centro de Investigación en Alimentación y Desarrollo, A.C.

"LA PORCIÓN CATALÍTICA F_1 DE LA ATP-SINTASA DEL CAMARÓN BLANCO (*Litopenaeus vannamei*): TRANSCRITOS, PROTEÍNAS Y RESPUESTA FRENTE A LA HIPOXIA"

POR:

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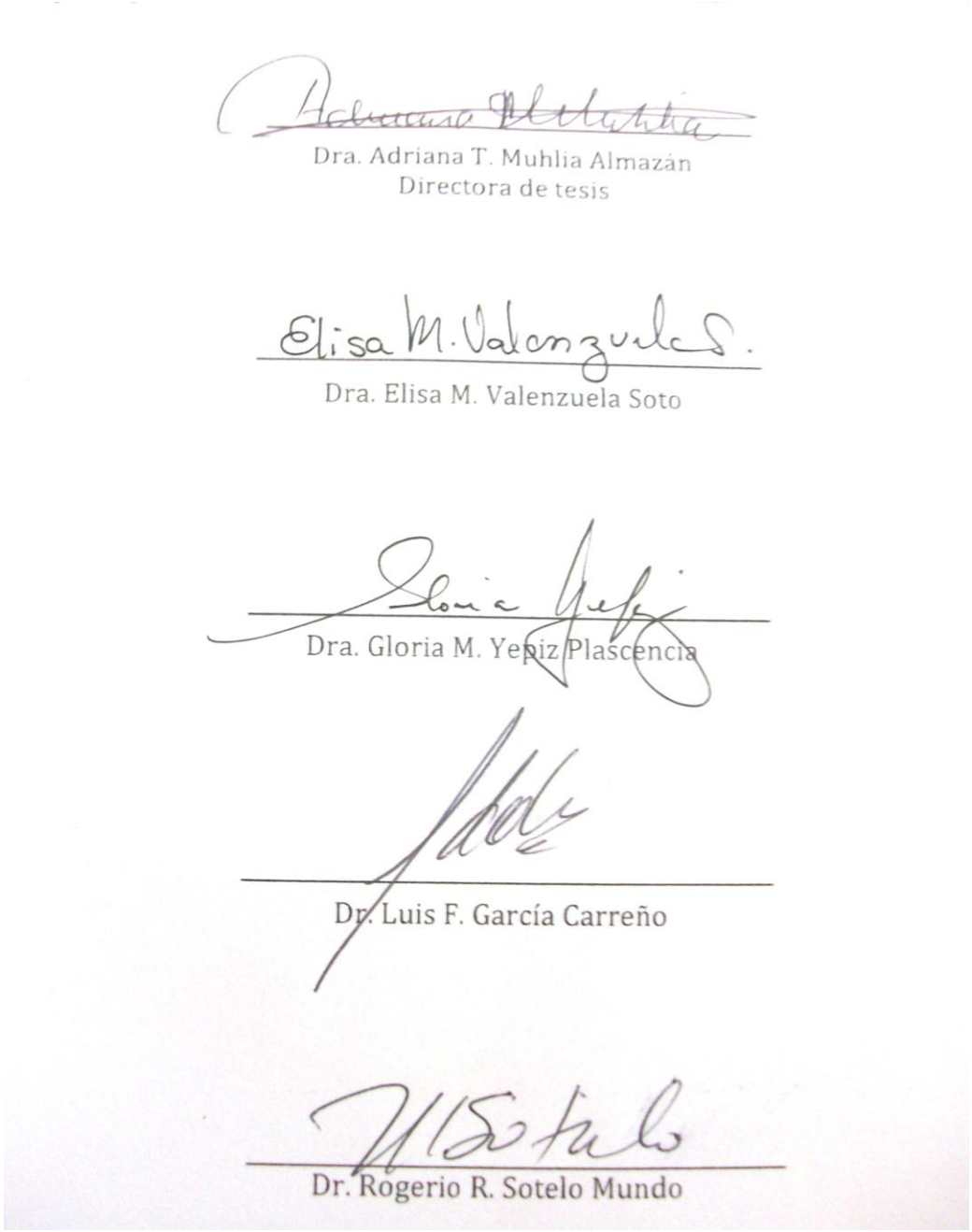
Los miembros del comité designado para revisar la tesis de Oliviert Martínez Cruz, la han encontrado satisfactoria y recomiendad que sea aceptada como requisito para obtener el grado de Doctor en Ciencias.



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ÍNDICE

LISTA DE FIGURAS

LISTA DE TABLAS

RESUMEN

La F_0F_1-ATP sintasa (EC 3.6.3.14) acoplada al gradiente electroquímico de protones generado en la cadena de transporte de electrones dentro de la mitocondria sintetiza el ATP. Además, esta enzima también hidroliza el ATP de acuerdo a los requerimientos energéticos de la célula. A la fecha los estudios realizados sobre la ATP-sintasa de los crustáceos son muy escasos. Así, en este trabajo se presenta el estudio de los cambios en la expresión génica y las proteínas de las subunidades de la F_0F_1-ATP sintasa del camarón blanco del Pacífico *Litopenaeus vannamei*. Adicionalmente se evaluó la actividad de la ATP sintasa en el camarón bajo condiciones de normoxia e hipoxia y se exploró mediante modelación molecular la posible estructura de la porción catalítica F_1 de la ATP-sintasa. En esta investigación, se caracterizaron las secuencias completas del ADNc de cada una de las 5 subunidades de la porción F_1 (*atpα*, *atpβ*, *atpγ*, *atpδ* y *atpε*). Las secuencias muestran regiones altamente conservadas y alta identidad con respecto a otras secuencias de invertebrados, sin embargo, se detectaron algunas substituciones en aminoácidos específicos. El modelo molecular teórico incluye las cinco subunidades y sugiere características funcionales específicas. También se realizó un bioensayo donde los organsmos fueron sometidos a diferentes concentraciones de oxígeno disuelto, confirmándose el efecto de la hipoxia al detectarse un incremento en la concentración de lactato en el plasma del camarón. La expresión génica no mostró diferencias significativas por efecto de la hipoxia, sugiriendo que la respuesta de los organismos no se encuentra regulada de manera transcripcional. Por primera vez se aislaron las mitocondrias del músculo del camarón con la finalidad de separar e identificar el complejo ATP-sintasa por técnicas inmunoquímicas. Asimismo, se evaluó la concentración de la subunidad ATPβ en el extracto mitocondrial, sin detectarse diferencias significativas entre tratamientos. Por otro lado, al evaluar la actividad ATPasa se detectó un incremento de la actividad por efecto de la hipoxia, lo que sugiere que la enzima cambia de sintetizar a hidrolizar ATP con la finalidad de preservar el potencial de membrana mitocondrial para mantener la homeostasis celular. Asimismo, la concentración de ATP en el músculo de camarón permaneció sin cambios durante la hipoxia, lo que puede sugerir que el organismo utiliza las reservas energéticas (fosfágenos como la arginina fosfato) para amortiguar la concentración de ATP permitiendo al organismo mantener las funciones celulares.

I. INTRODUCCIÓN

La hipoxia definida como un fenómeno en el cual la concentración de oxigeno disuelto en el agua es menor a 2.8 mg O_2/L, se observa de manera natural en diferentes ambientes acuáticos. Sin embargo, la intensificación de la acuacultura, la aplicación de fertilizantes, la deforestación y la descarga de desechos domésticos han potencializado este fenómeno en las últimas décadas en términos de frecuencia, severidad y número de áreas afectadas (Wu, 2002). Así, los organismos que habitan ambientes marinos han desarrollado mecanismos de respuesta altamente especializados para enfrentar los cambios en las condiciones ambientales. Debido a lo anterior, en la última década los estudios enfocados a evaluar y entender los efectos que causa la hipoxia en especies de invertebrados marinos como son los crustáceos y los bivalvos han incrementado de manera significativa (Gäde, 1983).

Para los crustáceos como para todos los organismos acuáticos, la disponibilidad de oxígeno disuelto en el agua es determinante para que se lleve a cabo la función vitalde respiración. Cada célula eucariota cuenta con su maquinaria propia, la mitocondria, para llevar a cabo este proceso a través de la cadena respiratoria. En la mitocondria ocurre una serie de procesos metabólicos precisos, coordinados y vitales en las células para la producción de hasta el 95% del ATP a través del transporte de electrones y la fosforilación oxidativa (Goffart y Wiesner, 2003).

La ATP-sintasa es el complejoenzimático que sintetiza o hidroliza el ATP. El complejo funcional se compone por dos porciones: la F_0 (porción transmembranal) y F_1 (porción catalítica). Durante el transporte de electrones, se bombean protones desde la matriz de la mitocondria hacia el espacio intermembranal, creando un potencial electroquímico. Los protones entran a favor del gradiente, pasan a través del complejo enzimático para volver a la matriz mitocondrial y la energía liberada es usada para sintetizar ATP. De esta manera sólo se sintetiza ATP cuando existe

este potencial electroquímico (síntesis acoplada al transporte de electrones). Por otro lado, la ATP-sintasa tiene la capacidad de hidrolizar ATP para mantener el potencial electroquímico en el espacio intermembranal y así mantener la homeostasis celular (Boyer, 2002).

Cuando existe suficiente oxígeno disponible para la fosforilación oxidativa, la F_1F_0-ATPsintasa mitocondrial actúa como el sitio de producción de ATP en las células animales y vegetales. Por el contrario, bajo condiciones de anoxia o hipoxia, la ATP-sintasa bombea protones desde la matriz mitocondrial, en un intento de mantener el potencial de la membrana mitocondrial (indispensable para el flujo de iones y la prevención de la lisis del organelo). Así, la ATP-sintasa cambia su función a ATPasa (Di Lisa et al., 1998). Lo estudios de la enzima ATP-sintasa se encuentran enfocados en modelos como bovinos, humanos, bacterias y levaduras, sin embargo en aquellos modelos animales menos estudiados pero potencialmente explotables los avances en el conocimiento son limitados, como el camarón, objeto de este estudio.

A pesar de que en la ATP-sintasa se han descrito elementos estructurales muy conservados, estudios recientes en especies como las levaduras han mostrado la existencia de nuevas proteínas con funciones aún no descritas, formando parte de los subcomplejos de manera especie-específica (Vazquez-Acevedo et al., 2006). A su vez, los estudios a nivel de genes, como de sus respectivas proteínas es escasa en modelos de invertebrados marinos y no se cuenta con información que permita relacionar la actividad de la enzima con los cambios en la concentración de ATP en los tejidos.

El objetivo principal de esta tesis doctoral fue caracterizar las cinco subunidades principales (α, β, γ, δ y ε) que conforman la porción catalítica F_1 del complejo ATP-sintasa del camarón blanco *Litopenaeus vannamei* y tratar de establecer una relación entre la cantidad de mRNA de cada una de las subunidades,

la actividad de la porción catalítica de la enzima y la concentración de ATP en el músculo, para evaluar la función de la enzima como una proteína de respuesta a las bajas concentraciones de oxígeno disuelto en el agua o hipoxia.

II. ANTECEDENTES

2.1 La Hipoxia en el Medio Acuático y su Efecto en los Crustáceos

Existe discrepancia entre diferentes autores cuando se trata de establecer las concentraciones de oxígeno disuelto en agua que definen al término hipoxia (Mugnier y Soyez, 2005; Zhang et al., 2006). En términos generales la hipoxia se presenta cuando el consumo de oxígeno excede el suministro del mismo (Diaz, 2001; Li y Brouwer, 2007). Así, la concentración de oxígeno disuelto en el agua en la cual se ven afectadas las funciones de los organismos puede variar entre especies, pero generalmente los efectos empiezan a aparecer cuando la concentración de oxígeno disminuye por debajo de los 2 mg/L (Diaz, 2001; Vaquer-Sunyer y Duarte, 2008). De acuerdo con Herried (1980), los mecanismos en respuesta a la hipoxia dependen de las condiciones ambientales y el estado fisiológico de los animales. Algunos de los factores que determinan los cambios en el metabolismo de los animales expuestos a la hipoxia son la temperatura, la salinidad, el pH, los contaminantes, la actividad y tamaño del organismo, entre otros (Zhang et al., 2006).

Existen publicaciones que describen el efecto de la hipoxia en el crecimiento, sobrevivencia, alimentación, muda, comportamiento, capacidad respiratoria de los camarones peneidos (Le Moullac et al., 1998; McGraw et al., 2001; McMahon, 2001; Wu et al., 2002; Perez-Rostro et al., 2004; Mugnier y Soyez, 2005). Por ejemplo se ha reportado que durante periodos cortos o largos de hipoxia en el camarón blanco *Litopenaeus vannamei*, se induce hiperglicemia debido a la movilización de la glucosa, desde el glucógeno almacenado en los tejidos, a través de la hemolinfa, con

la finalidad de satisfacer las demandas de substrato requerido para la glucólisis anaerobia (Racotta et al., 2002; Perez-Rostro et al., 2004).

Algunas especies de decápodos responden a condiciones hipóxicas usando mecanismos compensatorios cardio-respiratorios como la capacidad de la hemolinfa para transportar el oxígeno (Harper y Reiber, 1999). En el langostino *Macrobachium rosenbergii*, el camarón enano *Triops longicaudatus* y en el cangrejo azul *Callinectes sapidus,* la respuesta compensatoria a la reducción en la concentración de oxígeno se manifiestan como un aumento en la concentración de la hemocianina circulante, dando lugar a un incremento en la capacidad de la hemolinfa para unir y trasportar el oxígeno. Además, se han reportado un aumento en productos de la respiración como CO_2 y HCO_3, los cuales provocan incremento en el pH de la hemolinfa (Cheng et al., 2003; Harper y Reiber, 2006; Bell et al., 2009).

Aunado a estas respuestas fisiológicas, en *Callinectes sapidus* se ha reportado que en respuesta a hipoxia por 5 dias, ocurre una disminución de la expresión génica de varias proteínas como la hemocianina, la criptocianina (una proteína homóloga a la hemocianina pero sin cobre) y la citocromo c oxidasa subunidad 1 (indicador de que el metabolismo aeróbico está deteniéndose; Brouwer et al., 2004). En el camarón *Palaemonetes pugio*, expuesto a la hipoxia por un periodo de 7 días, se reportó que la cantidad del ARNm de las proteínas mitocondriales como la citocromo c oxidasa subunidad 2, la proteína ribosomal S2, la ATP sintasa subunidades d y f, y la acil-CoA deshidrogenasa se reducen tanto que son indetectables. Además, en este mismo crustáceo se detectó un incremento en la cantidad de ARNm de la manganeso superóxido dismutasa mitocondrial (MnSOD)(Brouwer et al., 2008; Brown-Peterson et al., 2008).

2.1.2 Factor inducible por hipoxia 1 (HIF-1)

Se ha demostrado que muchos genes son inducibles por hipoxia y son regulados por una proteína de unión al ADN conocida como el factor inducible por hipoxia 1 (HIF-1, por sus siglas en inglés). Este factor de transcripción es muy conservado, ya que se encuentra desde invertebrados hasta vertebrados, siendo su participación muy importante en la adaptación a la variación en la concentración de oxígeno (Semenza, 1999). HIF-1 regula la transcripción de muchos genes involucrados en el control celular y en las respuestas a la hipoxia a corto y largo plazo, incluyendo glucólisis, eritropoyesis, respiración, vasodilatación y angiogénesis (Ryan et al., 1998).

HIF-1 es un heterodímero compuesto por dos subunidades, la α de 120 kDa y la β de 94 kDa, cada subunidad contiene un motivo hélice-lazo-hélice y almenos un dominio Per y Sim (PAS, por sus siglás en inglés). HIF-1 reconoce y se une a una secuencia específica de DNA 5-TACGTGCT-3' y esta interacción con su sitio cognado es rápida (menos de 1 min). Además, se ha sugerido que la activación de ciertas cinasas se encuentra relacionada con la cascada de señalización de HIF-1 inducida por hipoxia (Berra et al., 2001; 2003). La expresión de HIF-1 es altamente regulada por la concentración de oxígeno celular y el nivel de la expresión se encuentra relacionado primeramente con la abundancia de la subunidad α. La subunidad β es expresada en todas las células de manera constitutiva, mientras que la subunidad α es sintetizada continuamente y degradada durante condiciones de normoxia, pero es acumulada rápidamente después de la exposición a baja concentración de oxígeno (Shih y Claffey, 1998; Li y Brouwer, 2007). Posteriormente, HIF-1 activa la transcripción de varios genes que son inducibles por hipoxia. Estos genes pueden agruparse en cuatro categorías diferentes: genes relacionados con la traducción del ARNm para generar una proteína, genes relacionados con el metabolismo de aminoácidos, genes relacionados con el metabolismo del ATP y genes relacionados con el crecimiento (Wu et al., 2002).

Soñanez-Organis et al. (2009) caracterizaron HIF-1 del camarón blanco *Litopenaeus vannamei* y reportaron que este factor de transcripción presenta los dominios importantes para su regulación y que la expresión de las subunides α y β es de manera tejido-específica bajo el efecto de la hipoxia. También que el silenciamiento de HIF-1 afecta la expresión de la hexocinasa (HK) de manera diferencial, debido a que se bloqueó la inducción de la expresión y la actividad enzimática de HK en branquias pero no en músculo (Soñanez-Organis et al., 2011).

Así, con la finalidad de mantener la homeostasis fisiológica en respuesta a condiciones ambientales desfavorables, cada especie de crustáceo ha desarrollado su propio mecanismo adaptativo, incluyendo respuestas conductuales y fisiológicas en respuesta a los cambios en la concentración de oxígeno disuelto en el agua.

2.2 La Estructura y Función de la Mitocondria

Las mitocondrias ocupan una fracción substancial (hasta el 20% en células hepáticas) del citoplasma y se pueden visualizar como cilindros alargados con un diámetro de 0.5 a 1 μm (Alberts et al., 1989). Además, cada mitocondria está integrada por dos membranas, la membrana externa e interna que forma a la matriz y el espacio intermembranal (Fig. 1).

En la membrana externa se pueden encontrar varias porinas, las cuales permiten el paso de otras proteínas a través de la bicapa lípidica. Estas porinas ceden el paso de moléculas hasta de 10,000 daltones, sin embargo, estas moléculas no pueden atravesar a la membrana interna (Low, 1956).

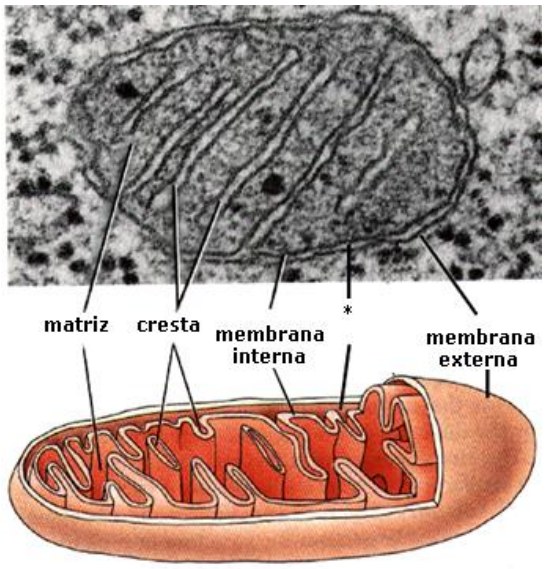


Figura 1. La mitocondria. Arriba, sección transversal de una mitocondria vista por microscopía electrónica de transmisión. Abajo, representación esquemática de una mitocondria. * Espacio intermembranal. Modificada de Alberts et al. (1989).

La membrana interna está compuesta del fosfolípido cardiolipina, el cual confiere a la membrana la capacidad de ser impermeable a los iones. Existen también proteínas incrustadas en la membrana que permiten el transporte de ciertas moléculas pequeñas que son metabolizadas o requeridas por las enzimas alojadas en la matriz (Low, 1956). Otra característica importante de la membrana interna es la capacidad de plegarse dando origen a las crestas. Este plegamiento le confiere a la membrana mayor superficie dentro de la mitocondria (Palade, 1952). En la membrana interna están embebidos los complejos enzimáticos que participan en la cadena de transporte de electrones y en la fosforilación oxidativa, la cual genera la mayor cantidad de ATP celular (Ryan y Hoogenraad, 2007).

La matriz contiene las enzimas necesarias para la oxidación del piruvato, ácidos grasos y el ciclo de Krebs. Además, contiene varias copias del DNA mitocondrial, ribosomas, tRNAs y su propia maquinaria enzimática para la expresión de los genes mitocondriales. Por otro lado, en el espacio intermembranal

se localizan algunas enzimas que utilizan el ATP y que fosforilan algunos nucleótidos (Alberts et al., 1989).

En la mitocondria, el metabolismo oxidativo es alimentado por los ácidos grasos y por el piruvato producido en el citosol durante la glucólisis. Estos compuestos son transportados desde el citosol hacia la matriz mitocondrial, en donde son hidrolizados hasta acetil-CoA; posteriormente, el grupo acetilo entra al ciclo de Krebs produciéndose $FADH_2$, NADH y CO_2. De tal manera que el $FADH_2$ y NADH participan en la cadena de transporte de electrones, cediendo sus electrones (Mathews et al., 2000).

2.3 La Cadena de Transporte de Electrones y la Fosforilación Oxidativa

Dentro de la mitocondria se lleva a cabo la fosforilación oxidativa, un proceso en el cual el gradiente de protones transmembranal formado por reacciones de oxidación y reducción de los cuatro complejos enzimáticos de la cadena respiratoria (complejos del I-IV) es utilizado por la ATP-sintasa (complejo V) para generar ATP (Fig. 2; Cardol et al., 2005). En mamíferos, la cadena de transporte de electrones está integrada por 4 complejos enzimáticos los cuales son: NADH deshidrogenasa (Complejo I), succinato coenzima Q reductasa (Complejo II), citocromo reductasa (Complejo III) y citocromo c oxidasa (Complejo IV)(Ryan y Hoogenraad, 2007).

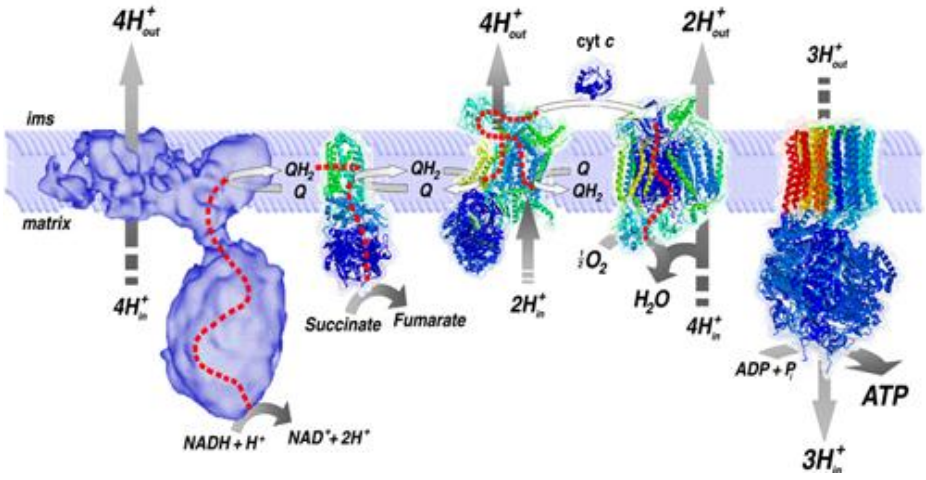


Figura 2. Complejos enzimáticos que participan en la cadena de transporte de electrones y la fosforilación oxidativa (Tomada de Ugalde, 2011).

La NADH deshidrogenasa o Complejo I (EC 1.6.5.3). Este complejo enzimático es el de mayor tamaño de las enzimas que translocan los protones en la mitocondria. Está integrada por 30 subunidades, de las cuales la mayoría están codificadas por el genoma nuclear y son importadas desde el citoplasma, aunque algunas son codificadas por el genoma mitocondrial y traducidas dentro de la mitocondria (Brandt, 2006). Esta enzima cataliza la transferencia de electrones desde el NADH hacia la coenzima Q y durante este proceso la enzima transloca 4 protones a través de la membrana interna (desde la matriz hacia el espacio intermembranal) por molécula oxidada de NADH (Weiss y Friedrich, 1991).

La succinato coenzima Q reductasa o Complejo II (EC 1.3.5.1), es la enzima que presenta menor masa molecular, ya que se encuentra integrado solo por cuatro subunidades que son codificadas por el núcleo (Cardol et al., 2005). Esta enzima no es una bomba de protones y se le considera una enzima bifuncional debido a que también participa en el ciclo de Krebs. La reacción que cataliza es la oxidación de succinato a fumarato con la reducción de la ubiquinona a ubiquinol (Rustin et al., 2002).

La enzima citocromo reductasa o Complejo III (EC 1.10.2.2), es una lipoproteína transmembranal compuesta de varias subunidades y en la mitocondria está integrada por un dímero de 9-11 subunidades y en las bacterias por un dímero de 3-4 subunidades (Covian y Trumpower, 2006). Este complejo cuenta con 3 grupos prostéticos distintos: dos grupos hemo del tipo b, un grupo hemo del tipo c y un grupo de moléculas del tipo fierro-azufre (2Fe-2S; Harnish et al., 1984). La citocromo reductasa cataliza la reducción del citocromo c por la oxidación de la coenzima Q al mismo tiempo que bombea 4 protones desde la matriz mitocondrial al espacio intermembranal (Covian et al., 2004).

La citocromo c oxidasa o Complejo IV (EC 1.9.3.1), que en los mamíferos, está integrada por varios grupos prostéticos y 13 subunidades, de las cuales 10 subunidades son codificadas por el genoma nuclear y 3 por el genoma mitocondrial. El complejo tiene dos grupos hemo (citocromo a y a_3) y dos centros de cobre (Cu_A y Cu_B) que forman un centro binuclear (Brunori et al., 1987). Esta enzima es el último aceptor de electrones y reduce el oxígeno molecular a agua acoplado al bombeo de protones desde la matriz mitocondrial hacia el espacio intermembranal (Tsukihara et al., 1996).

Por ultimo, la ATP-sintasa o Complejo V (EC 3.6.3.14), que en bovinos, se encuentra formado por 16 polipéptidos distintos, de los cuales dos subunidades son codificadas por el genoma mitocondrial y el resto de las subunidades son codificadas por el genoma nuclear (Rubinstein et al., 2003). Esta enzima puede dividirse en dos porciones, la porción catalítica F_1 y la porción transmembranal F_O. La ATP-sintasa utiliza el gradiente de protones que son translocados desde espacio intermembranal hacia la matríz, para la síntesis de ATP a partir de ADP y P_i (Boyer, 1993).

$$ADP + P_i + 4H^+_F \rightleftharpoons ATP + H_2O + 4H^+_D$$

Los subíndices F y D se refieren a fuera (espacio intermembranal) y a dentro (matriz mitocondrial), respectivamente.

2.4 La ATP-sintasa

Las ATP sintasas, también conocidas como bombas de protones ATPasas, funcionan como bombas de cationes a expensas de ATP o sintetizan el ATP utilizando el gradiente trasmembranal de protones (Lapierre et al., 2006). Han sido divididas en tres tipos dependiendo de su distribución filogenética como: 1) A-ATPasas localizadas principalmente en Archaea; 2) las V-ATPasas en Eukarya, predominantemente en vacuolas y en otras vesículas de la ruta endo o exocitosólica y finalmente, 3) F-ATPasas localizadas en la mitocondria, los plástidos (de algas y plantas) y en las bacterias (Lapierre et al., 2006).

La F_0F_1-ATP sintasa (EC 3.6.3.14), cataliza la fosforilación de ADP con fosfato inorgánico utilizando el gradiente electroquímico generado en el transporte de electrones. En general, el complejo F_0F_1-ATP sintasa en bovinos, tiene una masa molecular de aproximadamente 600 kDa y está integrado por 16 polipéptidos distintos (Mueller et al., 2004). Morfológicamente la enzima se divide en dos porciones: F_0 y F_1. La porción F_0 es hidrofóbica, se encuentra embebida en la membrana interna y contiene el canal de protones (Walker y Collinson, 1994). Esta porción en *Escherichia coli* está formada por tres subunidades (a, b_2 y $c_{10\text{-}12}$), y en los mamíferos se han descrito por lo menos siete subunidades distintas b (o F_oI), d, 6, A6L, OSCP, F_6 y DCCD (subunidad 9) (Hekman y Hatefi, 1991); mientras que en la levadura *Saccharomyces cerevisiae*, la porción F_0 se encuentra conformada por diez subunidades diferentes: 6, 7, 8, e, f, g, h, i, j y k (Tabla 1; Mueller, 2000).

La porción prácticamente esférica F_1 (hidrofílica) queda suspendida hacia la matriz mitocondrial e incluye los sitios catalíticos (Rubinstein et al., 2003). Esta porción en procariotes y eucariotes está compuesta básicamente por α_3, β_3, γ, δ y ε. La porción F_1 puede ser separada de la membrana y tener actividad ATPasa (Leslie y Walker, 2000). Ambas porciones se encuentran formadas por múltiples subunidades codificadas tanto por el núcleo como por la mitocondria (Arselin et al.,

1996) y existe una variabilidad en las subunidades que conforman a la enzima (Tabla 1 y Fig. 3).

Como parte del subcomplejo F_1 de la ATP-sintasa mitocondrial, la subunidad α en unión a la β forman el sitio catalítico de la enzima (Walker et al., 1985). En células animales y hongos ambas subunidades son sintetizadas como un precursor conteniendo un péptido líder en el extremo NH_2 terminal, el cual es procesado durante la introducción en la mitocondria (Walker et al., 1991). En los bovinos (*Bos taurus*), la levadura (*S. cerevisiae*) y la rana (*Xenopus leavis*), la pre-secuencia que dirige a la proteína hacia la mitocondria tiene aminoácidos hidroxilados y un gran número de residuos de aminoácidos cargados positivamente. En las tres especies, la homología entre las secuencias deducidas de aminoácidos del péptido líder es menor que la homología entre las proteínas maduras (Walker et al., 1989).

Tabla 1. Subunidades de la F_0F_1- ATP sintasa en bovinos, bacterias y levaduras

Bovino	Alias	*E. coli*	Levadura	Genoma	Importancia
α	Sub. 1	α	α	Nuclear	Esencial
β	Sub. 2	β	β	Nuclear	Esencial
γ	Sub. 3	γ	γ	Nuclear	Esencial
δ		ε	δ	Nuclear	Esencial
ε		Ausente	ε	Nuclear	importante
OSCP	Sub. 5	δ	OSCP	Nuclear	Esencial
Sub. a	Sub. 6	Sub. A	Sub. a	Mitocondrial	Esencial
Sub. B	Sub. 4	Sub. B	Sub. b	Nuclear	Esencial
Sub. c	Sub. 9	Sub. C	Sub. c	N (bovino) M(levadura)	Esencial
Sub. 8	A6L	Ausente	Sub. 8	Mitocondrial	Esencial
Sub. D	Sub. 7	Ausente	Sub. d	Nuclear	Esencial
Sub. e	TIM11	Ausente	Sub. e	Nuclear	Prescindible
Sub. f		Ausente	Sub. f	Nuclear	Esencial
Sub. g		Ausente	Sub. g	Nuclear	Prescindible
?		Ausente	Sub. h	Nuclear	Esencial
?	Sub. j	Ausente	Sub. i	Nuclear	Esencial
?		Ausente	Sub. k	Nuclear	Prescindible
F6		Ausente	?	Nuclear	Esencial
IF_1		Ausente	Inh1p	Nuclear	Prescindible
?		Ausente	Stf1p	Nuclear	Prescindible

Modificado de Mueller (2000)

La subunidad γ de la ATP-sintasa mitocondrial es parte de la porción extrínseca de la enzima conocida como tallo. Durante la hidrólisis de ATP la subunidad γ rota dentro del pseudohexágono formado por las subunidades α y β (Walker y Dickson, 2006). La rotación de la subunidad γ sucede en 3 pasos de 120°, probablemente a expensas de una molécula de ATP por cada movimiento, además la subunidad γ está en contacto con la subunidad ε y el anillo que forman las subunidades c para formar el "rotor" (Gibbons et al., 2000). Durante la síntesis de ATP, la translocación de los protones a través de la interfase entre las subunidades a y c de la porción F_O genera rotación de las subunidades $\gamma\varepsilon c_{10\text{-}12}$ y la rotación de γ forza a la subunidad β a que se abra y libere el ATP (Weber et al., 2000). La subunidad γ es codificada por el genoma nuclear y por lo tanto tiene un péptido señal que es removido al entrar a la membrana externa mitocondrial (Dyer et al., 1989).

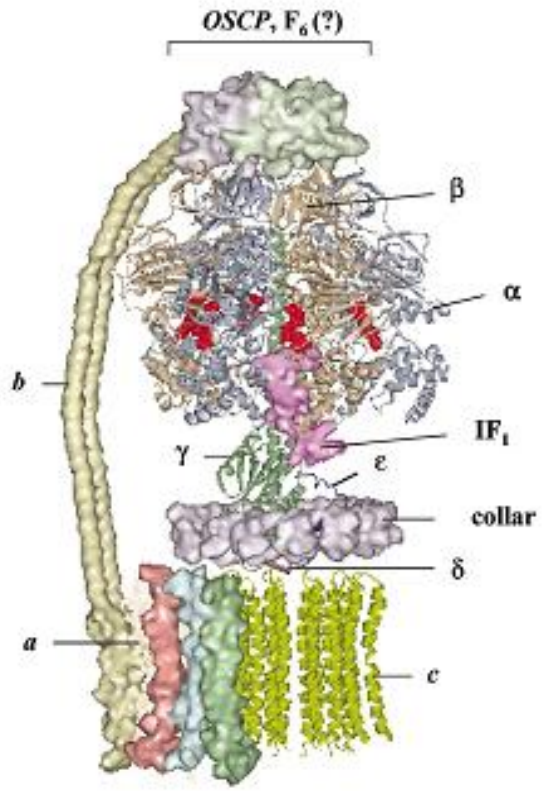


Figura 3. La ATP sintasa mitocondrial (Hong y Pedersen, 2004).

Finalmente, la estructura cristalográfica de la ATP-sintasa de levadura indica que la subunidad δ se encuentra ligada a la subunidad γ, por lo que se ha postulado que la subunidad δ es parte del rotor y gira con la subunidad γ (Rubinstein et al., 2003). Por otro lado, la subunidad ε es requerida para la unión de F_1 con F_0 e inhibe la actividad ATPasa de la porción F_1, disminuyendo la liberación del producto (Rodgers y Wilce, 2000). La subunidad δ forma un heterodímero con la subunidad ε y a su vez se asocian a la subunidad γ. Ambas subunidades son codificadas por el genoma nuclear y posteriormente importadas hacia la mitocondria (Hermans et al., 1988; Viñas et al., 1990).

El papel de las subunidades δ/ε es el de extender el rotor pero también es posible que actúen como un "embrague molecular". El embrague gira con el rotor del motor y se encuentra unido al rotor con la turbina, en este caso F_0 (Feniouk et al., 2006). Se ha sugerido que el embrague formado por δ/ε puede soltarse o unirse durante el tiempo de vida de la ATP-sintasa. Sin embargo, hasta este punto no existe evidencia de que la célula regule la eficiencia de la ATP sintasa adicionando o substrayendo subunidades δ o ε (Mueller, 2000).

Durante el proceso de respiración, en la membrana interna mitocondrial se transportan electrones y se bombean protones desde la matriz hacia el espacio intermembranal, creando un potencial electroquímico. Los protones entran a favor del gradiente, pasan a través de la ATP sintasa para volver a la matriz mitocondrial y la energía liberada es usada para sintetizar ATP. De esta manera sólo se sintetiza ATP cuando existe este potencial electroquímico (síntesis acoplada al transporte de electrones; Fillingame, 2000). Por otro lado, la ATP-sintasa tiene la capacidad de hidrolizar ATP cuando se intenta solo mantener el potencial electroquímico en el espacio intermembranal y así mantener la homeostasis celular (Di Lisa et al., 1998; Boyer, 2002).

Estudios bioquímicos, genéticos y cristalográficos de la F_1 en bovinos demostraron que el sitio catalítico se encuentra en la interfase entre las subunidades β y α. De tal manera, que existen tres sitios catalíticos formados por tres pares de subunidades α/β agrupadas en segmentos formando una especie de esfera (Lai Zhang y Mueller, 2000). Durante la catálisis interviene la subunidad γ, que al rotar produce cambios conformacionales secuenciales de los pares de subunidades α/β, estos cambios en la conformación del sitio de unión al nucleótido se conocen como conformación laxa (L), compacta (T) y abierta (O), las iniciales corresponden a las palabras en inglés: "Loose", "Thight" y "Open" (Walker et al., 1982). En este modelo, el ATP se sintetiza a partir de ADP y P_i y la liberación del ATP se produce por la rotación de γ y con la unión de ADP y Pi a otro sitio de ensamblaje. De acuerdo a la Figura 4 en el paso 1 de la reacción, la rotación de 120° de γ es impulsada por el paso de los protones a través de los canales de F_O y abre el sitio T produciendo la liberación de ATP, mientras el ADP y el P_i se unen a un sitio abierto. En el segundo paso, una rotación adicional de 120° hace que el ADP + P_i, que se había unido previamente, se unan a un sitio T para experimentar una condenzación a ATP, mientras que el sitio O, del que se liberó el ATP, une otro ADP y P_i para empezar un nuevo ciclo (Abrahams et al., 1994; Boyer, 2000; Cross y Müller, 2004).

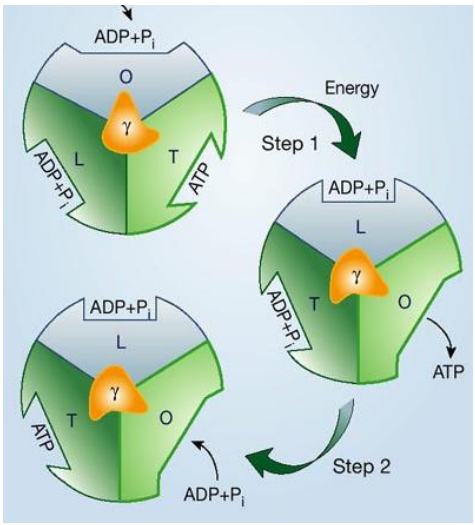


Figura 4. Modelo de unión secuencial para la síntesis de ATP (Cross, 2004).

Hasta el momento los estudios de la enzima ATP-sintasa se encuentran enfocados en modelos como bovinos, humanos, bacterias y levaduras, por consiguiente, los estudios en crustáceos son muy escasos. En el 2001, Li y Neufeld aislaron y caracterizaron bioquimicamente a la ATP-sintasa de las branquias del langostino *Orconectes virilis*, siendo hasta ese momento el único reporte para la ATP-sintasa de un crustáceo. En el 2007, Martínez-Cruz realizó estudios evaluando la cantidad de ARNm de los genes de las subunidades ATP6 (codificada por el genoma mitocondrial) y ATP9 (codificada por el genoma nuclear), de la glándula digestiva y el músculo del camarón blanco *Litopenaeus vannamei*. Dicho estudio reportó que en la glándula digestiva de los camarones existen cambios coordinados en la concentración de los transcritos de ambas subunidades en organismos sometidos a diferentes concentraciones de oxígeno disuelto en el agua desde la normoxia (6 mg/L) hasta condiciones de hipoxia (1.5 mg/L). Sin embargo, en la evaluación de la expresión génica de la subunidad mitocondrial ATP9 en el músculo no se detectaron cambios significativos. Lo anterior sugiere que a nivel transcripcional la coordinación de la expresión de los genes ATP6 y ATP9 de la ATP-sintasa, es tejido-especifica. Dicha coordinación se encuentra ligada con la función que realizan la glándula digestiva y el músculo, y la capacidad de estos órganos para movilizar las reservas energéticas cuando el camarón es sometido a hipoxia (Martinez-Cruz, 2007).

III. HIPÓTESIS

El camarón blanco *Litopenaeus vannamei* en condiciones de hipoxia es capaz de mantener un estado de homeostasis, incrementando la expresión de los genes de las subunidades básicas de la porción catalítica F_1 de la ATP-sintasa, la proteína de la subunidad catalítica ATPβ y la actividad ATPasa.

IV. OBJETIVO GENERAL

Caracterizar las subunidades de la porción catalítica F_1 de la ATP-sintasa, y evaluar los cambios en la expresión de los genes y las proteínas, así como la actividad de la enzima en el camarón blanco en condiciones de hipoxia.

V. OBJETIVOS ESPECÍFICOS

1) Obtener la secuencia nucleotídica del ADN complementario (ADNc) de cada subunidad de la porción F_1 y con la secuencia deducida de aminoácidos proponer un modelo estructural.
2) Cuantificar el ARNm de cada una de las subunidades α, β, γ, δ, y ε de la porción F_1 en el músculo del camarón en condiciones de hipoxia por PCR en tiempo real.
3) Investigar la relación entre la cantidad de ARNm y la proteína de la subunidad catalítica ATPβ de la ATP-sintasa por inmunodetección, en el músculo del camarón.
4) Evaluar los cambios en la actividad de la enzima y en la cantidad de ATP en músculo del camarón en hipoxia.

VI. MATERIALES Y MÉTODOS

6.1 Caracterización de las Secuencias de ADNc de las Cinco Subunidades de la Porción F_1 de la ATP-sintasa del Camarón Blanco *L. vannamei* y Generación del Modelo Estructural Predictivo

6.1.1 Aislamiento del ARNm total de branquias

Para la obtención de las secuencias de ADNc se aisló el ARN total de las branquias de cinco camarones. El tejido fue disectado y homogenizado en 1 mL del reactivo TRIzol® (Invitrogen, CA), que tiene su fundamento en la metodología descrita por Chomczynski y Sacchi (1987); siguiendo las instrucciones del fabricante. Brevemente, una vez homogenizado el tejido, se adicionaron 200 µL de cloroformo, se incubó por 5 min a temperatura ambiente y se centrifugó a 13000 x *g* por 15 min a 4 °C. Se colectó la fase acuosa y se repitió la extracción adicionando 500 µL de TRIzol®, posteriormente se obtuvo la fase acuosa de nuevo y se adicionó 1 volumen de isopropanol frío, se incubó por 10 min y se centrifugó a 13000 x *g* por 15 min a 4 °C. Se descartó el sobrenadante y el pellet se lavó con 1 mL de etanol al 75% en agua tratada con el 0.1% dietilpirocarbonato (DEPC, por sus siglás en inglés) frío. Se centrifugó a 8500 x *g* por 5 min a 4 °C, se descartó el sobrenadante, se eliminó el exceso de etanol remanente y se adicionaron 50 µL de agua DEPC al 0.1%.

6.1.2 Cuantificación y evaluación de la integridad del ARN total

El ARN total obtenido de las muestras se cuantificó por triplicado a una longitud de onda de 260 y 280 nm en un espectrofotómetro NanoDrop ® ND-1000 v 3.2, USA. Posteriormente, la integridad del ARN se evaluó por electroforesis en gel de agarosa-formaldehido al 1% en condiciones desnaturalizanes y libre de RNasas (Fourney et al., 1988), utilizando 5 µg de ARN total.

6.1.3 Eliminación del ADN genómico contaminante presente en las muestras de ARNm total y síntesis de ADNc

Para eliminar la contaminación por ADN genómico (ADNg) en las muestras de ARN total, se trataron 8 μg de cada muestra con DNasa I (1 U/μg de ARN) (Sigma, USA) como lo indican las instrucciones del fabricante. Se incubaron las muestras a 37 °C por 20 min y se inactivó la enzima adicionando 1 μL de EDTA 50 mM y elevando la temperatura a 70 °C por 10 min. Una vez terminado el tratamiento se realizó un PCR con la finalidad de confirmar la ausencia del ADNg. El ADN complementario (ADNc) se sintetizó a partir de 5 μg de ARN total de branquias libres de ADNg empleando el kit comercial Superscript First Strand Synthesis System for RT-PCR (Invitrogen, CA), siguiendo las indicaciones del fabricante.

6.1.4 Obtención de las secuencias de ADNc de las subunidades de la porción F_1

Para obtener la secuencia completa del ADNc de cada una de las subunidades, se diseñaron los oligonucleótidos específicos, mediante el programa Primer3 del sitio web http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi, para cada subunidad (Tabla 2) basados en secuencias no etiquetadas (ESTs por sus siglas en inglés) de *L. vannamei* depositadas en la base de datos GenBank y las secuencias completas de ADNc de las subunidades fueron obtenidas usando la estrategia "*primer walking*".

El extremo 3' de las secuencias nucleotídicas de cada una de las subunidades se obtuvo empleando la técnica de amplificación rápida de los extremos de ADNc (RACE, por sus siglas en inglés). Esta técnica consistió en sintetizar el ADNc a partir de ARN libre de ADNg mediante transcripción reversa con el kit comercial Superscript First Strand Synthesis System for RT-PCR (Invitrogen, CA), siguiendo las indicaciones ya descritas en el apartado 5.1.5 y con la siguiente modificación: en

la primera reacción se utilizó el oligonucleótido CDSIII/3'PCR del sistema BD-SMART (Clontech, USA) en lugar del oligo dT. Posteriormente, se realizó un PCR convencional empleando un oligonucleótido específico (sentido) que se encontraba situado hacia el extremo 3' y el oligonucleótido CDSIII/3'PCR (5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30N-1N-3'; N = A, G, C, o T; N-1 = A, G o C).

Las reacciones de PCR se realizaron usando 30 µL de Platinum ® PCR SuperMix, 1 µL de cada oligonucleótido (0.8 µM) y 1 µL de ADNc (con 238 ng equivalentes de ARN total). Las condiciones de amplificación fueron las siguientes: 1 min a 95 °C (un ciclo); 30 s a 95 °C, 1 min a 60 °C y 1 min a 72 °C (40 ciclos) y una extensión de 10 min a 72 °C en un termociclador DNA Engine ® Peltier Thermal Cycler (Bio-Rad, USA). Posteriormente, los productos de PCR obtenidos fueron analizados en geles de agarosa al 1 % teñidos con SYBR Safe ® DNA gel stain (Invitrogen, CA), purificados por columnas GFX (General Electric, CA) y su secuencia fue obtenida como servicio del Laboratorio de Evolución Molecular y Sistemática de la Universidad de Arizona.

Las secuencias nucleotídicas y las secuencias deducidas de aminoácidos fueron comparadas con las bases de datos de nucleótidos no redundantes y de proteínas utilizando el algoritmo BLAST (Altschul et al., 1990); los alineamientos fueron realizados con el algoritmo Clustal W (Thompson et al., 1994). Las secuencias deducidas de aminoácidos se obtuvieron empleando las herramientas del sitio web Expasy (http://au.expasy.org/tools/) y el software Mitoprot (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html) fue empleado para el análisis de las secuencias de las proteínas mitocondriales (Claros y Vincens, 1996).

Tabla 2. Oligonucleótidos específicos diseñados para la amplificación de los transcritos del complejo F_1-ATPsintasa

Gen	Oligonucleótido	Secuencia (5'-3')	Posición en el ADNc (nucleótidos)
atpα	ATPAFw1CB	CTCTTCTTTGGCTCGTCAC	26-44
atpα	ATPAFw2CB	ACAACATGGCTCTCGTCTCC	-5-15
atpα	ATPAFw3CB	TCAACTTGGAGCCCGATAAC	311-330
atpα	ATPARv1CB	CCATAGACACGGGCAATACC	248-228
atpα	ATPARv5	GGAGCAGCATCAGAGGCAGTGGCAGAC	842-815
atpα	ATPARv9CB	TTAGCAATCTACCCTAGCCCAC	1733-1711
atpβ	ATPBFw1CB	GGTGCTGGTGTAGGAAAGAC	613-632
atpβ	ATPBFw5	ATCATGTTGGGAGCTGCACAG	-3-18
atpβ	ATPBFw6	GGTAATGCTGTCGTTGATACT	358-377
atpβ	ATPBRv1CB	TACGGTCAGATGAATGAGCC	817-798
atpβ	ATPBRv6	ATGCGGCCAAGAGTACCAGGACCAACAG	425-398
atpγ	ATPGFw1	AGTGCATTGTGGGCCATC	-44-27
atpγ	ATPGRv1	GACCACTACTTCCCTGAT	924-942
atpγ	ATPGFw2	CTGGCTTCCCTCATGTTC	676-693
atpγ	ATPGRv2	CTTCAAAGGTTGCTGGC	453-470
atpγ	ATPGFw3	GATCAGGGGAAGTAGTGGTC	923-942
atpγ	ATPGFw4	CTGCAAGCACATCAAGGC	315-332
atpγ	ATPGRv4	CCGGTAATCACAGCCTG	814-830
atpδ	ATPDFw1	CATGGCCTTCACCTTTGC	75-92
atpδ	ATPDRv1	GCCTTGGAGAGGAGGTCAC	356-374
atpδ	ATPDFw2	GTTAGGCAGGTTGATGTCC	127-145
atpδ	ATPDRv2	CGTTAATGGTGACAATGCC	268-286
atpδ	ATPDFw3	CTTCCCTAGCCCGATTTG	-37-20
atpε	ATPEFw1	GTTCCTCCGCCATCTTCTG	-71-52
atpε	ATPERv1	CATCTTTACATCTGCAGG	188-205
atpε	ATPEFw2	CCTGCAGATGTAAAGATG	188-205
atpε	ATPERv2	CAGACTTCATGGGCTCCTTC	78-97

6.1.5 Generación del modelo estructural predictivo

La estructura de la porción F_1-ATPsintasa fue obtenida por modelación molecular, con base en la homología de secuencias primarias de las proteínas con respecto a una estructura obtenida experimentalmente y utilizada como templado. La estructura templado para la modelación fue seleccionada mediante la búsqueda de un homólogo en la base de datos del PDB (Banco de Datos de Proteínas, por sus siglas en inglés) utilizando el algoritmo BLASTp (Altschul et al., 1990).

La estructura cristalográfica de la porción F_1 de *Bos taurus* con una resolución de 2.4 Å (Gibbons et al., 2000), depositada en el PDB con el código **1E79**, fue utilizada como templado. Para llevar a cabo la modelación se empleó el

programa Molecular Operating Environment (MOE) en la versión 2009.10 (Chemical Computing Group).

Previo a la construcción del modelo, se realizó un alineamiento por pares de las secuencias de aminoácidos para cada una de las 5 subunidades de la porción F_1 de camarón blanco con la porción F_1 de bovino, implementada en MOE. Se construyeron un total de 25 modelos moleculares intermedios para cada una de las subunidades, las cuales fueron modeladas de forma independiente bajo el campo de fuerza CHARMM27.

Para cada subunidad se obtuvo un modelo final, el cual fue resultado de un esquema de minimización de energía partiendo del mejor modelo intermediario, seleccionado en base a las funciones de evaluación del MOE. El modelo final de la porción F_1 completa se obtuvo después del ensamblaje del modelo de cada subunidad seguido por una minimización de energía para optimizar las interacciones entre las cadenas.

6.2 Evaluación de la Expresión Génica de las Cinco Subunidades de la Porción F_1 en el Músculo del Camarón en Hipoxia

6.2.1 Organismos experimentales y bioensayo en condiciones de hipoxia

El bioensayo se realizó en las instalaciones del Centro de Investigaciones Biológicas del Noroeste, S.C., localizado en la ciudad de La Paz, B.C.S., México. Usando 240 camarones de la especie *L. vannamei*, con un peso aproximado de 30 g, que fueron distribuidos al azar en seis tanques con 300 L de agua (en cada tanque se colocaron 30 organismos). Los camarones se aclimataron por un periodo de 8 días a condiciones controladas de laboratorio: salinidad (35 ppm), temperatura (28 °C) y oxígeno disuelto en agua (OD) a una concentración de 6 mg/L (normoxia), y fueron alimentados hasta saciedad con alimento pelletizado (con un contenido de proteína del 35%) dos veces por día. Los restos de alimento y heces fueron

retirados por sifoneo y se realizaron recambios del 70% del volumen de agua marina diariamente.

Después del periodo de aclimatación, todos los organismos fueron ayunados 24 h antes del bioensayo, con el objetivo de asegurar que todos estuvieran bajo las mismas condiciones energéticas y se muestrearon solo aquellos que estuvieran en intermuda seleccionados por el método de setogénesis (Chan et al., 1988). Tres tanques se mantuvieron en condiciones de normoxia como controles. En los tres tanques experimentales restantes se removieron las piedras aireadoras y se burbujeó nitrógeno gaseoso para desplazar el oxígeno, además, se cubrieron los tanques con plástico para evitar el intercambio de gases. La concentración de oxígeno fue controlada y monitoreada constantemente utilizando un oxímetro digital durante todo el experimento.

Tres camarones fueron muestreados de cada tanque cuando la concentración de OD fue de 6, 2 y 1.5 mg/L (expuestos 3 h a cada condición) y cuando los animales fueron re-oxigenados (a la concentración de OD de 2 y 7 mg/L); simultáneamente se muestrearon los organismos controles, obteniendo un total de 90 muestras individuales.

Cada camarón se pesó y se extrajeron 400 µL de hemolinfa a partir de la base del quinto pereiópodo, utilizando una jeringa de 1 mL conteniendo dos volúmenes de solución anticoagulante fría (NaCl 450 mM, KCl 10 mM, NA_2-EDTA 10mM, HEPES 10 mM, pH 7.3) reportada por Vargas-Albores et al. (1993). Cada muestra de hemolinfa se centrifugó a 700 x *g* por 10 min a 4 °C, el plasma y los hemocitos fueron separados y almacenados a -80 °C hasta su uso. Posteriormente, los camarones se sacrificaron y se disectó el músculo. El tejido se sumergió en nitrógeno líquido y se almacenó a -20 °C.

6.2.2 Cuantificación de las concentraciones de lactato en plasma

Se cuantificó el lactato en el plasma obtenido de cada uno de los organismos experimentales, para ello se utilizó el kit comercial L-LACTATE-PAP (Randox, USA) ajustando las instrucciones del fabricante para su evaluación en microplaca. Brevemente, se colocaron 10 µL de plasma y 190 µL del reactivo del kit en cada pozo, se incubó por 30 min a 37 °C. Posteriormente, se realizó la medición en el lector de microplaca modelo 680 (Bio-Rad, USA) a una longitud de onda de 550 nm. Para todas las muestras las concentraciones de plasma se determinaron por triplicado.

6.2.3 Aislamiento, cuantificación, evaluación de la integridad y eliminación del ADNg contaminante presente en las muestras de ARN total

El ARN total se extrajo del músculo, se cuantificó, evaluó su integridad en geles de agarosa-formadehido en condiciones desnaturalizantes y se eliminó el ADNg contaminante de cada una de las muestras como se describió anteriormente en los apartados 6.1.1, 6.1.2 y 6.1.3.

6.2.4 Cuantificación del ARNm de cada subunidad por PCR en tiempo real

La cuantificación del ARNm de las subunidades *atpα*, *atpβ*, *atpγ*, *atpδ* y *atpε* se realizó usando sondas TaqMan® y un sistema de detección de PCR en tiempo real iQ5 multicolor (BioRad, Hercules, CA). Además, se evaluó la expresión del gen de la proteína ribosomal *L8* (**DQ316258**) para normalizar la expresión génica de cada una de las subunidades. La cuantificación se llevó a cabo por triplicado y para cada una de las reacciones consistieron se usaron 10 µL de mezcla Brilliant® II QRT-PCR 1-Step 2× Master Mix (Stratagene, USA), 1 µL de TaqMan probe (incluyedo 5 µM de oligonucleótidos específicos para cada subunidad; Tabla 3), 0.8 µL de la enzima RT/block, 300 ng de ARN total (libres de ADNg contaminante) y

agua hasta un volumen final de 20 µL. Por otro lado, las condiciones de amplificación fueron: 30 min a 50 °C, 15 min a 95 °C, seguido por 45 ciclos de 15 s a 95 °C y 1 min a 60 °C; la fluorescencia fue tomada a los 60 °C después de cada ciclo de amplificación.

Tabla 3. Características de las sondas TaqMan ® utilizadas en la evaluación de la expresión génica de las 5 subunidades de la porción F_1 -ATPsintasa del músculo de camarón

Subunidad	Oligonucleótido sentido (5'-3')	Oligonucleótido antisentido (5´-3')	Secuencia del reportero (5'-3')	Tipo del reportero	Tipo de opacante
atpα	GGGAGCCCATGCAGACT	GCCACGACCAATAGGCACAA	AAGGCCGTAGACTCTC	FAM	NFQ
atpβ	GGTGTCGGAAAGACTGTACTTATCA	CCAGCAAATACTGAGTAACCACCG	ACGTTGCCAAGGCTCA	FAM	NFQ
atpγ	GTTCTACACACTTAAGGAAGGAGCAT	CATCTCGCCGGCATTCTTG	CTGGCGGCATCCATG	FAM	NFQ
atpδ	GCCTGCTCACGTACCATCTC	GCAGTGCCATCACCTTCAAAA	CCAGGCTTCAGAACTG	FAM	NFQ
atpε	CTGAAGGAGCCCATGAAGTCT	GCCATCCTTCCACTTAATGATCCT	CACGTTTGGCAGCTTC	FAM	NFQ
L8	ATTTGCAACCTTGAGGAGAAGACT	TGGGCAATGACCTGAGCATAATT	ATCCACGGGCAATACG	HEX	NFQ

El método del $2^{-\Delta\Delta CT}$ se utilizó para calcular los cambios en la expresión génica de cada subunidad (Livak y Schmittgen, 2001). Brevemente, el cálculo está basado en el valor del C_T (entendiéndose como el ciclo en el que la fluorescencia sobrepasa la fluorescencia basal) de cada muestra durante la amplificación y la fórmula general consiste en: $2^{-((CTatpx - CTL8)\text{X mg/L} - (CT\ atpx - CT\ L8)6\text{ mg/L})}$ en donde:

- CTatpx = C_T de la subunidad evaluada
- CTL8 = C_T del gen constitutivo *L8*
- X mg/L = Condición experimental (durante hipoxia)
- 6 mg/L= Normoxia (controles)

Los resultados se expresaron como el cambio en la expresión génica de la subunidad evaluada (*atpα*, *atpβ*, *atpγ*, *atpδ* y *atpε*) normalizada con respecto a la expresión del gen constitutivo (*L8*) y relativa a la condición de normoxia (6 mg/L OD). En cada corrida de PCR en tiempo real se incluyeron controles negativos sin templado y sin oligonucleótidos. La eficiencia de la amplificación (E) fue determinada para cada gen siguiendo la fórmula $E = 10^{(-1/\text{pendiente})}$ debido a que en el método del $2^{-\Delta\Delta CT}$ las pendientes y las eficiencias de amplificación deben ser similares.

Se realizaron pruebas de homogeneidad de varianzas a los datos obtenidos mediante la prueba de Bartlett empleando el estadístico χ^2. Debido a la normalidad de los datos en el análisis estadísticos se utilizaron pruebas paramétricas. Los datos fueron analizados estadísticamente por análisis de varianza (ANOVA) de una vía y para discriminar entre las medias se utilizó la prueba de comparaciones múltiples de Tukey utilizando el programa Statistica v. 8.0 considerando un intervalo de confianza del 95% (p=0.05).

6.3 Inmunodetección de la Subunidad ATPβ de la ATP-sintasa en los Extractos Mitocondriales del Músculo de Camarón en Hipoxia

6.3.1 Aislamiento de las mitocondrias

El aislamiento de las mitocondrias del camarón se inició con 50 g de músculo de la cola y 400 mL de buffer de aislamiento A (sacarosa 250 mM, Tris 5 mM, EDTA 15 mM, pH 7.4), la mezcla se homogenizó en una licuadora a velocidad media por 1 min y rápidamente se ajustó el pH a 7.4 ± 0.1 con Tris saturado, ya que el pH ácido afecta a la mitocondria.

El homogenizado se filtró a través de dos capas de gasa y se centrifugó a 1055 x g por 10 min a 4 °C con la finalidad de precipitar los restos celulares y otros organelos. El sobrenadante se centrifugó a 16887 x g por 15 min a 4 °C y el precipitado se resuspendió en 20 mL de buffer B (sacarosa 250 mM, Tris 5 mM, pH 7.4) y se centrifugó nuevamente a 1055 x g por 10 min a 4 °C; se tomó el sobrenadante y se centrifugó a 16887 x g por 15 min a 4 °C. Finalmente, el precipitado se resuspendió en 0.5 mL de buffer B. Todo el proceso se realizó a 4 °C.

6.3.2 Separación del complejo ATP-sintasa por electroforesis nativa-azul en geles de gradiente de poliacrilamida

La concentración de la proteína mitocondrial fue evaluada por el método del ácido bicinconínico (BCA) usando seroalbúmina bóvina como estándar (Pierce, USA) ajustando las indicaciones del fabricante a microplaca. De manera breve, se adicionaron al pozo de la microplaca 10 µL de muestra y 190 µL de la solución reactiva (50 partes del reactivo A con 1 parte del reactivo B del kit), se incubó por 30 min a 37 °C y se midió la absorbancia a 550 nm en un lector de microplacas modelo 680 (Bio-Rad, USA).

Los complejos mitocondriales fueron separados por electroforesis nativa-azul en gel de poliacrilamida (BN-PAGE, por sus siglas en inglés) en gradiente lineal del 3.5 al 11%. Ochenta µg de proteína mitocondrial, previamente solubilizada con digitonina (10 mg/mg proteína) y centrifugada a 60462 x *g* por 2 h a 4 °C, se cargaron al gel. El buffer cátodo se preparó con Tricina 50 mM, Bis-Tris 15 mM, 0.01% Serva G, pH 7 y el buffer ánodo con Bis-Tris 50 mM, pH 7. Las condiciones de la electroforesis fueron a 35 v por 15 h a 4 °C en el sistema Mini-PROTEAN® Tetra Cell (Bio-Rad, USA) (Wittig et al., 2007) y se usó como marcador de peso molecular el kit de calibración de alto peso molecular (General Electric, USA).

6.3.3 Evaluación de la actividad ATPasa en gel

Se evaluó la actividad ATPasa en gel para identificar al complejo ATP-sintasa. La solución de reacción consistió en glicina 50 mM, $MgCl_2$ 3 mM, ATP 3 mM, $Pb(NO_3)_2$ 0.15%, pH 8.4. Primeramente, se mezcló la glicina y el $MgCl_2$, se ajustó el pH con etanolamina al 50%, se adicionó el ATP y finalmente se agregó el $Pb(NO_3)_2$. El pH se ajustó cada vez que se agregó un nuevo compuesto a la solución. Esta solución se adicionó al gel de poliacrilamida y se incubó a 36 °C por 24 h. Para acelerar la reacción puede adicionarse oxido de laurildimetilamina (LDAO, por sus siglas en inglés).

6.3.4 Separación de las subunidades de la porción F_1 por electroforesis en condiciones desnaturalizantes y reductoras

Para separar e identificar las subunidades que componen a la porción F_1 de la ATP-sintasa, realizó una la electroforesis en condiciones desnaturalizantes y reductoras (SDS-PAGE, por sus siglas en inglés) (Laemmli, 1970). Se prepararon geles conteniendo 3% y 12% de poliacrilamida (gel preparativo y separador, respectivamente). En estos geles la concentración final de Tris-HCl fue 0.375 M (pH 8.8) y 1% de SDS. Se adicionó 0.025% de tetrametiletilendiamina (TEMED) y persulfato de amonio (PSA) para la polimerización química de los geles.

El buffer cátodo y ánodo consistieron en Tris 0.025 M, glicina 0.192 M y 0.1% de SDS, y la muestra fue la tira de gel del BN-PAGE que contenía al complejo F_1 ATP-sintasa (identificada como una banda de masa molecular mayor a los 660 kDa) y mezclada con buffer muestra (Tris-HCl 0.0625 M (pH 6.8), 2% SDS, 10% glycerol, 5% 2-βmercaptoetanol y 0.001% azul de bromofenol). Las muestras se sumergieron en agua a 100 °C por 5 min para disociar completamente a las proteínas. La electroforesis se corrió a un amperaje constante de 15 mA hasta que el frente de corrido llegó al final del gel. Los geles fueron teñidos con 0.1% Coomassie brilliant blue R250.

6.3.5 Generación de anticuerpos policlonales conejo Anti-ATPβ de camarón

Se sintetizó un péptido de 23 aminoácidos de longitud con una pureza del 90% en la compañía PEPTIDE 2.0 (USA) basado en la secuencia deducida de aminoácidos de la subunidad *atpβ* de *L. vannamei* (**GQ848644**) (Martinez-Cruz et al., 2011). La secuencia del péptido (FDGELPPILNALEVANRSPRLVL) corresponde a la secuencia localizada en el extremo amino de la proteína. Un mg del péptido sintético se resuspendió en una solución de urea 3.5 M y NaCl al 0.9% y se diluyó 1:1 con adjuvante completo de Freund; con esta mezcla se inmunizó a un conejo

subcutáneamente en la parte superior de las piernas y la zona supraescapular (1 mL en total, 200 µL en cada inyección). Después de 28 días, se administró una segunda dosis del antígeno (0.5 mg del péptido sintético en 500 µL y el mismo volumen de adjuvante incompleto de Freund) en las mismas zonas. La sangre del conejo se tomó a los 7 y 14 días post inmunización de la vena marginal de la oreja. La sangre se dejó a temperatura ambiente por 30 min y se centrifugó a 2500 rpm por 5 min, se tomó el suero y se almacenó a 4 °C.

6.3.6 Purificación de los anticuerpos por cromatografía de afinidad a la proteína A

La purificación de los anticuerpos por cromatografía de afinidad consistió en diluir el suero 1:1 en buffer de dilución (Tris-HCl 0.2 M, NaCl 2 M, pH 8.6) y cargar la mezcla a la columna (agarosa/proteína A de *Staphylococcus aureus,* Bio-Rad) a un flujo de 1 mL/min. Después se realizó un lavado con una solución de Tris-HCl 0.1 M, NaCl 1 M, pH 8.6 y las IgG se eluyeron con 0.1 M de citrato de sodio a pH 3 y se neutralizaron con K_2HPO_4 0.2 M, pH 9. Se ultrafiltró con NaCl 0.1M usando una membrana de 10 kDa y aplicando una presión de 60 psi hasta un volumen final de 1 mL; se agregaron 100 µL de NaN_3 5 mM y los anticuerpos fueron almacenados a 4 °C.

6.3.7 Transferencia e inmunodetección de la subunidad ATPβ

Las proteínas mitocondriales previamente sepadas por SDS-PAGE se transfirieron a una membrana Immobilon PVDF (Millipore, USA) en un buffer conteniendo CAPS 100 mM y metanol 10%, pH 11; con un voltaje de 200 mA por 105 min. La membrana se bloqueó con Tween-20 al 2% en Tris 20 mM, NaCl 500 mM, pH 7.5 por 2 min y se lavó 4 veces con buffer TTBS (Tris 20 mM, NaCl 500 mM, 0.05% Tween-20, pH 7.5). Posteriormente, la membrana se incubó toda la noche con el anticuerpo policlonal conejo anti-ATPβ de camarón diluido 1:500 en TTBS a

4 °C. Al día siguiente, se lavó 4 veces con buffer TTBS y se incubó por 2 h con el anticuerpo secundario cabra anti-conejo conjugado con fosfatasa alcalina (Bio-Rad, USA) diluido 1:3000 en buffer TTBS. La reacción de la fosfatasa alcalina se desarrolló de acuerdo a las instrucciones del kit Immuno-Blot Alkaline Phophate Assay (Bio-Rad, USA), después del desarrollo del color, la membrana fue lavada con agua Milli-Q y se dejó secar.

6.3.8 Cuantificación de la subunidad ATPβ por el ensayo inmunoabsorbente ligado a enzima

La cuantificación de la subunidad ATPβ de los extractos mitocondriales se realizó mediante un ensayo por inmunoabsorción ligado a enzimas (ELISA, por sus siglas en inglés). Se cubrió el fondo de la microplaca con 2 µg de muestra del extracto mitocondrial (en 100 µL de buffer de cubierta) y 100 µL de buffer de cubierta (Na_2CO_3 15 mM, $NaHCO_3$ 35 mM, pH 9.6), toda la noche a 4 °C; después se realizaron cuatro lavados con buffer de lavado (Tris-HCl 0.10 M, Tween-20 0.05%, NaN_3 5 mM, rojo de fenol 0.05%, pH 7.4).

La placa se bloqueó con gelatina al 3% durante 1 h, se realizaron cuatro lavados y se incubó toda la noche a 4 °C con el anticuerpo policlonal conejo anti-ATPβ de camarón en una dilución 1:500 en buffer de lavado. Después de cuatro lavados, la placa se incubó por 2 h con el anticuerpo cerdo anti-IgG de conejo conjugado con peroxidasa de rábano (Bio-Rad, USA) diluido 1:2000 en buffer de lavado. Se realizaron tres lavados con buffer de lavado y uno más con buffer PBS ($K_2HPO_4.3H_2O$ 1 M, KH_2PO_4 1 M, NaCl 0.9%, pH 7.2).

La actividad peroxidasa se desarrolló al adicionar 100 µL del sustrato a cada pozo, preparado con 1 mg de 3, 3',5, 5'-tetramethilbenzidina (TMB) mezclado con 100 µL de CH_3COOH 2 M, 10 µl de H_2O_2 al 30% y CH_3COONa 0.1 M a pH 5.2 hasta un volumen final de 10 mL. La reacción se detuvo después de 2 min con 50 µL de

H_2SO_4 1M y la absorbancia se leyó a 450 nm en el lector de microplaca modelo 550 de Bio-Rad (USA).

Además, se incluyó una curva estándar con el péptido sintético para conocer la concentración de la proteína en los extractos mitocondriales. Se inició con una concentración de 2.5 µg/mL de péptidos y se realizaron diluciones seriales 1:2 quedando las siguientes concentraciones finales: 1.25, 0.625, 0.3125 y 0.15625 µg/mL.

6.4 Evaluación de la Actividad Enzimática ATPasa y Cuantificación de ATP en el Músculo de Camarón por Efecto de la Hipoxia

6.4.1 Evaluación de la Actividad ATPasa

La actividad ATPasa fue medida siguiendo la metodología descrita por Itoi et al. (2003), usando un espectrofotométro CARY 50 BIO equipado con un sistema de control de temperatura tipo "Peltier CARY single cell", a una longitud de 340 nm. La actividad de la enzima F_OF_1-ATPsintasa se evaluó en la dirección de hidrólisis del ATP, el medio de ensayo consistió en Tris 25 mM, sacarosa 300 mM, $MgCl_2$ 13 mM, KCl 133 mM, fosfoenolpiruvato 20 mM, NADH 0.13 mM, ATP 6.7 mM, 2 U de piruvato cinasa (Sigma-Aldrich, USA), y 3 U de lactato deshidrogenasa (Sigma-Aldrich, USA). Los ensayos fueron realizados con 60 µg de proteína mitocondrial, pH 8.2, un volumen final de 1.5 mL y cada una de las muestras se evaluó por triplicado.

Por otro lado, se evaluó la actividad citrato sintasa de los extractos mitocondriales. El ensayo se basa en la producción de la coenzima A (CoA.SH) proveniente del oxaloacetato, midiendo la liberación de los grupos sulfhidrilos usando el reactivo ácido 5,5'-ditriobis (2-nitrobenzóico) (DTNB, por sus siglas en inglés). El DTNB reacciona espontáneamente con los grupos sulfhidrilos para producir anión 5-tio-2-nitrobenzoato, el cual tiene un color amarillo y absorbe a 412 nm.

Citrato sintasa
Oxaloacetato + Acetil coenzima A → citrato + CoA.SH
CoA.SH + DTNB→CoA.S-S-nitrobenzoato + anión 5-tio-2-nitrobenzoato

La mezcla de reacción consistió en DTNB 10 mM (en Tris-HCl 1M, pH 8.1), Acetil CoA 10 mM, 10% Triton X 100, oxaloacetato 10 mM (en Tris-HCl 0.1 M, pH 8.1), 60 µg de extracto mitocondrial y H_2O hasta un volumen final de 1.5 mL. El ensayo consistió en mezclar el DTNB, la Acetil CoA, el Triton X 100 y el H_2O e incubar por 5 min a 30 °C para equilibrar la reacción; posteriormente, se adicionó la muestra y el oxaloacetato, y se monitoreó la reacción a 412 nm.

6.4.2 Cuantificación de ATP por cromatografía líquida de alta resolución

Se evaluó la concentración de ATP en el músculo de los camarones (Ryder, 1985), de la siguiente manera: a 1 g de músculo se le agregaron 5 mL de $HClO_4$ 0.6 M, se homogenizó y centrifugó a 3000 x *g* por 10 min a 0 ± 2 °C. Se tomaron 2 mL de sobrenadante y se ajustó el pH de 6.5 a 6.8 con KOH 1 M y 0.1 M. La solución fue incubada por 30 min en hielo, se filtró, se aforó a un volumen final de 10 mL con agua Milli-Q y la solución se almacenó a -20 °C hasta futuros análisis. Veinte µL de cada muestra fueron inyectadas y analizadas por HPLC en fase reversa (GmbH, Hewlett Packard, Alemania) y una columna ODS C18 Beckman ultrasphere (4.6 mm x 250 mm) con un tamaño de partícula de 5 µM (Beckman, Alemania). Las condiciones del análisis consitieron en una fase móvil de KH_2PO_4 0.04 M y K_2HPO_4 0.06 M, el flujo fue constante a 2 mL/min y la columna se mantuvo a una temperatura de 28 °C. La detección se realizó a una longitud de onda de 254 nm utilizando un detector UV Hewlett Packard 1100 GmbH (Alemania). Veinte µL de ATP 0.166 mM fueron inyectados como estándar.

6.5 Análisis Estadístico

Se realizaron pruebas de homogeneidad de varianzas a los datos obtenidos mediante la prueba de Bartlett empleando el estadístico χ^2. Debido a la normalidad de los datos en el análisis estadísticos se utilizaron pruebas paramétricas. Los datos fueron analizados estadísticamente por análisis de varianza (ANOVA) de una vía y para discriminar entre las medias se utilizó la prueba de comparaciones múltiples de Tukey utilizando el programa Statistica v. 8.0 considerando un intervalo de confianza del 95% (p=0.05).

VII. RESULTADOS Y DISCUSIÓN

7.1 Caracterización de la Porción Catalítica F_1 de la ATP-sintasa de Camarón Blanco y Análisis del Modelo Estructural Predictivo

7.1.1 Subunidad α (ATPα): secuencia de ADNc y proteína deducida

Se obtuvo una secuencia completa del ADNc de la subunidad alfa (*atp*α) de 1653 pb incluyendo el codón de inicio (ATG) y el codón de término (TAA). La secuencia que codifica al péptido señal de 46 residuos (6 cargados positivamente y ninguno cargado negativamente; Fig. 5) es de 138 pb. La proteína madura es de 504 residuos correspondiendo a una masa molecular calculada de 54.49 kDa y un punto isoeléctrico de 7.8 (Tabla 4), es codificado en 1515 pb (posición 139 - 1653). Finalmente, se incluye una región no traducida de 465 pb que corresponde al extremo 3', incluyendo la señal de poliadenilación y la cola poli A (Fig. 5). La secuencia completa de ADNc (2118 pb) se depositó en la base de datos GenBank con el número de acceso: **GQ848643**. Esta secuencia presenta una alta identidad con algunas especies de insectos como *Bombyx mori* (89%, **NP_001040233**), *Apis mellifera* (88%, **XP_392639**), *Aedes aegypti* (85%, **XP_001655906**) y con *Bos taurus* (84%, **NP_777109**).

En la secuencia de la subunidad ATPα del camarón se localizaron tres dominios conservados: a) el dominio ATP-synt_ab_N de la posición 66 a la 132, b) el domino ATP-synt_ab (dominio de unión a nucleótidos) de la posición 188 a la 412, el cual incluye el motivo Walker A de la posición 209 a la 216 (GDRQTGKT) y el dominio Walker B de la posición 305 a la 309 (LIIYD) y c) el dominio ATP-synt_ab_C de la posición 424 a la 528 (Fig. 6).

Además, en el dominio ATP-synt_ab se localizaron 15 residuos altamente conservados e involucrados en la unión al ATP en las posiciones 211, 215, 216, 217, 243, 248, 309, 310, 313, 368, 384, 397, 402, 403 y 413. También se localizaron 41 aminoácidos en la interface con la subunidad ATPβ en las posiciones 155-156,

172-173, 175-176, 179, 181, 211-212, 249-252, 254-255, 258, 319-320, 326-328, 330-331, 336, 340, 343, 347, 384, 387, 398-399, 402 y 413 (Marchler-Bauer et al., 2009). Asimismo, se detectaron algunas substituciones de aminoácidos en la secuencia de la proteína: T50, S63, N64, R123, A142, G163, G164, L165 y algunos residuos que son específicos para invertebrados como: A60, P61, K62, C487, E507, T514 y A524 (Fig. 6).

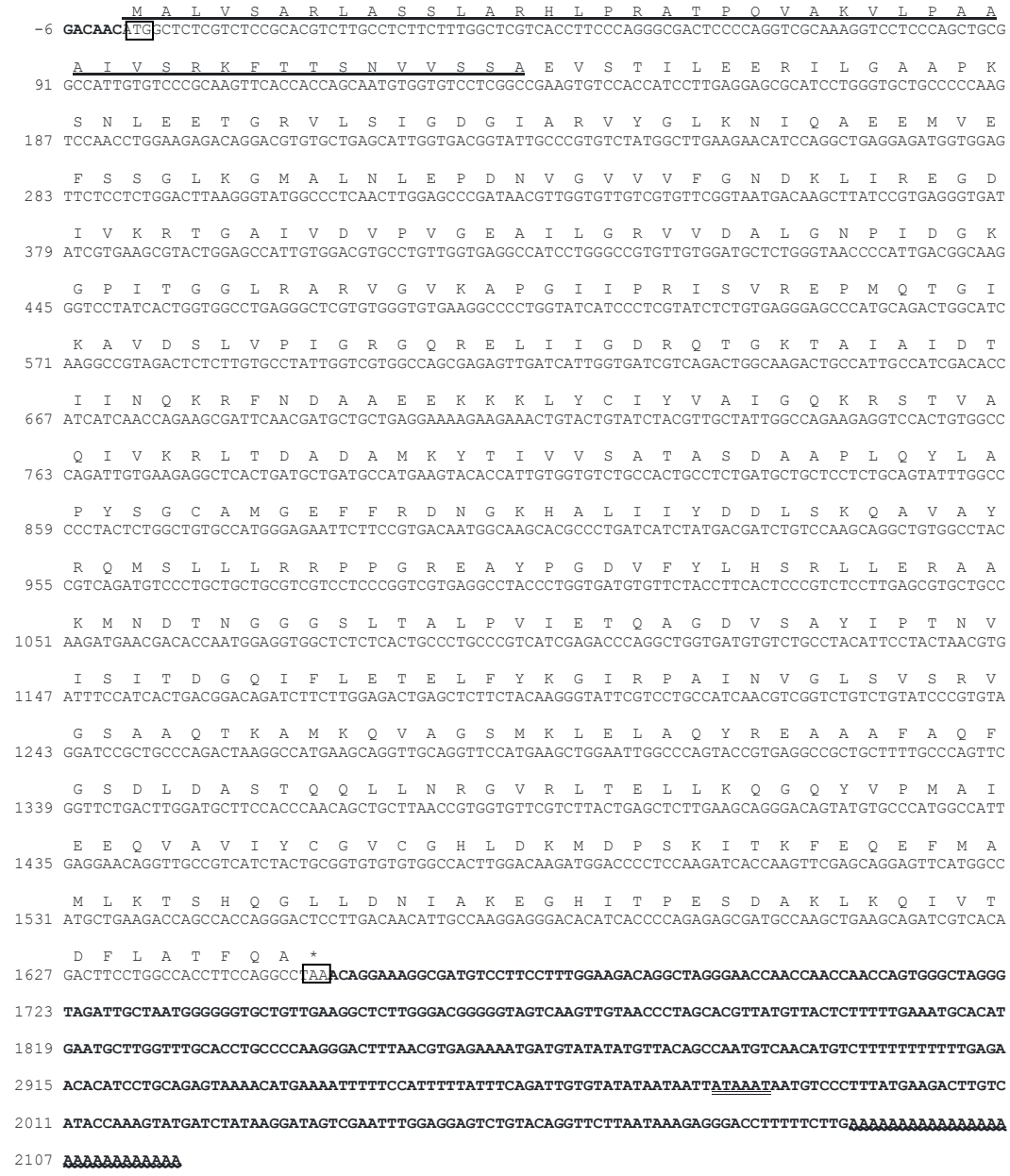
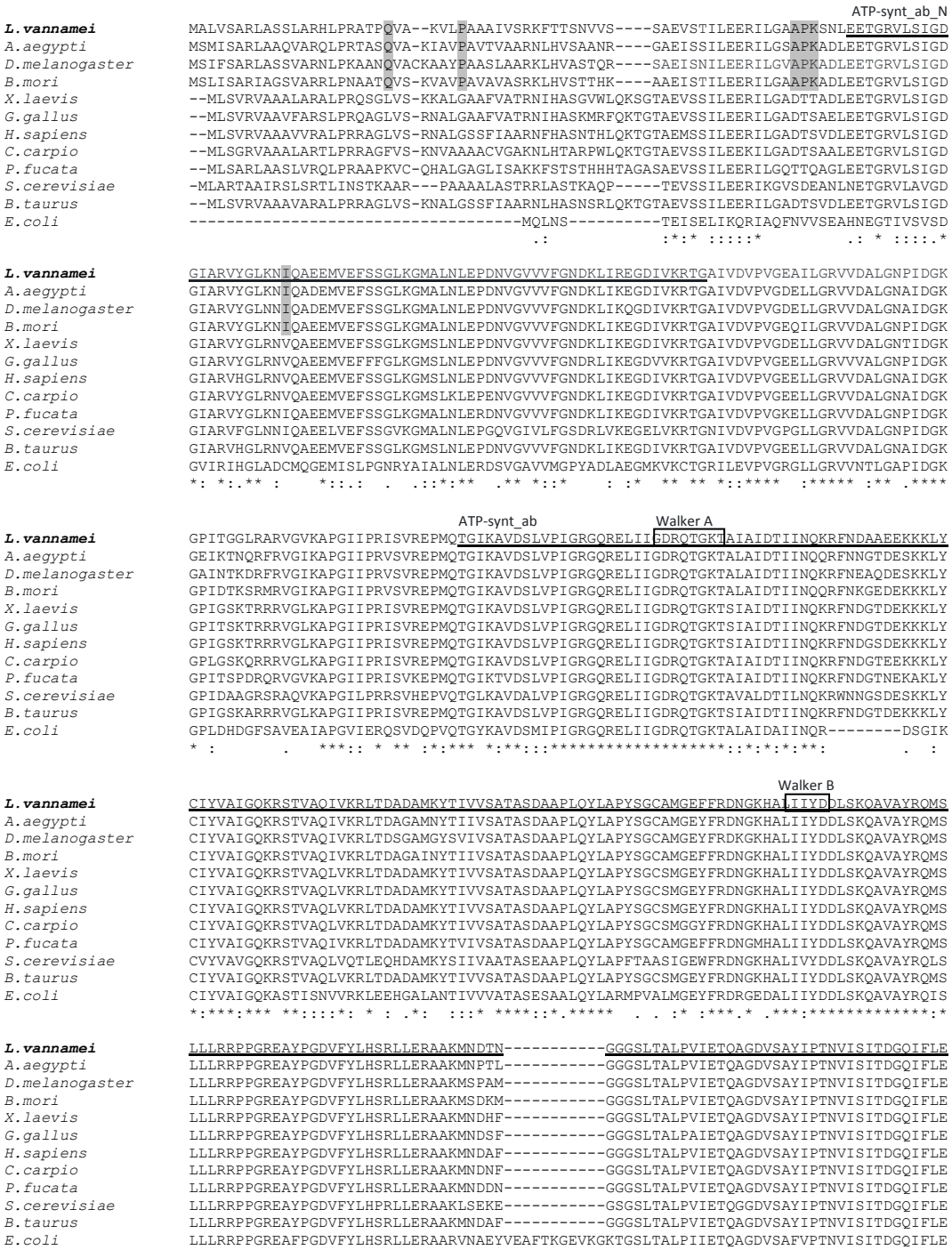


Figura 5. Secuencia de ADNc y deducida de aminoácidos de la subunidad ATPα de camarón blanco *L. vannamei*. Los codones de inicio y término se encuentran enmarcados; la señal de poliadenilación se encuentra doblemente subrayada y la cola poli A está subrayada con ondas; los aminoácidos que corresponden al péptido señal se encuentran subrayados.



Continuación...

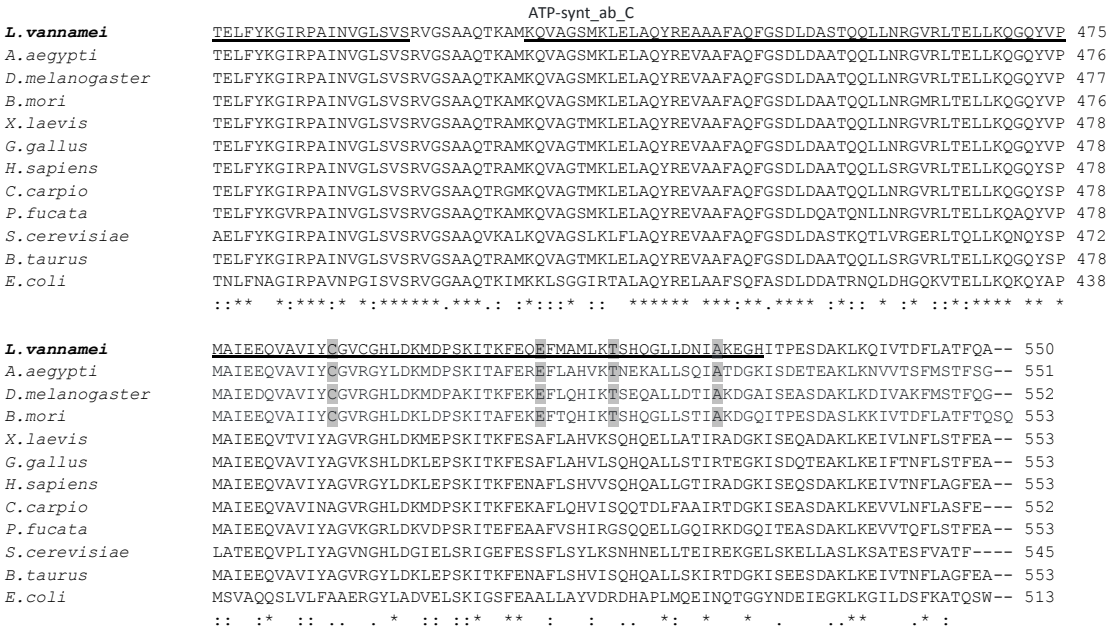


Figura 6. Alineamiento de la subunidad ATPα de diversas especies de vertebrados e invertebrados y localización de los dominios y motivos conservados. Los residuos subrayados indican los tres dominios conservados; los residuos enmarcados representan los motivos y los residuos resaltados en gris representan las substituciones encontradas en invertebrados.

7.1.2 Subunidad β (ATPβ): secuencia de ADNc y proteína deducida

La secuencia completa de ADNc de la subunidad *atpβ* de las branquias del camarón blanco es de 1820 pb, e incluye el codón de inicio en la posición 1 y el codón de termino en la posición 1579. En esta secuencia también se localizan las regiones no traducidas 5' y 3' las cuales constan de 3 y 242 pb respectivamente; en la región 3' UTR se incluye la señal de poliadenilación y la cola poliA (Fig. 7). Esta secuencia completa de ADNc se depositó en la base de datos GenBank con el número de acceso: **GQ848644**. El análisis de la secuencia nucleotídica de esta subunidad muestra un alto porcentaje de identidad con otras secuencias reportadas para crustáceos, obteniéndose hasta un 98% con las secuencias del camarón chino *Fenneropenaeus chinensis* (**ACM91675**) y el camarón japonés *Marsupenaeus japonicus* (**AMC91676**), un 91% con el langostino *Pacifastacus leniusculus* (**ABI34071**) y un 83% con el bovino *Bos taurus.*

La secuencia deducida de la preproteína es de 525 aminoácidos y presenta una masa molecular de 55.96 kDa y un punto isoeléctrico calculado de 5.03. El péptido señal predicho es de 43 aminoácidos, el cual incluye 6 residuos cargados positivamente y ninguno cargado negativamente (igual que la subunidad ATPα; Fig. 7). La proteína madura tiene 482 aminoácidos y una masa molecular calculada de 51.45 kDa y un punto isoeléctrico calculado de 4.78 (Tabla 4). Dentro de la secuencia de la proteína se localizan los dominios: a) ATP-synt_ab_N de los residuos 59 – 126, b) ATP-synt_ab entre los residuos 182 – 402 e incluye los motivos Walker A (GGAGVGKT) de los residuos 203 – 210 y Walker B (LLFID) de los residuos 299 – 303 involucrados en la unión al ATP y c) ATP-synt_ab_C de los residuos 415 – 525.

En esta secuencia se localizaron algunas regiones conservadas como DELSEED (residuos 441 – 447), el cual forma un bucle cargado negativamente cercano al sitio en donde el inhibidor endógeno IF_1 interactúa con la ATP-sintasa, para regular la actividad hidrolítica (Cabezon et al., 2001). También se localizó la secuencia GERXXE en la posición 234 – 239 que se ha sugerido participa en el proceso de hidrólisis de ATP al interactuar con la subunidad ATPα en el sitio catalítico (Wada et al., 2000; Fig. 8).

En la proteína ATPβ se detectaron 11 sitios de unión al ATP en las posiciones: 206, 209, 210, 211, 235, 236, 239, 303, 307, 392 y 393, los cuales son altamente conservados entre eucariotes. También, se detectaron 39 residuos en la interfase con la subunidad ATPα en las posiciones: 151, 168, 170, 171, 173, 175, 236 – 238, 241, 269 – 271, 276, 307, 314, 320 – 322, 324, 325, 330, 334, 341, 358, 361, 362, 366, 373, 375 – 377, 388, 389, 398, 399, 401 y 403.

Se identificaron algunas substituciones presentes solo en las secuencias de crustáceos en los residuos: G74, E75, N120, S130, G136, E158, S161, D169, S194, D258, S263, I401, H428, S487, E490 y P503. Además, se identificaron

substituciones presentes solo en la secuencia de *L. vannamei* que se sugiere pudieran estar implicadas en posibles cambios estructurales o funcionales como: S59, A86, I117, V123, H159, A407, H412, S420, P482, D483, P484, R486, D498, A502, Q507, S509, I514, A522 y P524 (Fig. 8).

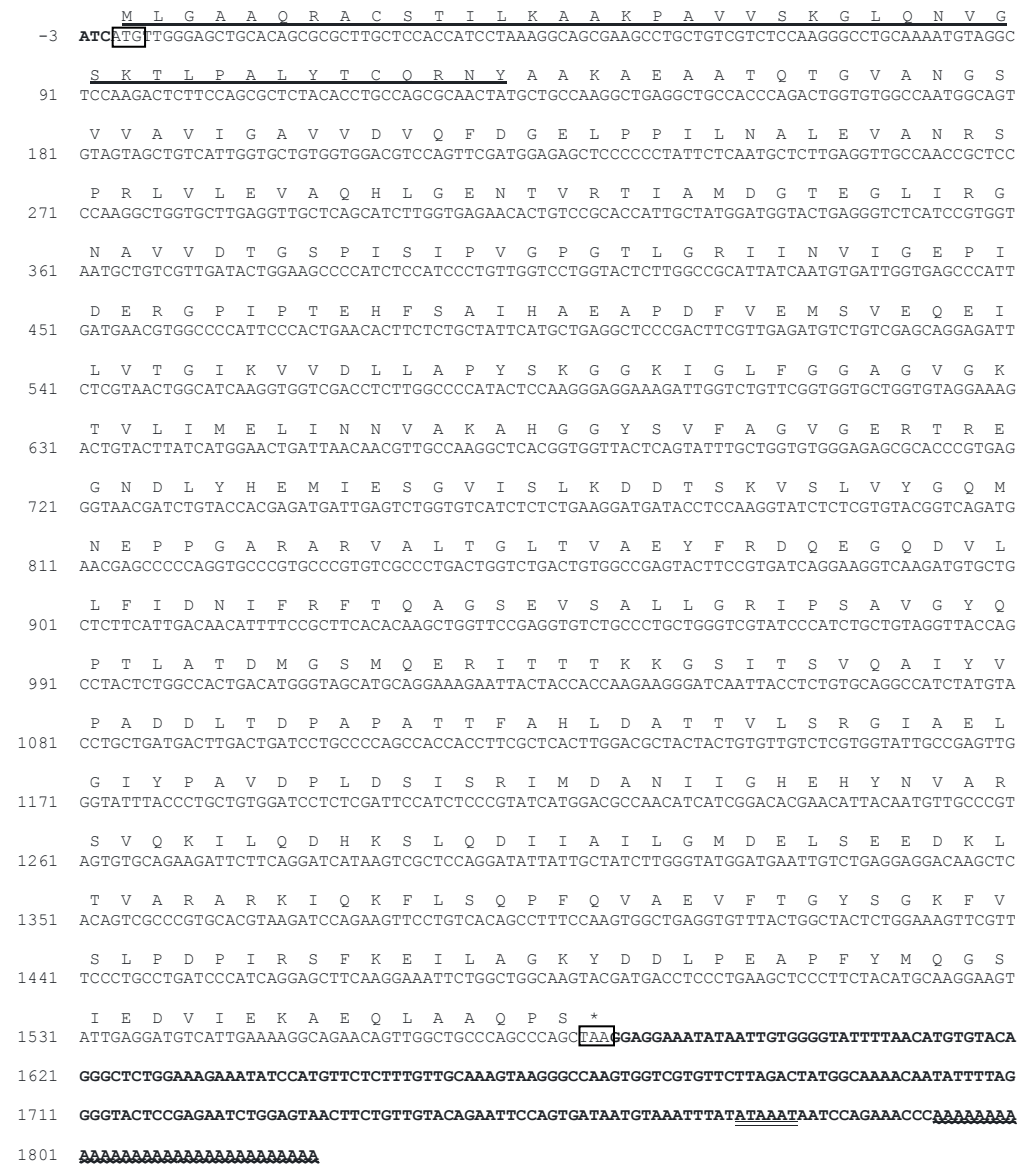
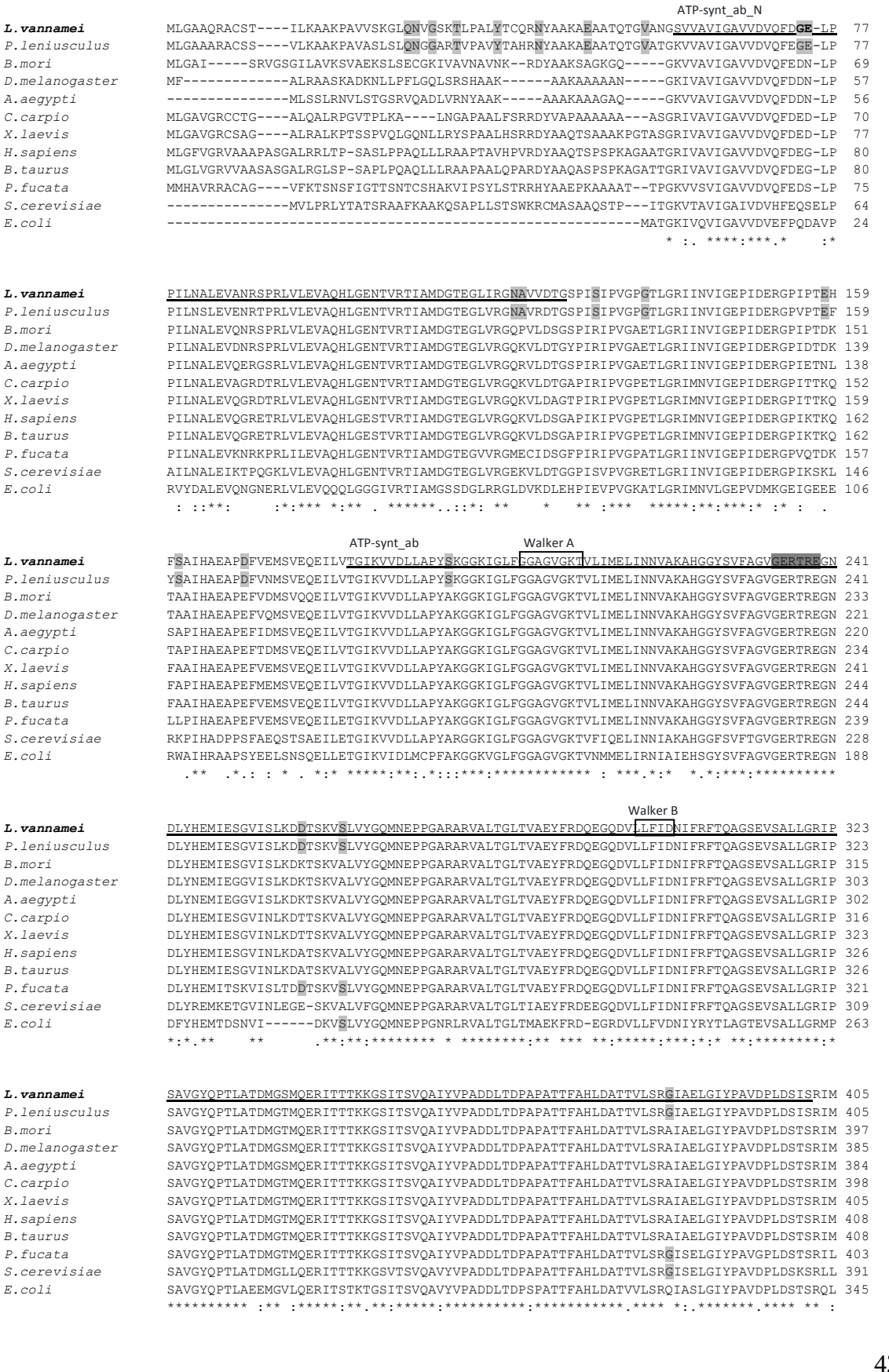


Figura 7. Secuencia de ADNc y deducida de aminoácidos de la subunidad ATPβ de camarón blanco *L. vannamei*. Los codones de inicio y término se encuentran enmarcados; la señal de poliadenilación se encuentra doblemente subrayada y la cola poli A está subrayada con ondas; los aminoácidos que corresponden al péptido señal se encuentran subrayados.


ATP-synt_ab_N
ATP-synt_ab
Walker A
Walker B
L.vannamei
P.leniusculus
B.mori
D.melanogaster
A.aegypti
C.carpio
X.laevis
H.sapiens
B.taurus
P.fucata
S.cerevisiae
E.coli

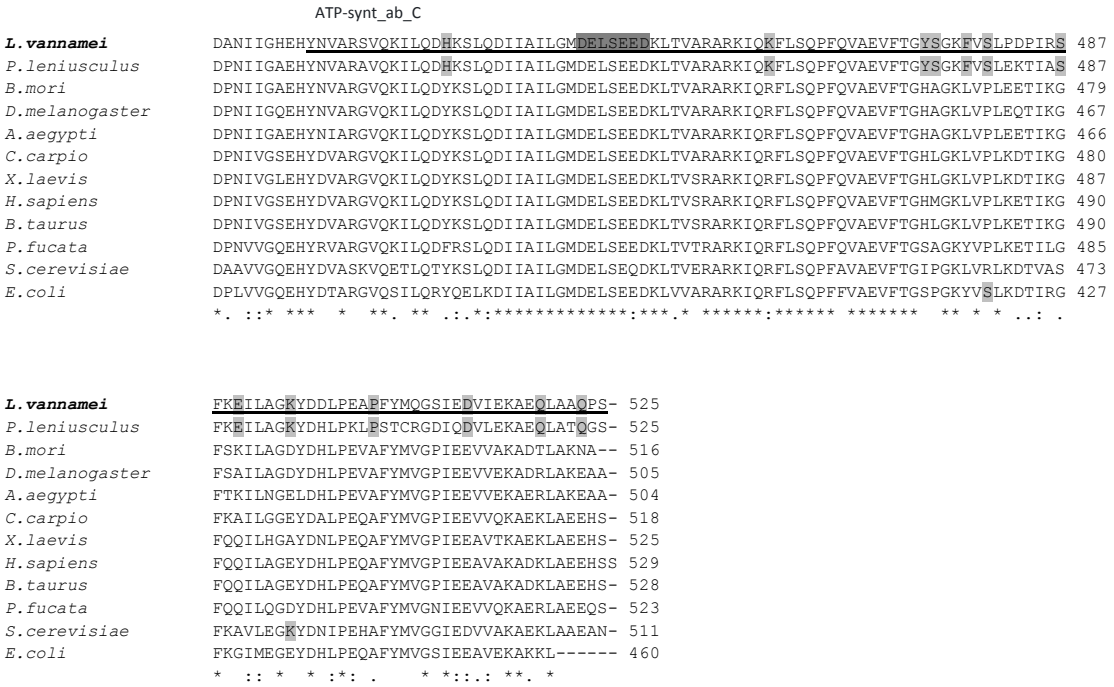


Figura 8. Alineamiento de la subunidad ATPβ de diversas especies de vertebrados e invertebrados y localización de los dominios y motivos conservados. Los residuos subrayados indican los tres dominios conservados; los residuos enmarcados representan los motivos y los residuos resaltados en gris representan las substituciones encontradas en invertebrados.

7.1.3 Subunidad γ (ATPγ): secuencia de ADNc y proteína deducida

La secuencia completa del ADNc de la subunidad *atpγ* de *L. vannamei* consta de 1382 pb, misma que incluye la región codificante de 867 pb incluyendo el codón de inicio ATG y el codón de término TAA. La secuencia que codifica al péptido señal de 31 residuos (10 cargados positivamente y 10 aminoácidos negativamente) es de 93 pb. La proteína madura es de 258 residuos correspondiendo a una masa molecular calculada de de 27.8 kDa (Tabla 4), codificado en 774 pb. Finalmente, se localiza una región no codificante de 515 pb hacia el extremo 3' (Fig. 9). El alineamiento múltiple de la secuencia de *atpγ* de *L. vannamei* arrojó un porcentaje de identidad del 65% con la secuencia reportada de *Aedes aegypti* (**XP 001653466**), un 67% con *Bombyx mori* (**XP 001040428**), un 64% con *Culex quinquefasciatus* y un 81% con *Bos taurus* (**AAA30398**). La

secuencia completa de ADNc se depositó en la base de datos GenBank con el número de acceso: **HM036579**.

En el alineamiento múltiple de la secuencia ATPγ del camarón con otras especies se detectaron sustituciones en varios residuos de la secuencia de *L. vannamei* como: Q→K85, Q→K86, C→A106 and A→K121, Q → I132, L→ Y137, G→ K139, S→ N140, A→ M143, Q→ V144, G→ N146, A→ P153, E→ L156, T→ N163, G→ T173, F→ I195, P→ S197, N→ E202 y A→ N251 (Fig. 10).

Figura 9. Secuencia de ADNc y deducida de aminoácidos de la subunidad ATPγ de camarón blanco *L. vannamei*. Los codones de inicio y término se encuentran enmarcados; la señal de poliadenilación se encuentra doblemente subrayada y la cola poli A está subrayada con ondas; los aminoácidos que corresponden al péptido señal se encuentran subrayados.

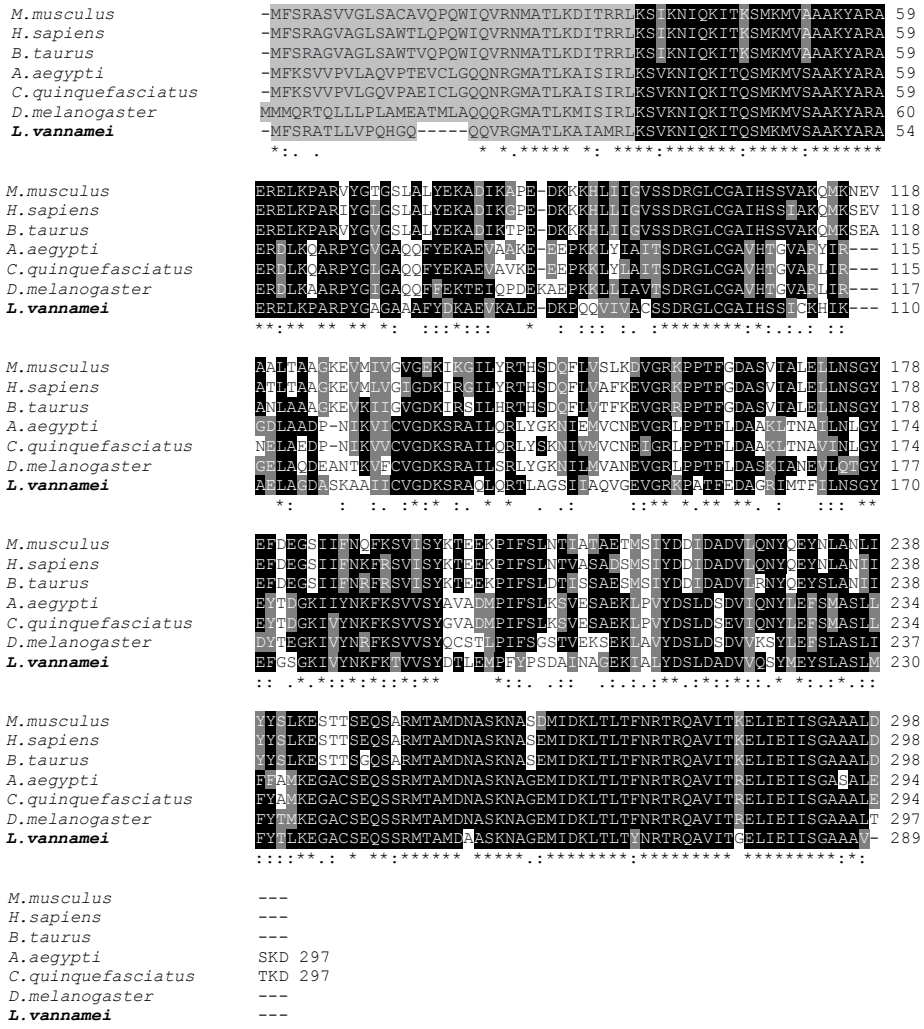


Figura 10. Alineamiento múltiple de secuencias de la subunidad ATPγ en diversas especies. Los residuos con identidad alta se encuentran marcados con asterisco (negro), los que presentan similitud alta con dos puntos (gris) y los residuos con similitud débil con punto (blanco). Además, al inicio del alineamiento se encuentra marcado el péptido señal hacia el extremo amino.

7.1.4 Subunidad δ (ATPδ): secuencia de ADNc y proteína deducida

La secuencia completa de ADNc de la subunidad *atpδ* de camarón es de 762 pb e incluye una región codificante de 474 pb (Fig. 11). Dentro de esta región codificante se localiza el péptido señal de 22 residuos (6 presentan carga positiva y 2 carga negativa) y la proteína madura de 136 residuos tiene una masa molecular calculada de 14.07 kDa (Fig. 12, Tabla 4). Finalmente, la secuencia no traducida hacia el extremo 3' está compuesta por 288 pb. La secuencia del ADNc de *atpδ* de *L.*

vannamei presenta un alto porcentaje de identidad con algunas especies de insectos como *A. aegypti* (60%, **XP_001655447**), *Tribolium castaneum* (71%, **XP_968285**), *Apis mellifera* (67%, **XP_625060**) y *Bos taurus* (56%, **NP_788843**). La secuencia completa de ADNc de *L. vannamei* se depositó en la base de datos GenBank con el número de acceso: **HM036580**.

Figura 11. Secuencia de ADNc y deducida de aminoácidos de la subunidad ATPδ de camarón blanco *L. vannamei*. Los codones de inicio y término se encuentran enmarcados; la señal de poliadenilación se encuentra doblemente subrayada y la cola poli A está subrayada con ondas; los aminoácidos que corresponden al péptido señal se encuentran subrayados.

En el alineamiento múltiple de secuencias de ATPδ del camarón con otras especies, se detectaron sustituciones en varios residuos de la secuencia de *L. vannamei* que se sugiere pudieran estar implicadas en posibles cambios estructurales como: A→E24, Q→A33, A→L129, N→S130, A→S131 y E→A150 (Fig. 12).

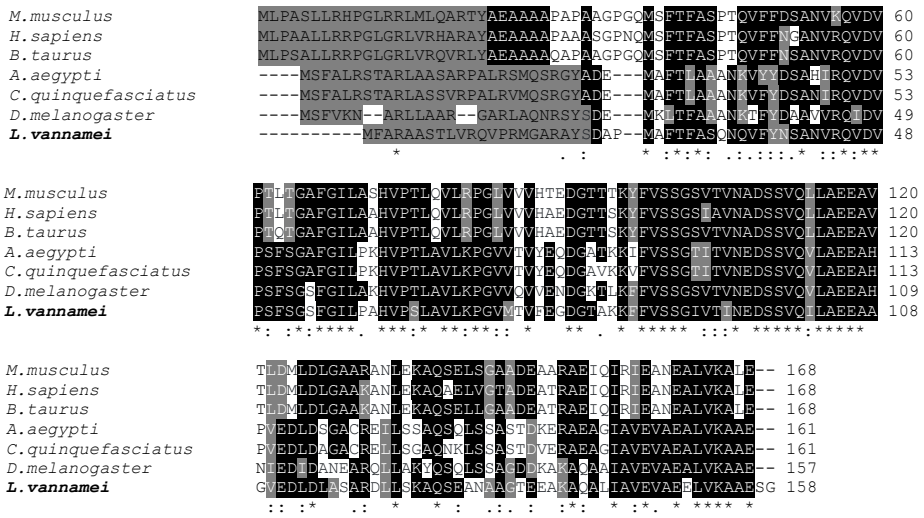


Figura 12. Alineamiento múltiple de secuencias de la subunidad ATPδ en diversas especies. Los residuos con identidad alta se encuentran marcados con asterisco (negro), los que presentan similitud alta con dos puntos (gris) y los residuos con similitud débil con punto (blanco). Además, al inicio del alineamiento se encuentra marcado el péptido señal hacia el extremo amino.

7.1.5 Subunidad ε (ATPε): secuencia de ADNc y proteína deducida

La secuencia completa de ADNc de la subunidad *atpε* en camarón es de 277 pb e incluye una región codificante de 168 pb y una región no traducida de 109 pb hacia el extremo 3' (Fig. 13). Está secuencia de ADNc presenta un alto porcentaje de identidad con algunas especies de insectos como *T. castaneum* (64%, **NP_001164282**), *Anopheles gambie* (63%, **XP_313974**), *Daphnia pulex* (59%, **EFX79623**) y con bovino *B. taurus* (54%, **NP_001137213**). La secuencia completa de ADNc se depositó en la base de datos GenBank con el número de acceso: **HM036581**.

La secuencia deducida de aminoácidos de la proteína consta de 55 residuos y representa una masa molecular calculada de 6.2 kDa (Tabla 3). En el alineamiento múltiple de secuencias de ATPε con otras especies se detectaron sustituciones en varios residuos de la secuencia de *L. vannamei* como: F→ A3, A→ N16, R→ K43, D→ N49 y A→ P52 (Fig. 14).

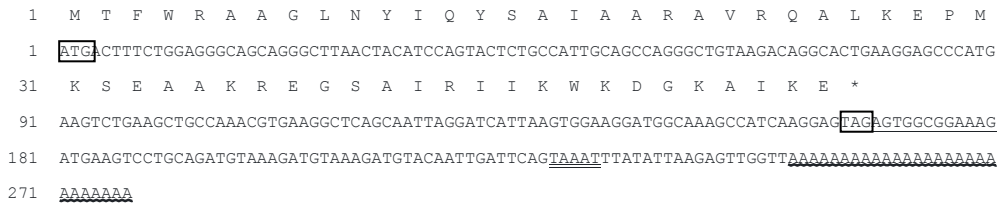


Figura 13. Secuencia de ADNc y deducida de aminoácidos de la subunidad ATPε de camarón blanco *L. vannamei*. Los codones de inicio y término se encuentran enmarcados; la señal de poliadenilación se encuentra doblemente subrayada y la cola poli A está subrayada con ondas; los aminoácidos que corresponden al péptido señal se encuentran subrayados.

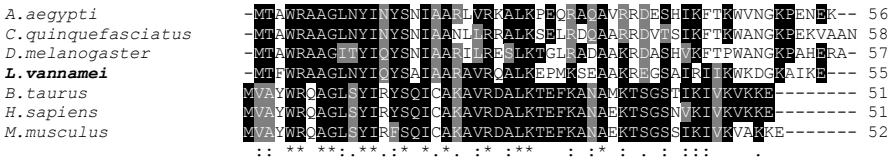


Figura 14. Alineamiento múltiple de secuencias de la subunidad ATPε en diversas especies. Los residuos con identidad alta se encuentran marcados con asterisco (negro), los que presentan similitud alta con dos puntos (gris) y los residuos con similitud débil con punto (blanco).

Tabla 4. Características del ADNc y la secuencia deducida de aminoácidos de las 5 subunidades de la porción F_1-ATPsintasa.

Subunidad	Secuencia de ADNc (pb)	Identidad[a]	Péptido señal (pb)	Proteína madura (pb)	3' UTR (pb)	Secuencia de la pre-proteína	Péptido señal (aa.)	Proteína madura (aa.)	Masa molecular calculada (kDa)[b]
ATPα	2118	*B. mori* 87% *A. aegypti* 84%	138	1515	465	550 aa	46 (8.36%)	504	54.49
ATPβ	1820	*P. leniusculus* 82%	129	1449	242	525 aa	43 (8.19%)	482	51.45
ATPγ	1382	*B. mori* 62% *A. aegypti* 65%	93	774	515	289 aa	31 (10.72%)	258	27.8
ATPδ	762	*A. aegypti* 66% *B. mori* 66% *C. quinquefasciatus* 66%	66	408	288	158 aa	22 (11.67%)	136	14.07
ATPε	277	*N. vitripennis* 68% *O. coriaceus* 54% *P. humanus corporis* 54%	--------	168	109	55 aa	----------	55	6.2

[a] Porcentaje de identidad arrojado por el algoritmo Blast (Altschul et al., 1990).

[b] Masa molecular calculada obtenida con las herramientas del sitio web Expasy (http://au.expasy.org/tools/).

En base a los resultados obtenidos para cada una de las subunidades de la porción F_1 de la ATP-sintasa, se tiene que en este complejo enzimático el tamaño de las subunidades decrece, sugiriendo que la complejidad de cada polipéptido se encuentra relacionada con la función que realiza en la enzima. Así, las subunidades que realizan directamente la catálisis enzimática, ATPα y ATPβ son las de mayor tamaño. En la tabla 3 la subunidad ATPγ se localiza en la tercera posición con 1382 pb y le siguen la subunidad ATPδ y ATPε con 762 y 277 pb, respectivamente.

El análisis de las secuencias nucleotídicas de cada subunidad arrojó porcentajes de identidad más altos con respecto a las secuencias pertenecientes a los insectos que a los vertebrados, lo que sugiere una estrecha relación filogénetica con este grupo, siendo el más cercano a *L. vannamei* (Martinez-Cruz et al., 2011). Las secuencias nucleotídicas fueron utilizadas para la construcción de un modelo estructural predictivo, que podrá brindar mayor información sobre la enzima y hacer inferencias sobre las interacciones entre subunidades e incluso con otros complejos enzimáticos.

7.1.6 Análisis del modelo estructural predictivo de la ATP-sintasa

La ATP-sintasa ha sido ampliamente estudiada en diferentes organismos como mamíferos, levaduras y bacterias (Runswick y Walker, 1983; Walker et al., 1985; Dyer et al., 1989; Runswick et al., 1990; Viñas et al., 1990; Chen et al., 2007), y se ha atribuido su alto porcentaje de identidad, entre las especies, debido a su capacidad de sintetizar el ATP.

En las Figuras 15 y 16 se muestra el modelo estructural predictivo de la porción F_1 de la ATP-sintasa de camarón blanco construido por homología con la porción F_1 de *Bos taurus*. El modelo molecular generado para la porción F_1 de la ATP-sintasa mostró una alta identidad con el modelo de bovino. En este modelo las secuencias de aminoácidos de las subunidades presentaron los siguientes porcentajes de identidad: ATPα (87.6%), ATPβ (86.1%), ATPγ (62%), ATPδ

(58.8%) y ATPε (53.2%). Las subunidades que conforman a la porción F_1 de la ATP-sintasa presentan alta identidad con especies de artrópodos y vertebrados, sin embargo, se detectaron diferencias en la composición de la secuencia deducida de aminoácidos, lo que sugiere características especie específicas del camarón *L. vannamei* que le han permitido a la enzima llevar a cabo su función en las condiciones de vida del organismo.

Se sabe que los complejos que participan en el transporte de electrones y en la fosforilación oxidativa no se encuentran distribuidos al azar en la membrana interna mitocondrial, sino que están ordenados en estructuras supramoleculares llamados respirosomas (Schägger y Pfeiffer, 2000). Se ha reportado que en la levadura *Saccharomyces cerevisiae* el complejo IV esta unido al complejo III de tres maneras distintas: 1) en dímeros, 2) en dos supercomplejos unidos a otro y 3) la interacción de dos monómeros del complejo VI, siendo la distribución de las diferentes formas dependiente de las condiciones de crecimiento. En los mamíferos la mayoría de los complejos I están ensamblados en supercomplejos con el complejo III y con hasta cuatro complejos IV (Schägger y Pfeiffer, 2000, 2001). Finalmente, se ha descrito en mamíferos, plantas (*Arabidopsis spp.)* y algas (*Chlamydomonas* y *Polytomella)* que la ATP-sintasa se encuentra formando dimeros, tetrámeros y hasta oligómeros del mismo complejo (Van Lis et al., 2003; Eubel et al., 2004; Dudkina et al., 2005; van Lis et al., 2005), y aunque a la fecha no se cuenta todavía con evidencia de estos supercomplejos en invertebrados marinos, se sugiere que la ATP-sintasa en el camarón está organizada de la misma manera dentro de la mitocondria, debido al alto porcentaje de identidad del modelo estructural de la enzima del camarón con respecto al modelo de bovino, sin embargo, se requiere de análisis más profundos y otro tipo de herramientas bioquímicas para corroborar esta afirmación.

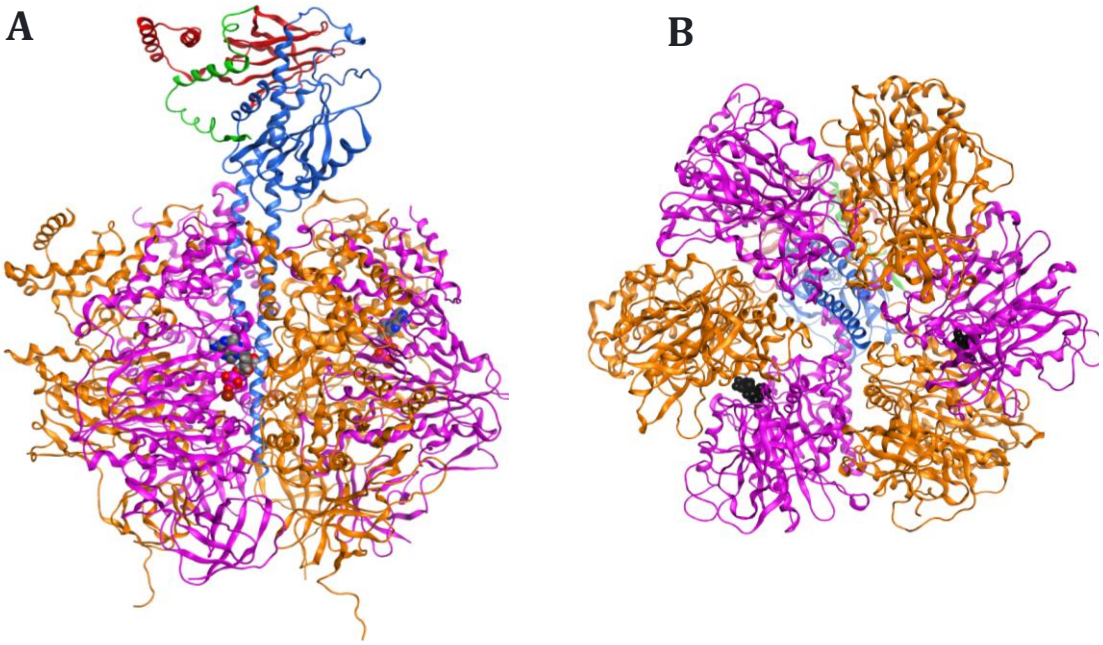


Figura 15. Diagrama de listones de la porción F_1 de ATP-sintasa de camarón blanco *L. vannamei*. A)vista frontal y B)vista desde la matriz mitocondrial. Se muestran las subunidades ATPα (anaranjado) y ATPβ (rosa) alternadas, y las subunidades que forman el rotor central ATPγ, ATPδ y ATPε (azul, verde y rojo, respectivamente).

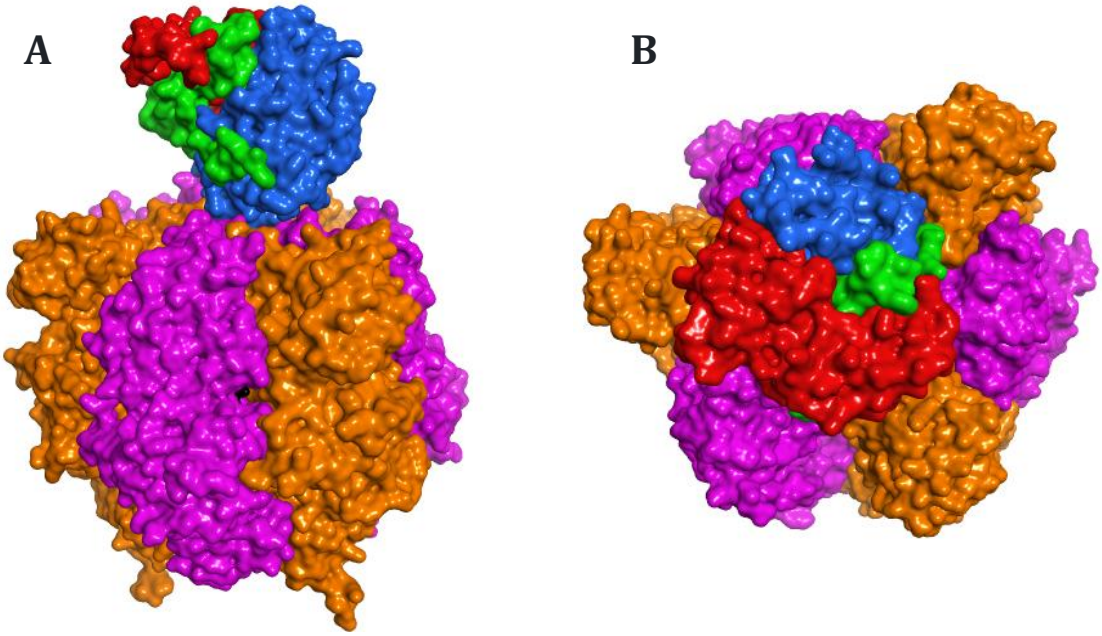


Figura 16. Representación espacial tridimensional de la porción F_1 de la ATP-sintasa de camarón blanco *L. vannamei*. A) vista frontal y B) vista desde el espacio intermembranal. Se muestran las subunidades ATPα (anaranjado) y ATPβ (rosa) alternadas, y las subunidades que forman el rotor central ATPγ, ATPδ y ATPε (azul, verde y rojo, respectivamente).

7.2 La Hipoxia Influye en la Concentración de Lactato en Plasma y en la Expresión Génica de las Subunidades de la Porción F_1 de la ATP-sintasa del Músculo de Camarón

7.2.1 Cuantificación de las concentraciones de lactato en plasma

En esta investigación se detectó un incremento del 54% en la concentración de lactato en plasma de los camarones expuestos a 1.5 mg/L de OD, comparados con los camarones a 6 mg/L de OD. Cuando los organismos fueron reoxigenados, la concentración de lactato disminuyó 10% comparado con los organismos en hipoxia (1.5 mg/L). Sin embargo, este porcentaje no representa un cambio significativo ($p<0.05$)(Fig. 17).

Los crustáceos en hipoxia tienen mayor movilidad aumentar la ventilación, branquias con mayor superficie respiratoria y cambios en la concentración de glucosa, proteína y lactato en plasma (Cheng et al., 2003; Abe et al., 2007). El lactato es el producto principal en el metabolismo anaerobio en los crustáceos, por lo tanto, varias especies presentan una alta concentración de este metabolito cuando se enfrentan a condiciones hipóxicas. Sin embargo, cuando las concentraciones de oxígeno se reestablecen, el lactato puede ser oxidado a CO_2 y H_2O, excretado o puede convertirse en glucógeno (Ellington, 1983).

Se ha reportado que después de la reoxigenación, las respuestas metabólicas son rápidas, observándose una disminución de los metabolitos en el plasma. En especies de crustáceos como *Eriocheir sinensis* (Zou et al., 1996), *Penaeus setiferus* (Rosas et al., 1999), *L. vannamei* (Racotta et al., 2002), *Marsupenaeus japonicus* (Abe et al., 2007), *Parastacus defossus* y *Parastacus brasiliensis* (da Silva-Castiglioni et al., 2011), se han reportado cambios rápidos en la concentración de lactato en plasma. Por lo tanto, los camarones respondieron al efecto progresivo de la hipoxia, incrementando la concentración de lactato en plasma, como se ha reportado en otros crustáceos.

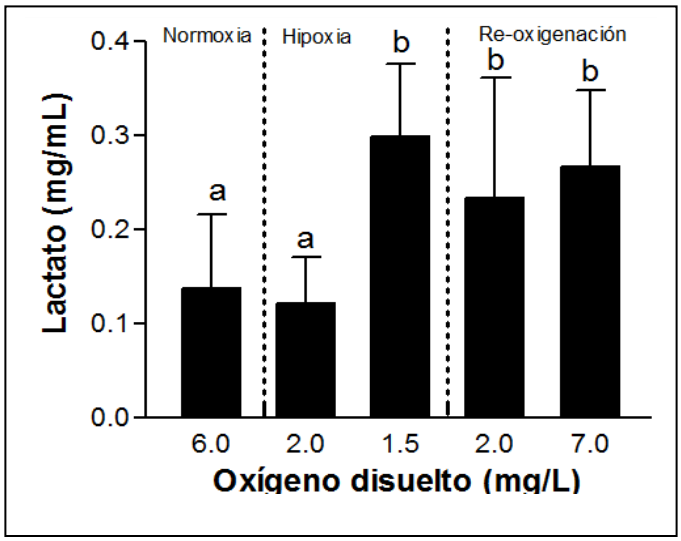


Figura 17. Concentración de lactato en el plasma del camarón blanco *L. vannamei* bajo el efecto de la hipoxia. Las diferentes literales representan diferencias significativas entre tratamientos ($p<0.05$).

7.2.2 Expresión relativa de las subunidades de la F_1-ATPsintasa

De manera general, en el músculo del camarón *L. vannamei* no se detectaron cambios significativos en la expresión relativa de las subunidades de la porción F_1 de la ATP-sintasa por efecto de la hipoxia. Sin embargo, los resultados muestran un patrón de expresión en respuesta a las diferentes concentraciones de oxígeno disuelto. Se identificó una reducción del 20-60% a 2 mg/L con respecto a la normoxia, un incremento de 110% a 1.5 mg/L (Fig. 18C) comparado con los camarones a 2 mg/L y finalmente durante la reoxigenación (7 mg/L) se detectó una reducción en la expresión del 15 hasta un 114% (Fig. 18).

El efecto de la hipoxia en la expresión génica de las subunidades que conforman el transporte de electrones y la fosforilación oxidativa ha sido poco estudiada en los crustáceos. Muhlia-Almazan et al. (2008) al evaluar la expresión génica de algunas subunidades de la ATP-sintasa en camarón blanco en diferentes

tejidos, reportaron que el incremento en la expresión de las subunidades es tejido-específica y que estas respuestas están ligadas a la función que desempeña el tejido. Aunado a esto, el que no se detecten cambios significativos en la expresión de las subunidades de la ATP-sintasa en el músculo de camarón, se debe a que el organismo cuenta con otras estrategias adaptativas que le permiten sobrevivir bajo condiciones de estrés.

Por ejemplo, en el camarón *Palaemonetes pugio* se evaluaron los cambios en la expresión de genes mitocondriales bajo el efecto de hipoxia crónica y los resultados mostraron que el camarón se adapta a los efectos de la hipoxia, conservando energía debido a la disminución de la síntesis de proteínas (Brouwer et al., 2007). En camarón chino *Fenneropenaeus chinensis* se evaluaron los perfiles proteómicos del hepatopáncreas en respuesta a la hipoxia, y reportó una disminución en la subunidad ATPα de la ATP-sintasa y de la citrato sintasa. El decremento de estas dos enzimas podría reflejar una depresión severa de la respiración aeróbica debido a ausencia del oxígeno en los camarones durante la hipoxia (Jiang et al., 2009).

Recientemente Martinez-Cruz et al. (2011) reportaron un incremento en la expresión en la subunidad *atpβ* en pleópodos del camarón blanco a 1.5 mg/L, sin embargo, en branquias la expresión tanto de la subunidad α como β disminuyó bajo el mismo estímulo. De tal manera, que se sugiere que los cambios en la expresión de las subunidades de la ATP-sintasa son parte de los mecanismos biológicos que permiten al camarón tolerar el efecto de la hipoxia.

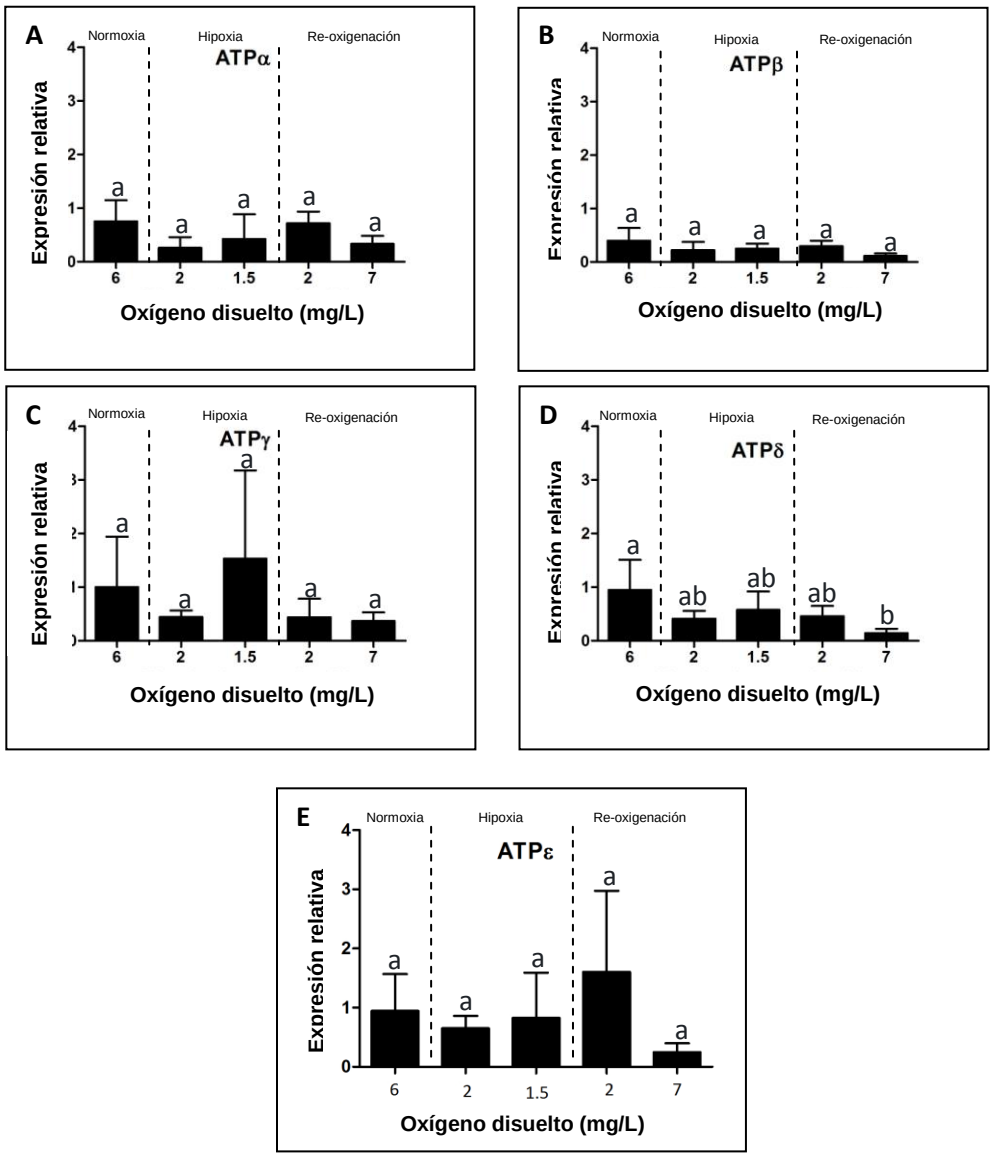


Figura 18. Expresión génica de las cinco subunidades de la porción F_1 de la ATP-sintasa en músculo de camarón en respuesta a la hipoxia. Las diferentes literales representan diferencias significativas entre tratamientos ($p<0.05$).

7.3 Efecto de la Hipoxia en la Subunidad ATPβ de la ATP-sintasa presente en los Extractos Mitocondriales del Músculo de Camarón

7.3.1 Aislamiento e identificación de la porción F_1 de la ATP-sintasa

Se aislaron las mitocondrias del músculo del camarón *L. vannamei*, obteniéndose concentraciones de proteína mitocondrial de alrededor de 10 µg/mL a partir de 50 g de tejido. Además, se separó el complejo ATP-sintasa presente en los extractos mitocondriales usando electroforesis en condiciones nativas (BN-PAGE). Está técnica permitió detectar una banda superior a los 669 kDa (Fig. 19), la cual coincide con la masa molecular reportada para la ATP-sintasa de algunos vertebrados incluyendo al bovino, la rata (Wittig y Schägger, 2008) y para procariotes como *E. coli* (Mueller et al., 2004).

También se detectó actividad de ATPasa en gel en una banda con tamaño superior a los 669 kDa. capaz de hidrolizar el ATP, dando como resultado la formación de precipitado, lo que sustenta la presencia de la porción F_1 catalíticamente activa en los extractos mitocondriales (Fig. 19A). En la Figura 19B se muestra el patrón electroforético en condiciones desnaturalizantes y reductoras, de las subunidades que conforman a la ATP-sintasa. La masa molecular de las proteínas se encuentra dentro de un rango de 40 a 66 kDa, correspondiente a la masa molecular calculada para las subunidad ATPβ (Martinez-Cruz et al., 2011).

Se identificó a la subunidad ATPβ por inmunodetección del conjunto de proteínas presentes en el gel de poliacrilamida en condiciones desnaturalizantes y reductoras. En la Figura 19C se muestra una sola banda de aproximadamente 51 kDa, que corresponde a la masa molecular calculada para la subunidad ATPβ (Martinez-Cruz et al., 2011). Con estos resultados se confirmó que los anticuerpos policlonales son específicos para la ATPβ de camarón, mismos que se utilizaron para cuantificar a la subunidad en los extractos mitocondriales de camarón bajo el efecto de la hipoxia.

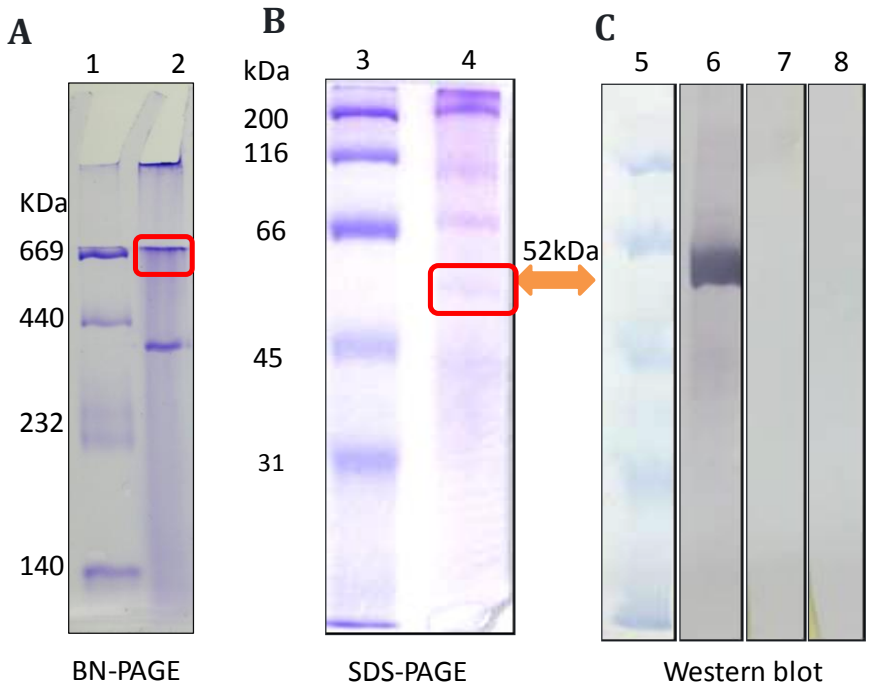


Figura 19. A) Identificación de la ATP-sintasa mitocondrial en condiciones nativas, B) Identificación de la subunidad ATPβ en condiciones desnaturalizantes y reductoras e C) Inmunodetección de la subunidad ATPβ en el músculo del camarón. 1) Marcador nativo, 2) extracto mitocondrial, 3) Marcador de amplio rango, 4) subunidades correspondientes a la ATP-sintasa, 5) Marcador de amplio rango transferido, 6) detección de la subunidad ATPβ del resto de las subunidades correspondientes a la ATP-sintasa, 7) control sin anticuerpo primario y 8) control sin anticuerpo secundario.

7.3.2 Cambios en la concentración de la subunidad ATPβ

No se detectaron diferencias significativas ($p>0.05$) en la concentración de la subunidad ATPβ en el músculo de camarón bajo el efecto de la hipoxia (Fig. 20). Esto concuerda con los resultados obtenidos en la evaluación de la expresión génica de la subunidad ATPβ, lo que sugiere una disminución en el consumo de energía necesaria para llevar a cabo la transcripción del ADN y traducción del ARNm. Aunado a la habilidad del camarón, y a varias especies de invertebrados, para reducir y cambiar su metabolismo a un nuevo estado hipometabolico, durante la hipoxia, y con ello reducir las demandas de ATP (Storey y Storey, 1990). Se ha reportado que en *Artemia franciscana* la síntesis de proteínas mitocondriales y

citoplasmáticas disminuye hasta un 90% en respuesta a la anoxia (Kwast y Hand, 1996a, b).

Se atribuye que la supresión coordinada de la síntesis de las proteínas es controlada por la combinación de factores como la concentración de oxígeno y la disminución del pH intracelular (Kwast y Hand, 1993, 1996a). Bailey y Driedzic, (1996) también reportaron una reducción de la síntesis de proteínas en la fracción mitocondrial de corazón de tortugas anóxicas, sin embargo, no se detectó ningún cambio en la capacidad respiratoria mitocondrial. Estos estudios previos revelan varias respuestas con respecto al impacto de la baja concentración de oxígeno en el metabolismo.

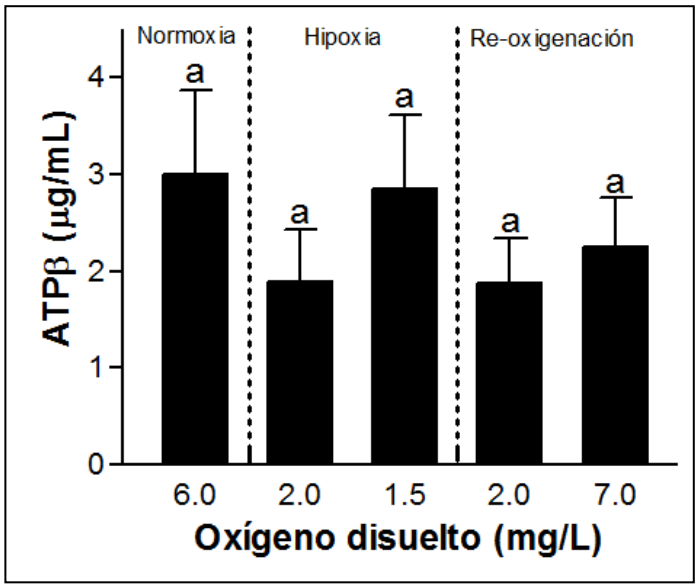


Figura 20. Concentración de la subunidad ATPβ en el extracto mitocondrial del músculo de camarón bajo el efecto de la hipoxia. Las diferentes literales representan diferencias significativas entre tratamientos ($p<0.05$).

7.4 La Actividad ATPasa y la Concentración de ATP en el Músculo de Camarón se Incrementan por Efecto de la Hipoxia

7.4.1 Efecto de la hipoxia en la actividad ATPasa

Se detectaron diferencias significativas en la actividad ATPasa de los extractos mitocondriales por efecto de la hipoxia. Se detectó un incremento del 70% en la actividad ATPasa de los camarones en hipoxia a 2 mg/L con respecto a los animales en normoxia (Fig. 21). Di Lisa et al. (1995) reportaron que en los organismos expuestos a la hipoxia, la producción de energía es suprimida dentro de la mitocondria debido a la reducción en la fosforilación oxidativa y con ello se reprime la síntesis de ATP. Además, bajo este mismo estímulo el ATP proveniente de la glucólisis es utilizado por la ATP-sintasa, la cual lo hidroliza con la finalidad de mantener el potencial intermembranal. Asimismo, la ATP-sintasa puede hidrolizar el ATP presente en la matriz mitocondrial, cuando el intercambio de ATP entre los compartimentos intracelulares cesa. De la misma forma, se detectó un decremento del 70% de la actividad en los organismos reoxigenados (7 mg/L) con respecto a normoxia (Fig. 21). Este resultado sugiere que la síntesis de ATP mitochondrial continua vía cadena de transporte de electrones acoplada a la fosforilación oxidativa, al restablecerse el gradiente electroquímico en el espacio intermembranal (Budinger et al., 1998).

Por otro lado, se evaluó la actividad citrato sintasa de los extractos debido a que su actividad en otros organismos no sufre fluctuaciones bajo condiciones de estrés o patológicas (Pon y Schon, 2007). Por esta razón, se ha reportado la normalización de la actividad con la citrato sintasa, cuando se utilizan homogenados o fracciones mitocondriales impuras para determinaciones enzimáticas, con la finalidad de prevenir artefactos debido a las diferencias en el contenido mitocondrial (Pon y Schon, 2007)(Fig. 22).

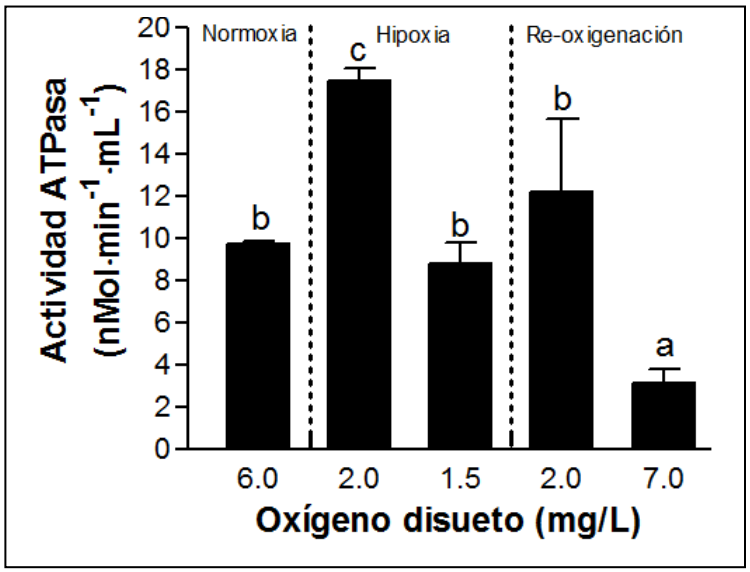


Figura 21. Actividad ATPasa evaluado en los extractos mitocondriales del músculo de camarón bajo el efecto de la hipoxia. Las diferentes literales representan diferencias significativas entre tratamientos ($p<0.05$).

En cada uno de los tratamientos se detectó un incremento en la actividad ATPasa con respecto a la actividad citrato sintasa, que fue empleada como control interno de la actividad. Lo que sugiere que la actividad ATPasa se ve modificada por el efecto de la concentración de oxígeno y que la actividad de la citrato sintasa puede usarse en el camarón como en otros organismos, como una enzima de control interno en la mitocondria.

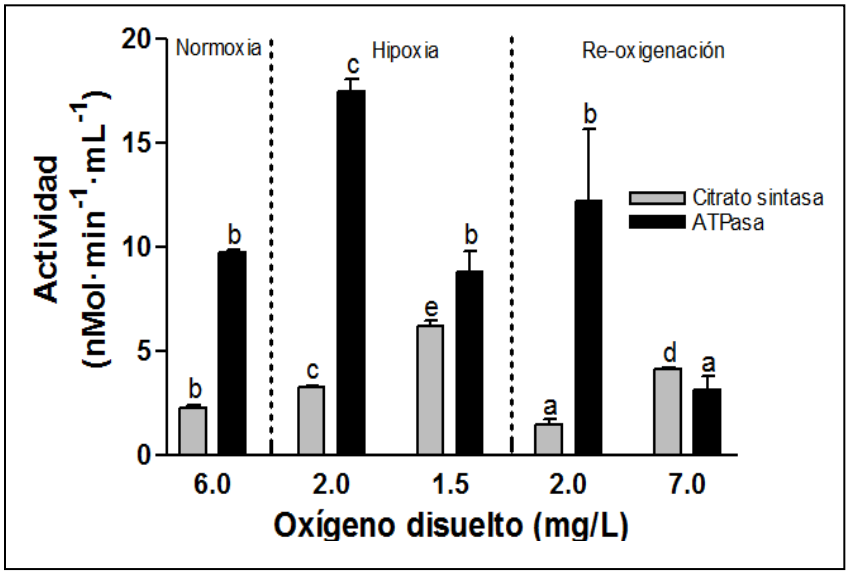


Figura 22. Evaluación de la actividad ATPasa y citrato sintasa en los extractos mitocondriales de camarón bajo el efecto de la hipoxia. Las diferentes literales representan diferencias significativas entre tratamientos para cada enzima ($p<0.05$).

7.4.2 Efecto de la hipoxia en la concentración de ATP

Las concentraciones de ATP pueden disminuir modesta o drásticamente dependiendo de la habilidad de las especies para ajustarse a los cambios en la concentración de oxígeno del ambiente (Hochachka et al., 1996). Cuando se evaluó la concentración de ATP en el músculo no se detectaron diferencias significativas por efecto de la hipoxia, sin embargo, una vez que el sistema fue reoxigenado (7 mg/L), la concentración de ATP disminuyó un 25% representando una diferencia significativa con respecto a los organismos expuestos a 2 mg/L (Fig. 23).

Esta reducción tardía de la concentración de ATP es resultado de la respuesta de los camarones al efecto de la hipoxia por periodos de tiempo largos, lo que produce una falla en la cadena respiratoria y provoca una reducción de la energía, como se ha reportado en vertebrados como mamíferos y peces (Di Lisa et al., 1995; Itoi et al., 2003), de tal manera que el organismo puede utilizar fuentes alternas de energía durante la hipoxia (Hill et al., 1991; Speed et al., 2001).

En invertebrados, la enzima arginino cinasa (EC. 2.7.3.3) es una cinasa fosfágeno que cataliza la transferencia reversible de un grupo fosforil del Mg^{2+}ATP a la arginina dando como resultado arginina fosfato y Mg^{2+}ADP (Pan et al., 2004). La arginina fosfato funciona como un reservorio de energía, generando ATP cuando se detecta una disminución aguda de este compuesto (Morris et al., 2005). Los camarones optan por otras estrategias que le permitan enfrentar déficits severos de energía causados por la represión de la respiración aerobia, como el consumo de arginina fosfato o incrementando la digestión de alimentos para generar suficiente glucosa que pueda ser metabolizada. Estas estrategias solo producen suficiente energía para resistir un cambio drástico de ATP mientras se reestablece el sistema (Hochachka et al., 1996).

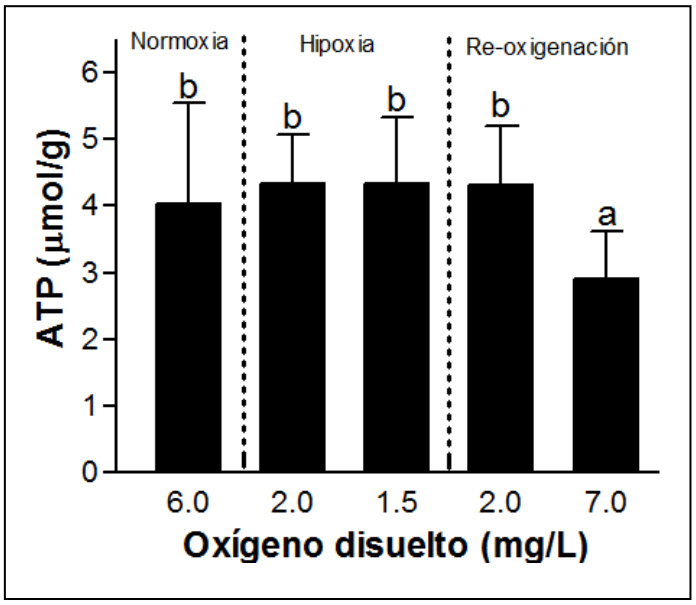


Figura 23. Cantidad de ATP presente en el músculo de camarón *L. vannamei* bajo el efecto de la hipoxia. Las diferentes literales representan diferencias significativas entre tratamientos ($p<0.05$).

VIII. CONCLUSIONES Y RECOMENDACIONES

- Se abordó el estudio de la porción F_1 de la ATP-sintasa en músculo de camarón blanco *L. vannamei*. Se obtuvieron las secuencias completas de ADNc y se evaluó la expresión génica de cada una de las subunidades que integran esta porción bajo el efecto de la hipoxia. Además, se propuso un modelo estructural de esta misma porción. Se aislaron mitocondrias del músculo de camarón y se identificó el complejo ATP-sintasa por técnicas bioquímicas e inmunológicas. También se evaluó el efecto de la hipoxia en la concentración de lactato en plasma, proteína mitocondrial (ATPβ), ATP y en la actividad enzimática de la ATP-sintasa.

- Las subunidades de la porción F_1 de la ATP-sintasa en camarón son muy conservadas y tienen los dominios y motivos (unión de ADP, Pi y ATP) necesarios para realizar la catálisis de ATP.

- La expresión génica de las subunidades bajo el efecto de la hipoxia es de manera tejido-específica, ya que no se detectaron cambios en el músculo, pero se sabe que la hipoxia afecta la expresión de las subunidades de la ATP-sintasa en otros tejidos como la glándula digestiva.

- El efecto de la hipoxia se ve reflejado en el incremento de la actividad ATPasa, por lo tanto la ATP-sintasa incrementa su capacidad de hidrolizar el ATP con la finalidad de mantener el gradiente electroquímico intermembranal en la mitocondria.

- Debido a que no se encontraron diferencias en la concentración de ATP durante la hipoxia, se propone que el camarón hace uso de reservas energéticas como la arginino fosfato con la finalidad de mantener las concentraciones de ATP en el músculo. Sin embargo, aun es importante evaluar la concentración de este fosfágeno y atribuir el efecto amortiguador del ATP.

- Con base en lo anterior, se da certeza a la hipótesis en lo referente a la hidrólisis del ATP por la ATP-sintasa para mantener el potencial electroquímico dentro de la mitocondria, y con ello la homeostasis, pero no en lo referente al efecto de la hipoxia en la cantidad de ARNm y en las subunidades de la enzima, debido a que no se detectaron cambios estadísticamente significativos.

- Es importante evaluar el inhibidor intrínseco IF1, debido a que se ha reportado que esta proteína interviene en la regulación de la enzima cuando la concentración de oxígeno disminuye. Además, resulta importante adentrarse en la caracterización bioquímica con la finalidad de incrementar el conocimiento de la ATP-sintasa de los crustáceos.

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ANEXOS

ANEXO I. Martinez-Cruz, O.; Garcia-Carreño, F.; Robles-Romo, A.; Varela-Romero, A. & Muhlia-Almazan, A. (**2011**). Catalytic Subunits *atpα* and *atpβ* from the Pacific White shrimp *Litopenaeus vannamei* FoF_1 ATP-synthase complex: ADNc Sequences, Phylogenies, and mRNA Quantification During Hypoxia. *J. Bioenerg. Biomembr.*, 43:119-133.

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Catalytic subunits *atpα* and *atpβ* from the Pacific white shrimp *Litopenaeus vannamei* F_OF_1 ATP-synthase complex: cDNA sequences, phylogenies, and mRNA quantification during hypoxia

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Abstract In the mitochondrial F_OF_1 ATP-synthase/ATPase complex, subunits α and β are part of the extrinsic portion that catalyses ATP synthesis. Since there are no reports about genes and proteins from these subunits in crustaceans, we analyzed the cDNA sequences of both subunits in the whiteleg shrimp *Litopenaeus vannamei* and their phylogenetic relationships. We also investigated the effect of hypoxia on shrimp by measuring changes in the mRNA amounts of *atpα* and *atpβ*. Our results confirmed highly conserved regions for both subunits and underlined unique features among others. The ATPβ deduced protein of shrimp was less conserved in size and sequence than ATPα. The relative mRNA amounts of *atpα* and *atpβ* changed in shrimp pleopods; hypoxia at 1.5 mg/L caused an increase in *atpβ* transcripts and a subsequent decrease when shrimp were re-oxygenated. Results confirm that changes in the mRNAs of the ATP-synthase subunits are part of the mechanisms allowing shrimp to deal with the metabolic adjustment displayed to tolerate hypoxia.

Keywords *atpα* subunit · *atpβ* subunit · F_OF_1 ATP synthase · Hypoxia · Shrimp

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Introduction

The mitochondrial F_OF_1 ATP-synthase complex is a multimeric enzyme that catalyzes the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i). This catalytic process is triggered by the electrochemical gradient of protons generated along the electron transport chain in the mitochondrion (Pedersen 2007). This enzyme synthesizes 95% of the ATP molecules in cells, and its function is closely related to the presence of oxygen that takes electrons yielded by this electron transport chain (Alberts et al. 2008). The F_OF_1 ATP synthase is formed by two major components, a catalytic headpiece F_1, and a base-piece/stalk membrane-embedded F_O. Minor components involve subunits which are encoded in the nucleus and in the mitochondrion genome (Walker et al. 1991). As other nucleus-encoded proteins, the ATP-synthase subunits posses a short N-terminal sequence that signals their importation into the mitochondrion (Schatz and Butow 1983). The catalytic building block of F_1 is formed by a subcomplex of 3α and 3β subunits (Abrahams et al. 1994; Boyer 1997); all of them are encoded in the nuclear genome, translated in the cytoplasm, and imported into the mitochondrion as pre-proteins (Walker and Runswick 1983; Breen 1988).

In eukaryotic organisms the main regulatory mechanisms that act in the oxidative phosphorylation system (OXPHOS) include substrate availability (NADH, ADP), membrane potential, allosteric regulation, reversible phosphorylation, and the expression of tissue-specific isoforms (Hüttemann et al. 2008). In the ATP-synthase complex, subunits α and β have been deeply studied in animal and plant models because they directly participate during catalysis and the existence of various isoforms from both genes have been confirmed in some species to participate during enzyme regulation in a tissue-function-specific manner (Kataoka and Biswas 1991; Lalanne et al. 1998; Hoskins et al. 2007).

Studies on the mitochondrial F_OF_1 ATP synthase from invertebrate species are scarce, especially in crustaceans. One complete mRNA sequence of *atpβ* in the crayfish *Pacifastacus leniusculus* has been reported in the GenBank (Accession Number DQ874396), and few partial sequences of *atpα* from crustaceans have been found as ESTs (expressed sequences tags) in the same data base.

Marine crustaceans are commonly exposed to fluctuating dissolved oxygen concentrations (OC) in water along their life cycle. Hypoxia is the condition that results from low oxygen concentrations in marine coastal waters affecting organisms fitness and survival (Wu et al. 2002). The biological responses of crustaceans to hypoxia include a metabolic reorganization, the switch to anaerobic metabolism, avoidance behaviour, increase in the frequency of ventilatory movements and metabolic rate depression (Morris et al. 2005).

In this study we analyzed, for the first time, the *atpα* and *atpβ* subunits of the mitochondrial F_OF_1 ATP-synthase complex from the Pacific white shrimp *Litopenaeus vannamei* to obtain basic information that includes the description of the complementary DNA (cDNA) sequences, phylogenetic relationships, the steady-state mRNA detection of both subunits in gills and pleopods, and the effect of hypoxia on the expression of the codifying genes to obtain new insights related to the organization of ATP synthesis in this marine invertebrate.

Materials and methods

Animals and bioassays

A bioassay was conducted in the laboratory with adult *L. vannamei* shrimp weighing 30±1 g. Shrimp were acclimatized to laboratory conditions for 8 days in marine water at 28 °C, 35 ppt salinity, constant aeration at 6 mg/L OC, and commercial food was supplied twice a day. After acclimation, five shrimp were decapitated and gills were dissected and submerged in 250 μL of TRIzol reagent (Invitrogen, Carlsbad, CA) for total RNA isolation and cDNA sequencing purposes.

A preliminary assay was conducted to determine the gradually decreasing of dissolved OC in water, as it occurs in coastal waters or aquaculture ponds, by removing air-stones from an experimental 1,000 L tank containing 30 shrimp at a constant water volume. Dissolved oxygen concentration in water was continuously measured with a digital oxymeter until water reached the lowest OC value recorded (1.5 mg/L), which remained constant.

The hypoxia assay was conducted using 270 adult shrimp that were randomly distributed in nine 1,000 L tanks (n=30 each) and kept under normoxia (6 mg/L). After acclimation, all shrimp were starved for 24 h, then three tanks were kept at normoxia as the control group and three shrimp were collected from each tank. The air supply was stopped in the six remaining tanks to induce hypoxia. Three shrimp were collected from each tank when OC decreased to 4.0, 2.0, and 1.5 mg/L with intervals of 4–5 h between samplings. Then, the six tanks were gradually re-oxygenated by placing air-stones in each tank, and when the OC reached 7 mg/L, three last shrimp were collected.

Molting stages were previously determined in all shrimp according to the setogenesis phase (formation of new seta in uropods) (Chan et al. 1988). All experimental organisms included in the assay were at intermolt stage. Once collected, shrimp were decapitated and gills and pleopods were dissected and stored in TRIzol reagent (Invitrogen, Carlsbad, CA) at −80 °C until used.

Each shrimp was weighed and 400 μL of hemolymph were extracted from the base of the fifth pereiopod with a 1-mL syringe containing two volumes of pre-cooled shrimp anticoagulant solution containing 300 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA at pH 7.3 (Vargas-Albores et al. 1993). Each hemolymph sample was centrifuged at 7,000 × g for 10 min at 4 °C. Plasma and hemocytes were separated, and both were stored at −80 °C until used. Glucose and lactate concentrations were measured with commercial kits for medical diagnosis (RANDOX, GL2614 and LC2389, Antrim, UK) as indicators of the hypoxia effect on shrimp plasma.

Atpα and *atpβ* cDNA sequencing

Total RNA was isolated from gills using TRIzol reagent following the manufacturer instructions. Five μg of total RNA were used to synthesize cDNA using the GeneRacer kit as specified by the manufacturer (Invitrogen, Carlsbad CA). The cDNA was used as a template for PCR amplifications. Various specific oligonucleotides were designed for each subunit based on mRNA and ESTs sequences available at the GenBank for *atpα* (BF024234, FE066413, and FE100354, Gross et al. 2001), and for *atpβ*

(DQ874396) from crustacean and other invertebrate species (Table 1).

PCR amplifications were carried out to obtain the full cDNA sequence of both subunits. A BD Advantage 2 polymerase mix (BD Biosciences Clontech, Palo Alto, CA) was used in a total volume reaction of 25 μL that included 1 μL of cDNA (equivalent to 250 ng total RNA), specific forward or reverse oligonucleotides (Table 1), and one of the forward or reverse oligonucleotides provided in the RACE kit to amplify 5′- and 3′- ends. A thermocycler (DNA Engine, BioRad, Hercules, CA) was used at the following conditions: 3 min at 94 °C (1 cycle); 30 s at 94 °C, 30 s at 60–62 °C, and 1 min at 68 °C for (30 cycles). The resulting PCR products were analyzed in 1.5% agarose gels (Sambrook and Russell 2001) and stained with SYBR Safe (Invitrogen, Carlsbad, CA). PCR products were purified and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced at Macrogene Inc. (Seoul, Korea).

The predicted amino acid sequence of both subunits was obtained in the web site http://au.expasy.org/tools/. Sequence analyses were performed using Blast (N, X and P), and Clustal W algorithms (Thompson et al. 1994; Altschul et al. 1997). The Mitoprot software was used to predict and analyze the mitochondrial protein sequences (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html; Claros and Vincens 1995).

Phylogenetic analysis

Phylogenetic relationships of ATPα and ATPβ were determined including both complete sequences from *L. vannamei* (ATPα GQ848643 and ATPβ GQ848644); we included available sequences of ATPα: *Drosophila melanogaster* (NP_726243); *Drosophila yakuba* (XP_002092136); *Bombix mori* (NP_001040233); *Pediculus humanus corporis* (EEB14270); *Aedes aegypti* (XP_001655906); *Mus musculus* (NP_031531); *Xenopus laevis* (NP_001081246); *Xenopus tropicalis* (NP_001025610); *Danio rerio* (NP_001070823); *Salmo salar* (ACN10935); *Caenorhabditis elegans* (NP_001021526); *Anopheles gambiae* (XP_314018); *Ixodes scapularis* (EEC16574); *Bos taurus* (NP_777109); *Homo sapiens* (NP_004037); *Escherichia coli* (AAA24735); *Pinctada fucata* (ABJ51956); *Gallus gallus* (NP989617); *Saccharomyces cerevisiae* (NP_009453), and ATPβ: *D. melanogaster* (CAA50332); *D. yakuba* (XP_002099634); *Drosophila pseudoobscura pseudoobscura* (EAL29273); *B. mori* (NP_001040450); *P. humanus corporis* (EEB19469); *A. aegypti* (XP001656737); *Culex quinquefasciatus* (XP_001847944); *M. musculus* (AAB86421); *B. taurus* (NP_786990); *H. sapiens* (AAA51808); *X. laevis* (NP_001080126); *X. tropicalis* (NP_001001256); *D. rerio* (NP_001019600); *C. elegans* (NP_498111); *I. scapularis* (EEC17118); *Pacifastacus leniusculus* (ABI34071); *P. fucata* (ABC86835); *S. cerevisiae* (NP_012655); and *E. coli* (AAA24737).

All sequences were aligned using Clustal W algorithm (Thompson et al. 1994) and construction of phylogenetic hypothesis from the dataset was done using the neighbor joining (NJ), and maximum parsimony (MP) methods. Both, nucleotide and amino acid sequences were used during constructions, and as the same topology was observed for both criteria, only the amino acid trees were analyzed.

Atpα and *atpβ* mRNA quantification by qRT-PCR

Dissected gills and pleopods from experimental groups were homogenized using TRIzol (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer. Total RNA concentration was evaluated spectrophotometrically (260/280 nm) and RNA integrity was analyzed in a 1.5% formaldehyde-agarose gel electrophoresis (Sambrook and Russell 2001). DNA was removed from RNA samples by

Table 1 Specific oligonucleotides used for PCR amplification of shrimp F_1 ATP-synthase subunits *atpα*, *atpβ*, and *L8* genes

Gene	Oligonucleotide name	Sequence (5′-3′)	cDNA positions (nt)
Atpα	ATPAFw1CB	CTCTTCTTTGGCTCGTCAC	27–45
Atpα	ATPAFw2CB	ACAACATGGCTCTCGTCTCC	−5–15
Atpα	ATPAFw3CB	TCAACTTGGAGCCCGATAAC	311–330
Atpα	ATPARv1CB	CCATAGACACGGGCAATACC	248–229
Atpα	ATPARv5	GGAGCAGCATCAGAGGCAGTGGCAGAC	842–816
Atpα	ATPARv9CB	TTAGCAATCTACCCTAGCCCAC	1733–1711
Atpβ	ATPBFw1CB	GGTGCTGGTGTAGGAAAGAC	613–632
Atpβ	ATPBFw5	ATCATGTTGGGAGCTGCACAG	−3–18
Atpβ	ATPBFw6	GGTAATGCTGTCGTTGATACT	358–378
Atpβ	ATPBRv1CB	GGCTCGTTCATCTGACCGTA	818–799
Atpβ	ATPBRv6	ATGCGGCCAAGAGTACCAGGACCAACAG	423–399
L8	L8Fw3	TAGGCAATGTCATCCCCATT	223–242
L8	L8Rv3	TCCTGAAGGAAGCTTTACACG	320–300

digestion with DNase I (Roche, Indianapolis, IN) according to manufacturer instructions. To evaluate mRNA concentration from shrimp samples, 4 μg of total RNA were reverse transcribed using the Superscript III first strand synthesis system (Invitrogen, Carlsbad, CA,) and oligo (dT) oligonucleotide.

We assessed the steady-state mRNA amount of *atpα* and *atpβ* using an iQ5 multicolor real-time PCR detection system (BioRad, Hercules, CA). Specific oligonucleotide pairs were used to amplify *atpα* (ATPAFw1 and ATPARv1), and *atpβ* fragments (ATPBFw1 and ATPBRv1). The ribosomal protein *L8* (DQ316258) was used as an internal control gene to normalize *atpα* and *atpβ* expression (Table 1). Real time PCR amplifications were done in duplicates. Total volume reactions of 25 μL included 12. 5 μL of 2X iQ SYBR Green supermix (BioRad, Hercules, CA), 1 μL (20 μM) of the corresponding forward and reverse oligonucleotides, cDNA synthesized from 250 ng of total RNA from each individual sample and water. PCR conditions were: 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s, 62 °C for 1 min, 68 °C for 55 s with a final melting curve program from 60 °C to 95 °C increasing 0.3 °C each 20 s. Fluorescence readings were taken at 68 °C after each amplification cycle.

To calculate changes in mRNA concentration the $2^{-\Delta\Delta CT}$ method reported by Livak and Schmittgen (2001) was used. The calculation is based on the C_T value of each sample during PCR amplification and the formula $2^{-((CTatpx-CTL8)\text{hypoxia}-(CTatpx-CTL8)\text{normoxia})}$. Results are expressed as the fold change in mRNA steady-state amount of the target gene normalized to the *L8* ribosomal protein and relative to normoxia conditions (6 mg/L). Data obtained were analyzed by one way ANOVA and for post hoc analysis the Tukey test was used. Statistical significance was considered when $p<0.05$. Analyses were performed using Statistica v 8.0 software.

Results

Atpα cDNA sequence

Two PCR fragments of expected size were amplified from gills cDNA using oligonucleotide pairs ATPAFw2/ATPARv5 and ATPAFw3/ATPRv9, the resulting fragments overlapped and produced a confirmed complete cDNA sequence including the coding region with an open reading frame which comprises 1,653 base pairs (bp) containing putative start (ATG) and stop (TAA) codons (GQ848643). This cDNA sequence comprises in the 5′-end, a 138 bp region that codes a putative signal peptide (46 residues), the mature protein of 1,515 bp at positions 139–1,653, and finally the 3′-UTR is 465 bp long including a polyadenylation signal and the poly A tail (Fig. 1). The *atpα* cDNA sequence of *L. vannamei* without the signal peptide shows high identity with those of insect species as the yellow fever mosquito *A. aegypti* (83%, DQ440037; Ribeiro et al. 2007), the fruit fly *D. melanogaster* (82%, Y07894), and the silkworm *B. mori* (81%, DQ311340).

The deduced amino acid sequence of the pre-protein ATPα is 550 residues long, with a predicted molecular weight of 59.23 kDa (Figs. 1 and 2). The putative signal peptide of 46 residues in the N-terminal region, which is supposed to be the mitochondrial import sequence, contains 6 positively charged amino acids and no negatively charged ones. The ATPα mature protein is 504 amino acids long with a predicted molecular weight of 54.49 kDa, and an isoelectric point of 7.80.

Three conserved domains were identified in shrimp ATPα subunit, (i) the ATP-synt_ab_N domain at positions 66–132, (ii) the ATP-synt_ab domain (or nucleotide binding domain) at positions 188–412, which also includes the Walker A motif at positions 209–216 (GDRQTGKT), and the Walker B motif at positions 305–309 (LIIYD), both of them commonly observed in those proteins including nucleotide binding sites, and finally, (iii) the ATP-synt_ab_C domain at positions 424–528 (Fig. 2; Walker et al. 1982).

Fifteen predicted amino acids which help to bind ATP (binding sites) were detected along the ATP-synt_ab domain at positions 211, 215, 216, 217, 243, 248, 309, 310, 313, 368, 384, 397, 402, 403, and 413 most of them highly conserved among species, and thirty four predicted sites of ATPα were located at the interface with ATPβ subunit at positions 155–156, 172–173, 175–176, 179, 181, 211–212, 249–252, 254–255, 258, 319–320, 326–328, 330–331, 336, 340, 343, 347, 384, 387, 398–399, 402, and 413 (Marchler-Bauer et al. 2009; Fig. 2). Some amino acid substitutions were detected in the *L. vannamei* protein sequence: T50, S63, N64, R123, A142, G163, G164, and L165, and some others were K62, C487, E507, T514 and A524.

Phylogenetic relationships of shrimp ATPα

For MP, we obtained trees with tree bisection-reconnection (TBR) branch-swapping heuristic searches in PAUP in which, all characters were equally weighted and starting trees were obtained by 1,000 random stepwise additions. Nodal support was estimated by calculation of non-parametric bootstrap (1,000 pseudo-replicates, 10 random addition replicates per pseudo-replicate) proportions (Felsenstein 1985).

Phylogenetic relationships were investigated for *L. vannamei* ATPα and other species sequences in the Protein Data Bank. One hundred and sixty nine of the 570 amino acid sequences aligned were parsimony informative. Amino acid NJ and MP trees (length=992 steps, c. i.=0.749, r. i.=0.630)

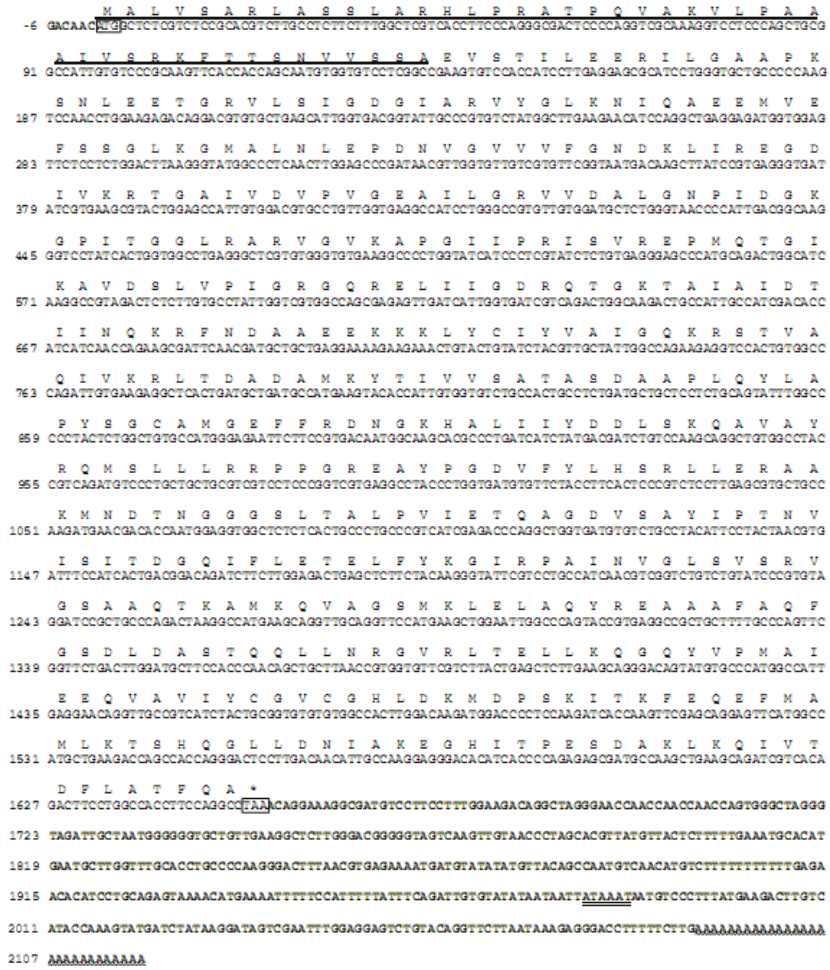

Fig. 1 Shrimp *atpα* cDNA and deduced amino acid sequences. Framed nucleotides indicate start, and stop codons. Untranslated sequence at 3′ -end in bold letters; double underlined sequence indicate the poly A signal, the poly A tail is shown at the end. Underlined amino acids at 5′-end show the signal peptide sequence

showed a similar topology for the main groups of Crustacean, Insecta and Vertebrata. Crustaceans were a close sister group of Insecta in NJ criteria, and close to Insecta + Vertebrata in MP (Fig. 3).

Atpβ cDNA sequence

The full length *atpβ* cDNA from shrimp gills (GQ848644) is 1,792 bp long. It includes a start codon (ATG) and a stop codon (TAA) at positions 1 and 1,579, respectively. Untranslated regions were 3 bp in the 5′- end, and 211 bp in the 3′-end which included the polyadenylation signal and the poly A tail (Fig. 4). It showed an 82% identity with the *atpβ* mRNA from the crayfish *Pacifastacus leniusculus* (DQ874396), which is the only cDNA sequence that has been reported from crustaceans to date. The deduced pre-protein ATPβ is 525 amino acids long with a predicted molecular mass of 55.96 kDa and a theoretical isoelectric

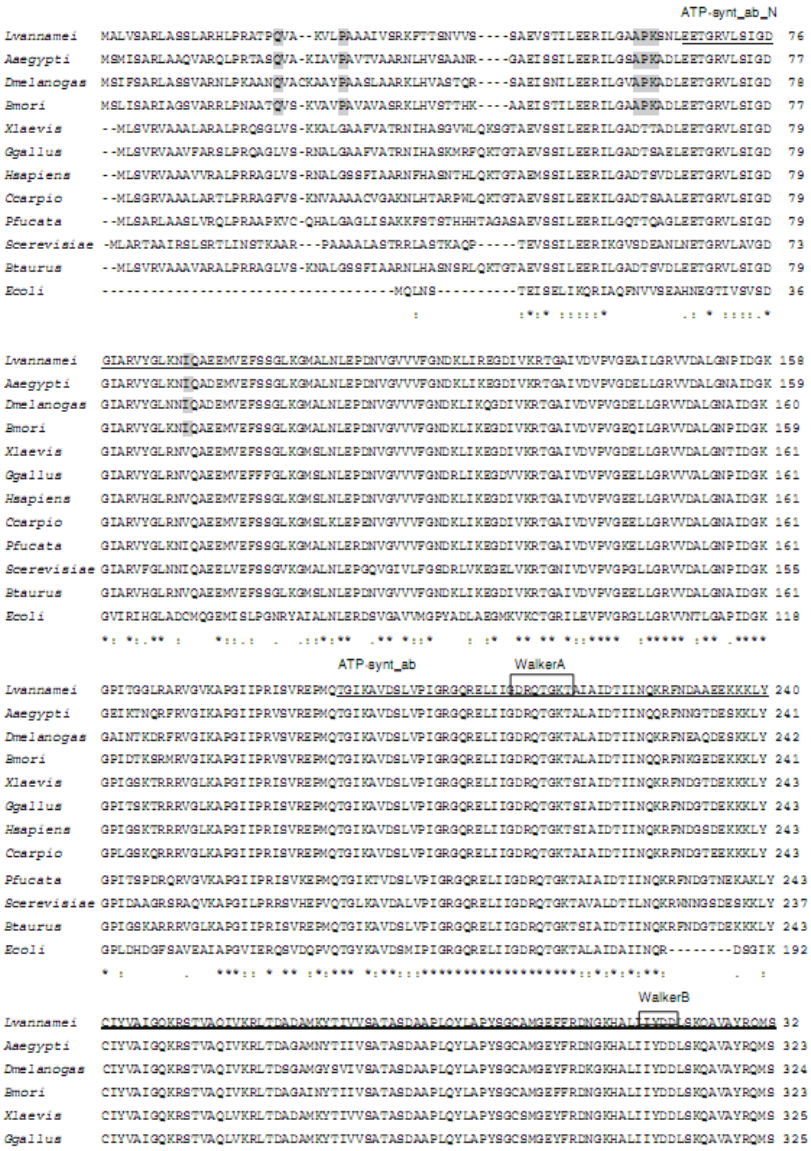

Fig. 2 *ATPα* multiple protein alignment. (*) identical residues; (:) conservative substitutions; and (.) semiconservative substitutions. Underlined residues indicate the three conserved domains. Black framed residues indicate motifs and grey highlighted amino acids are substitutions found in invertebrates

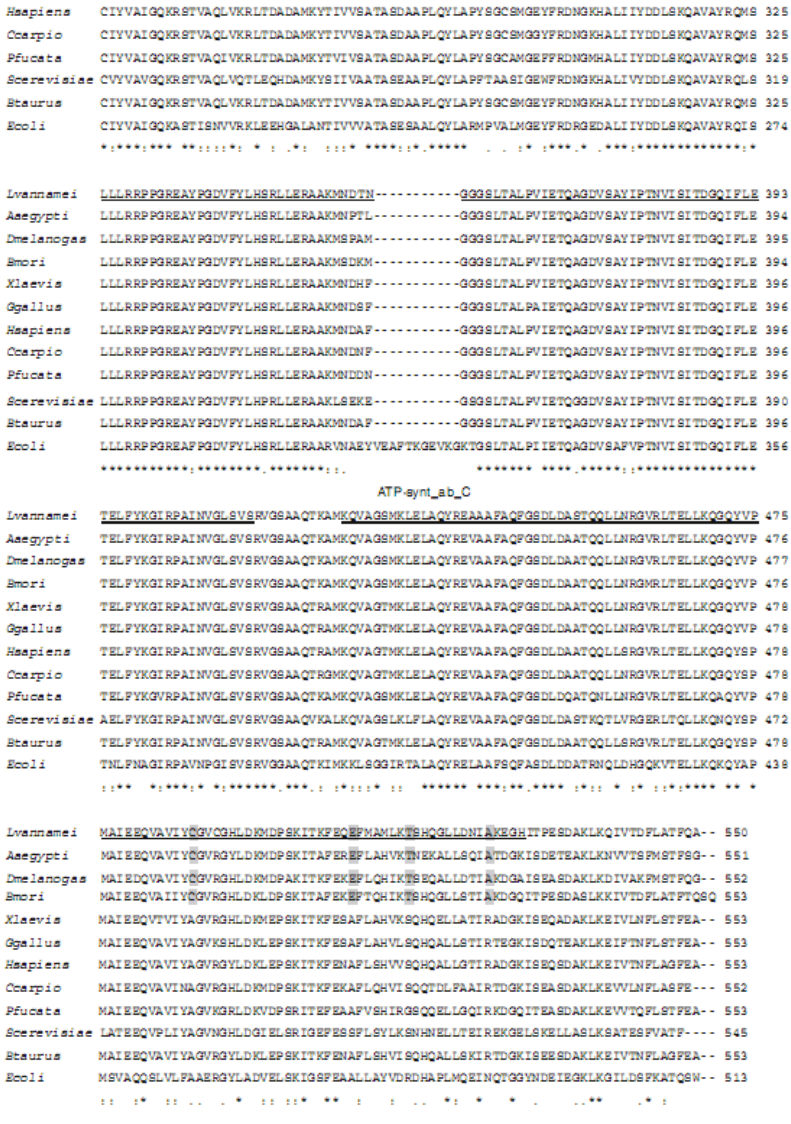

Fig. 2 (continued)

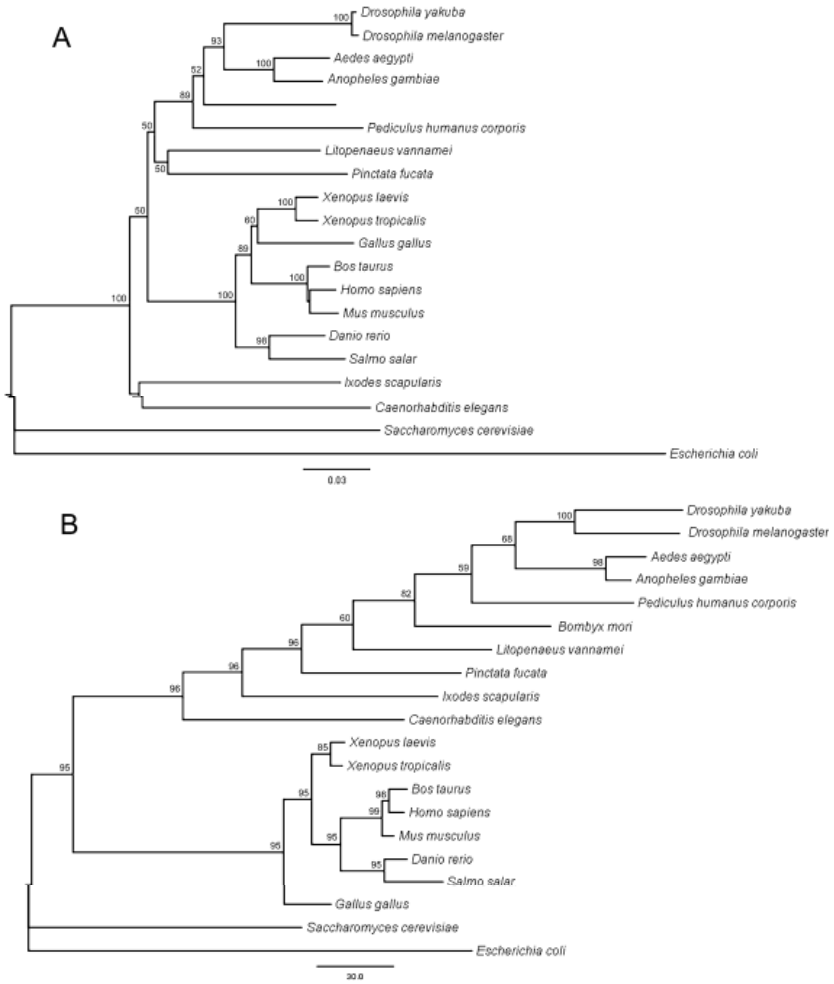

Fig. 3 Topology of phylogenetic trees recovered using neighbor joining (**a**), and maximum parsimony (**b**) from amino acid sequences of the white shrimp ATPα. Numbers above the nodes represent nonparametric bootstrap percentages

point of 5.03. The putative signal peptide contains 43 amino acids, 6 positively charged and no negatively charged residues as observed in the signal peptide of the ATPα subunit (Fig. 4). The mature protein comprises 482 amino acids, a predicted molecular mass of 51.45 kDa and an isoelectric point of 4.78.

The three conserved domains from the ATPβ protein were identified in this sequence as: (i) ATP-synt_ab_N domain which includes residues 59–126, (ii) the ATP-synt_ab domain between residues 182–402 containing the Walker A motif (GGAGVGKT), which forms a P-loop responsible for triphosphate binding at positions 203–210, and five residues (LLFID) that were identified as the Walker B motif at positions 299–303; the third domain, ATP-synt_ab_C was found at positions 415–525 (Fig. 5). Additional conserved sequences were found in shrimp ATPβ as DELSEED which includes residues 441–447, and forms a negatively charged loop near to the site where the IF_1 inhibitor, which regulates the hydrolytic activity of the

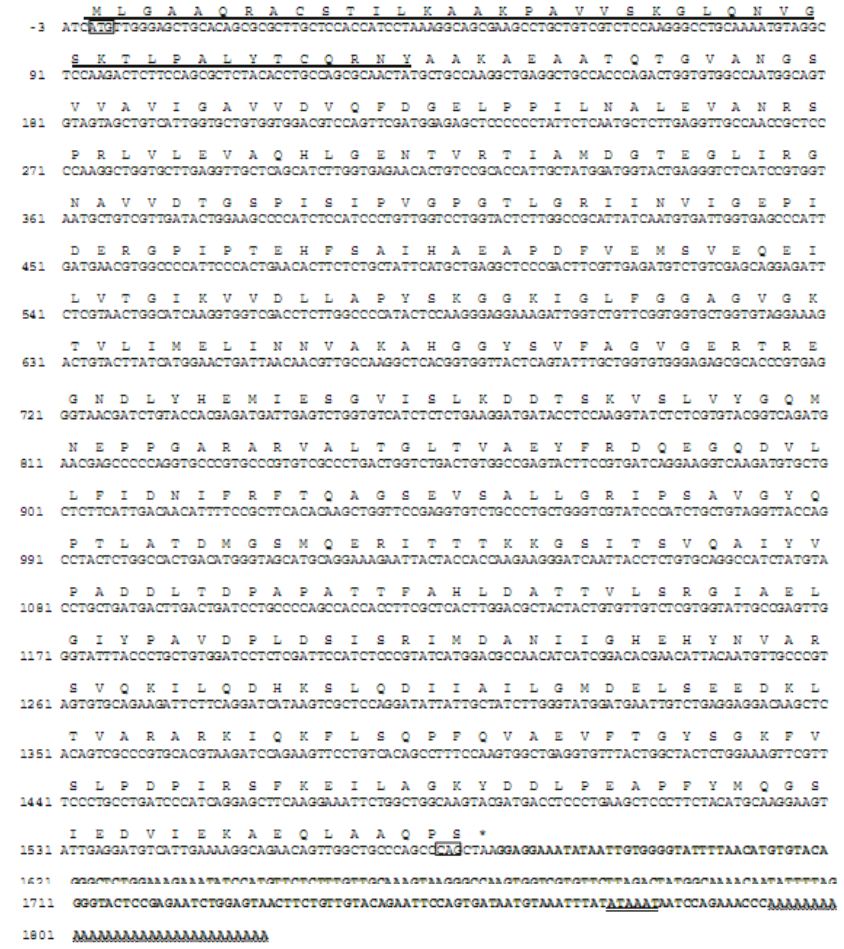

Fig. 4 Shrimp *atpβ* cDNA and deduced amino acid sequences. Framed nucleotides indicate start, and stop codons. Untranslated sequences at 5′- and 3′-ends in bold letters; double underlined sequence indicate the poly A signal, the poly A tail is shown at the end. Underlined amino acids at 5′-end show the signal peptide sequence

F_OF_1 ATP-synthase, interacts with the enzyme (Cabezon et al. 2001). The GERXXE sequence was detected at positions 234–239, this is suggested to play a role in the process of ATP hydrolysis by interacting with subunit alpha at the catalytic site (Wada et al. 2000; Fig. 5).

Eleven ATP binding sites were detected along the ATPβ protein at positions 206, 209, 210, 211, 235, 236, 239, 303, 307, 392, and 393, all of them are highly conserved between eukaryotes. Thirty nine sites were located at the interface with ATPβ subunit at positions: 151, 168, 170, 171, 173, 175, 236–238, 241, 269–271, 276, 307, 310, 314, 320–322, 324, 325, 330, 334, 341, 358, 361, 362, 366, 373, 375–377, 388, 389, 398, 399, 401, and 403. Along the ATPβ we identified amino acid substitutions shared only by crustacean species as *P. leniusculus*, and *L. vannamei* at specific sites as G74, E75, N120, S130, G136, E158, S161, D169, S194, D258, S263, I401, H428, S487, E490, and P503. Some other substitutions as S59, A86, I117, V123, H159, A407, H412, S420, P482,

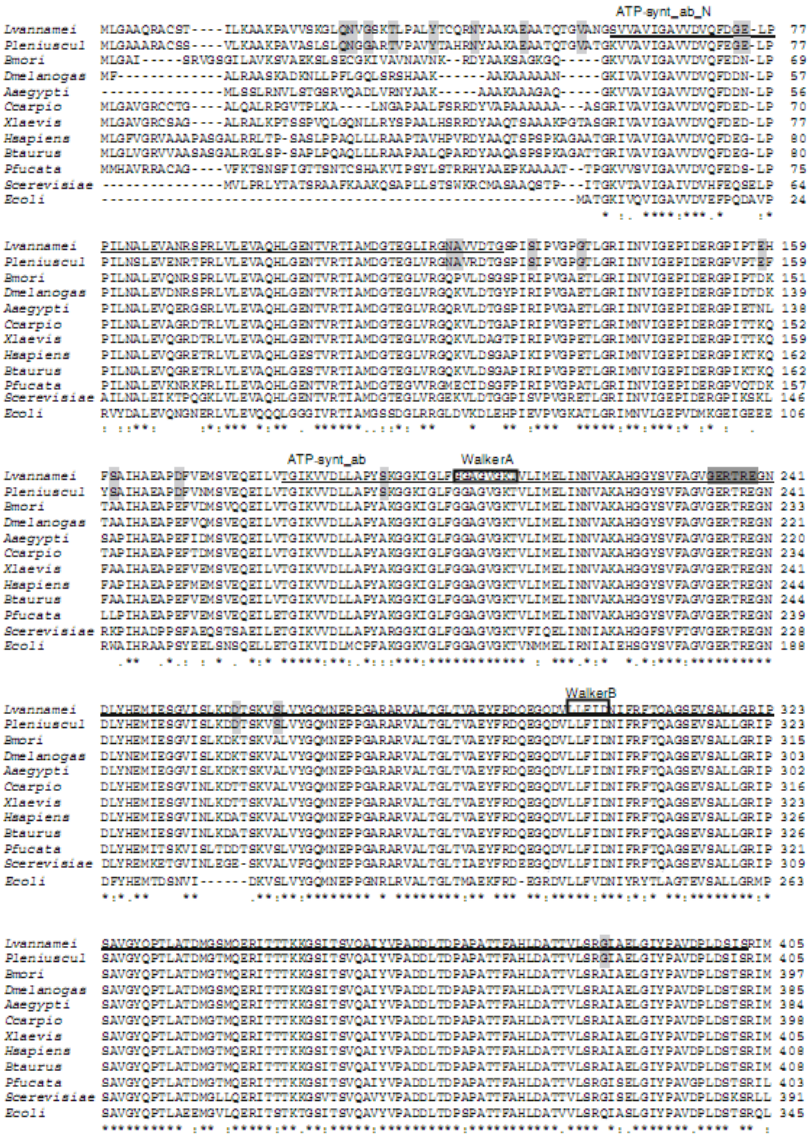

Fig. 5 *ATPβ* multiple protein alignment. (*) identical residues; (:) conservative substitutions; and (.) semiconservative substitutions. Underlined residues indicate the three conserved domains. Black framed residues indicate motifs and other conserved sequences. Grey highlighted amino acids are substitutions found in crustaceans

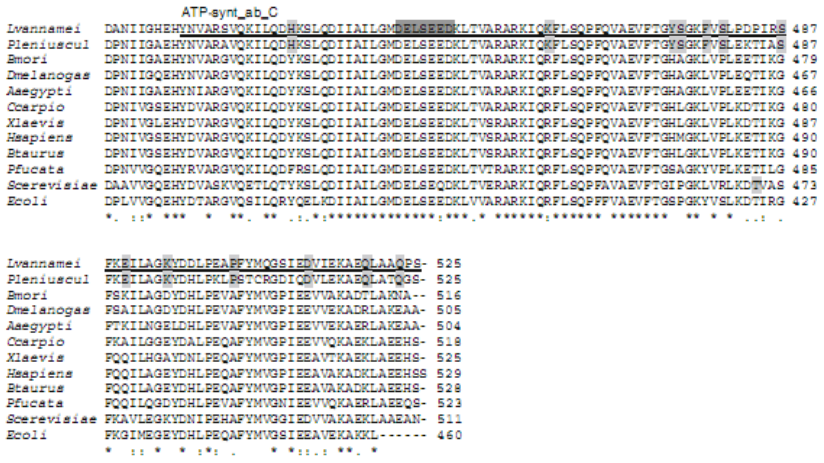

Fig. 5 (continued)

D483, P484, R486, D498, A502, Q507, S509, I514, A522, and P524 were only detected for *L. vannamei* ATPβ and could result in structural /functional changes (Fig. 5).

Phylogenetic relationships of shrimp ATPβ

Phylogenetic relationships among *L. vannamei* ATPβ and available sequences from related groups were investigated using complete amino acid sequences. One hundred and fifty five of the 601 amino acid sequences aligned were parsimony informative. Amino acid NJ and MP trees (length=883 steps, c. i.=0.695, r. i.=0.635) showed the same topology for the main groups of Insecta, Vertebrata, and Crustacean. Crustaceans are a close sister of the main groups of Insecta, Vertebrata, Acari, and Nematode for both criteria. Both criteria produced trees with higher similar topology and bootstrap (Fig. 6).

Atpα and *atpβ* steady-state mRNAs from shrimp at hypoxia

No differences were observed between controls and shrimp at 4 and 2 mg/L OC, but higher concentrations of lactate were found in shrimp exposed for 4 h to 1.5 mg/L (0.26 mg/mL), this value was 80% higher than the plasma lactate concentration of shrimp exposed to 2 mg/L (0.05 mg/mL; $P<0.05$). After re-oxygenation at 7 mg/L, lactate concentrations decreased 42% (0.15 mg/mL) compared with shrimp exposed to hypoxia at 1.5 mg/L. Glucose concentrations in plasma changed in a similar trend as lactate, and the highest values of glucose were detected in shrimp exposed to 1.5 mg/L (0.36 mg/mL), an 88% increase over that found in shrimp exposed to 2 mg/L (0.04 mg/ mL).

The steady-state mRNA concentrations of *atpα* and *atpβ* in gills and pleopods were evaluated as affected by OC in water (6, 4, 2, 1.5, and re-oxygenation at 7 mg/L). We found no statistical differences in the mRNA amounts of *atpα* in both tissues at hypoxia ($P>0.05$), even though a decrease of 56 and 47% was observed in gills and pleopods when OC in water declined to 1.5 mg/L, compared to those at normoxia 6 mg/L (Figs. 7a and 8a). Moreover, no statistical difference in the relative expression of *atpβ* was detected in both tissues ($P>0.05$), but conversely to that observed in the *atpα* variations, a slight increase of 28% in the hypoxia group at 2.0 mg/L was detected in gills, and an increase of 70% was detected in pleopods in the hypoxia group at 1.5 mg/L (Figs. 7b and 8b). Thus, no evidence of a coordinated change was observed between *atpα* and *atpβ* subunits.

Discussion

The mitochondrial multimeric complexes that are part of the oxidative phosphorylation system (OXPHOS) have scarcely been studied in crustaceans, regardless of their importance in the energetic metabolism that allows these organisms to surpass the environmental changes they face. To our knowledge this is the first report of a complete *atpα* cDNA sequence from crustaceans, and the second for *atpβ* subunit of the mitochondrial ATP-synthase complex. To date, there are only a few reports about the study of both

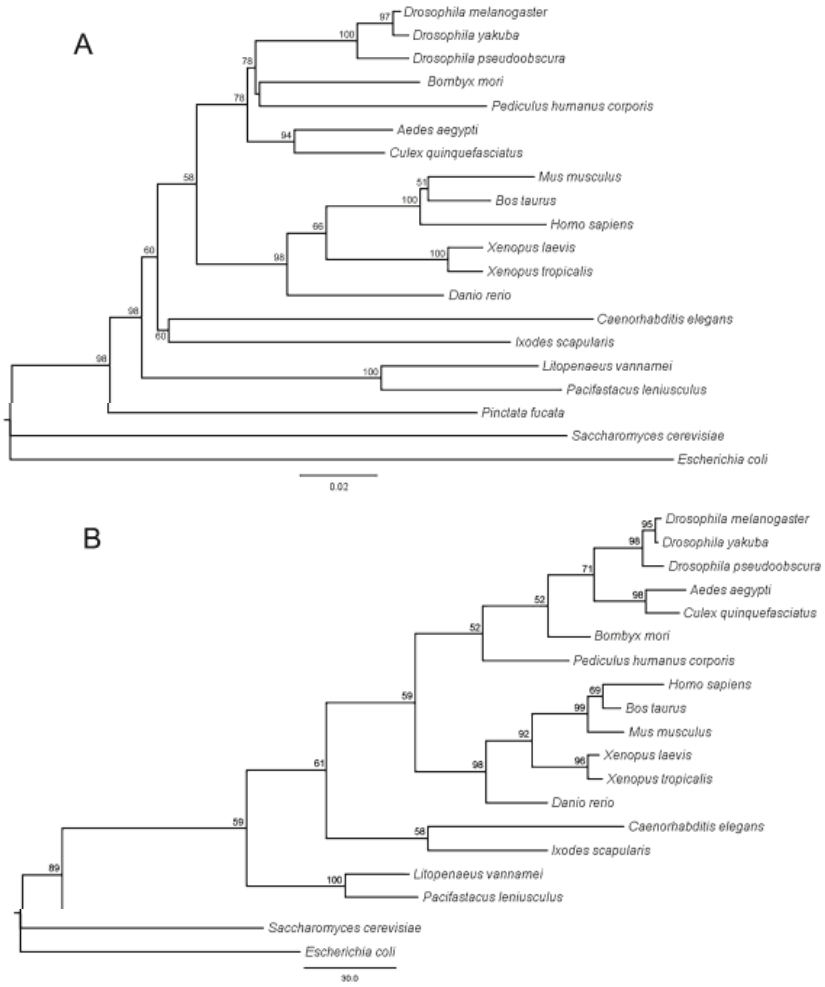

Fig. 6 Topology of phylogenetic trees recovered using neighbor joining (**a**), and maximum parsimony (**b**) from amino acid sequences of the white shrimp ATPβ. Numbers above the nodes represent nonparametric bootstrap percentages

shrimp subunits and/or other ATP-synthase subunits (Muhlia-Almazan et al. 2008).

Although mRNA isoforms have been described for bovine and human (Pierce et al. 1992), we did not find differences between analysed sequences from different shrimp tissues, neither for *atpα* transcripts, nor for *atpβ* (data not shown), suggesting that no additional mRNA isoforms are synthesized from genes encoding the mRNAs characterized in this study; however the possibility of additional genes encoding other transcripts for both *atpα* and *atpβ* already exists.

Analyses showed that the shrimp deduced proteins of ATPα and ATPβ subunits are highly conserved, both containing the three major domains, the two Walker motifs and specific ATP-binding sites characterizing these proteins (Walker and Runswick 1983; Walker et al. 1989; Abrahams et al. 1994; Atteia et al. 1997), and some other above

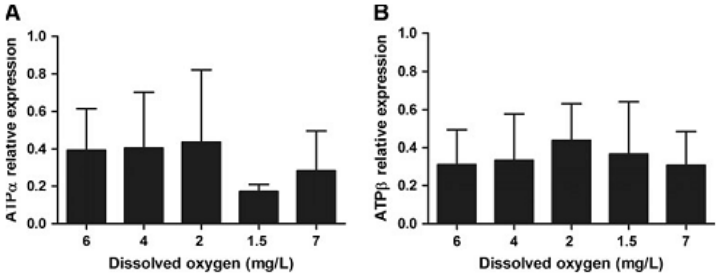

Fig. 7 Relative expression of **A** *atpα*, and **B** *atpβ* transcripts in the gills of shrimp at different oxygen concentrations. Data represent the mean and standard deviation value

mentioned elements that allow them to integrate the water-soluble F_1 motor that forms the catalytic portion of the enzyme (Von Ballmoos et al. 2009). Our results are in agreement with the current paradigm that the central role of these proteins in ATP synthesis is due to subtle interactions among subunits, which are only possible by complex complementarities. Hence selective pressure must punish mutants and what we see is highly conserved proteins (Peña et al. 1995).

In addition to conserved characteristics, we detected unique elements in the amino acid sequences of the N-terminal signal peptides of both shrimp subunits when compared with other species. The signal peptide of shrimp ATPα subunit shares length and gaps with those from insect species (residues 46 to 48; Peña et al. 1995); signal peptides from vertebrate species are usually 49 residues long and show different conserved residues (Walker et al. 1985). The shrimp ATPβ signal peptide sequence showed no identity with invertebrate or vertebrate species; however, it showed a high identity percentage, 82%, when compared to that of the crayfish *P. leniusculus* sharing length and gaps, thus both crustacean sequences were different from those of other invertebrate and vertebrate species. These results are in agreement with knowledge indicating that the signal peptide of mitochondrial proteins is highly variable, both in length and sequence, and that conserved elements are limited to their basic character and secondary structure (Hendrick et al. 1989). The predicted molecular weight of both shrimp ATP-synthase subunits also differed when compared to other taxa groups. The ATPα subunit is highly conserved in molecular mass among species, but ATPβ is larger than those proteins from other invertebrates and similar to those from vertebrates.

The results of phylogenetics based upon MP and ML analyses of the nucleotide and amino acid sequences provided similar basic topology branch support by both criteria, as the general topology of the trees shown in the entire analysis. Higher values were obtained for amino acid than for nucleotide trees due to the presence of synonymous codons. Genetic distances and parsimony bootstrap showed strong statistical bootstrap support for the association of *L. vannamei* to Insecta and Vertebrata clade. NJ ATPα showed monophyly to Insecta, but MP moves monophyly to a lower branch including Nematoda and Protist. This information confirms our previous observations about this subunit.

The ATPβ sequences were more reliable showing the clade of crustaceans as a sister of all the Insecta, Vertebrata and isolated from Nematode and Protist. NJ and MP of ATPβ showed monophyly to a main group of Insecta and Vertebrata fixing the monophyly to a lower branch close to Mollusca and constructing a big clade. ATPβ were more consistent in showing the phylogentic relationships in a broad scale as a result of the conservation of the sequences. Conservation of both units may not be an artifact to conclude the relationships of the main clades in this study given the limited number of taxa whose data were available to be included in this research.

Unique characteristics found in shrimp proteins ATPα and ATPβ in this research should be deeply analyzed since some of these registered substitutions could be related to specific enzymatic properties of the soluble F_1-ATP

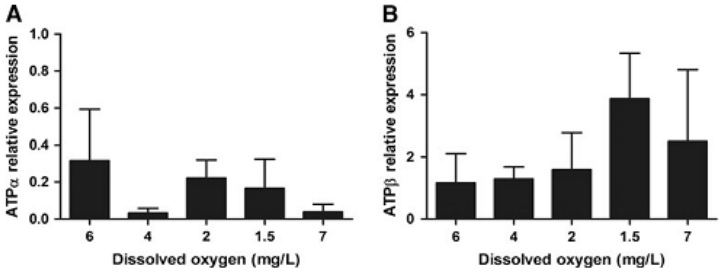

Fig. 8 Relative expression of **A** *atpα*, and **B** *atpβ* transcripts in the pleopods of shrimp at different oxygen concentrations. Data represent the mean and standard deviation value

synthase portion, as reported by Li and Neufeld (2001), after they isolated and characterized this portion from the gills of the crayfish *Orconectes virilis*. They emphasized the high ATPase activity of the enzyme, as well as sensitivities to inhibitors and modulators that distinguish the crustacean enzyme from all those previously examined. As observed in figures, crustacean proteins can share specific amino acid substitutions with microbial, vertebrate and invertebrate species, suggesting these species as interesting models to study.

Crustaceans exposed to hypoxia biochemical compensation evolve as a complex and highly integrated series of responses to maintain cellular homeostasis by adjusting various metabolic pathways, where ATP synthesis/hydrolysis is crucial (McMahon 2001). The exposure to oxygen depleted water increases ventilation frequency since gills O_2 diffusion is affected (Morris and Callaghan 1998), implying an increase in the energy demand of shrimp. In addition, studies state that a reduction in metabolism is a manner of avoiding anaerobiosis (Hochachka 1988). However, as hypoxia continues, shrimp produce lactate as the end product of anaerobic metabolism, and the concentration of cellular ATP decreases. All these physiological responses agree with those observed in our experimental shrimp exposed to increasing degrees of hypoxia, confirming the hypoxia-compensating status along the assay.

No coordinated changes were detected in the mRNA amounts of these two nucleus-encoded subunits along the assay. Previously, the presence of coordinated mechanisms was reported when comparing the nuclear and mitochondrial subunits of the enzyme (Muhlia-Almazan et al. 2008); however, it seems not to be the case between nuclear subunits from both evaluated tissues.

Concerning the relative expression of *atpβ* in shrimp pleopods, an up-regulation of 3-fold was observed in the lowest OC (1.5 mg/L). This increase was also observed for *atp6* and *atp9* mRNAs of shrimp at hypoxia in the digestive gland (Martinez-Cruz 2007); this observation supports the suggestion that the expression of the nuclear *atpβ* subunit is strictly coordinated with the expression of *atp6* (Peña et al. 1995), which is a mitochondrially encoded subunit in the fruit fly *D. melanogaster*. In the grass shrimp, *Palaemonetes pugio*, exposed to 1.5 mg/L OC for 3 days, an increase in the *atpf* mRNA concentration was detected and, in agreement with our results, this increase was not coordinated since no changes were detected in the mRNA concentration of both nuclear *atpβ* and *atpd* subunits (Brown-Peterson et al. 2008).

The increase of *atpβ* mRNAs in pleopods during hypoxia, and the subsequent decrease as response to normoxia during re-oxygenation agrees with our previous observations (Martinez-Cruz 2007). These changes are explained as a response to a decreasing OC of water by synthesizing new ATP-synthase complexes in the early phase of hypoxia, in order to maintain the mitochondrial membrane potential by using the still available intracellular ATP molecules. Then, after oxidative phosphorylation is reduced or absent, and the accumulation of lactate reduces tissues pH, ATP-synthases will change from ATP producers to powerful ATP consumers as observed in the ischemic myocardium of vertebrates (Di Lisa et al. 1998).

We propose that the increasing amount of mRNAs in response to hypoxia, and the existence of coordinated mechanisms of gene's expression, as we previously reported in shrimp (Muhlia-Almazan et al. 2008) are tissue-specific responses. Each of these responses are tightly related to tissues function since gills in this research, and muscle in the previous report (Martinez-Cruz 2007) were not affected by the oxygen reduction, and no coordinated expression was observed in this report between nuclear encoded genes of the ATP-synthase subunits.

Previous studies in shrimp confirm our proposal by demonstrating that the genes expression profile of a tissue is tightly related to its function (Clavero-Salas et al. 2007). To date, the amount of mRNA of some of the ATP-synthase subunits from crustaceans and other marine invertebrates have been reported to be affected by factors as viral infections, osmotic stress and hypoxia in different tissues as the midgut gland, gills, and hemocytes, which affects their primary role (De la Vega et al. 2007; Zhao et al. 2007; Soñanez-Organis et al. 2009).

The results of this study suggest that changes in the amount of mRNAs encoding the F_OF_1 ATP-synthase subunits are part of the mechanisms allowing shrimp to deal with metabolic adjustment to tolerate hypoxia. This new information about mitochondrial enzymes from marine invertebrates as crustaceans not only confirms predictable elements about the catalytic subunits of the ATP synthase, but also allowed us to detect those species-specific characters that could explain organism adaptive features to their aquatic environment. Future studies should be addressed to deeply analyse a predictive model of the F_1 portion of shrimp ATP synthase. The isolation and characterization of the subcomplex and its forming parts, and ATPase kinetics will help to understand and explain shrimp enzyme differences.

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Chapter

Invertebrates Mitochondrial Function and Energetic Challenges.

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1. Introduction to Invertebrates Biology.

The term "invertebrate" recalls all animal species lacking a backbone or a bony skeleton. Although "invertebrate" is not a scientific term that encloses a taxonomic rank, this group includes animal species represented by over 30 phyla and it includes the first animals that successfully inhabited the earth, an event that – according to the fossil evidence – dates back to around 600 million years ago. This group is composed of several different phyla, such as annelids, molluscs, sponges, cnidarians, echinoderms, and all species from the phylum Arthropoda – which is the largest among invertebrates and is comprised by insects, arachnids and crustaceans (nearly reaching 1,033, 160 species).

Since they appeared for the first time during the Cambrian period, invertebrates have played an important ecological role since they are frequently the key constituents of many trophic chains and they occupy virtually every available ecosystem on Earth, being characterised by notable variations in temperature, oxygen concentrations, food availability and food quality. Also, many species occupy highly specific and important roles in nature as pollinators, parasites or vectors for parasitic diseases affecting human and animal health.

It is clear that the ability of invertebrates to inhabit almost every ecosystem – as well as the diverse array of morphological and behavioural strategies used to obtain nutrients from the environment – is an accurate reflection of the enormous ability of these organisms to solve their most basic energetic requirements. From blood-suckers such as mosquitoes, intestinal nematodes and leeches (hirudin), to small plankton marine feeders such as cnidarians and marine benthic bivalves, all species face changes in food availability throughout their life cycle which affect their energy stores and growth rates (Peck, 2002; Popova-Butler & Dean, 2009). A beautiful example of highly specific energy stores – crucial during invertebrates' life cycle and important to human health – is that of the female mosquito (*Anopheles gambiae*), which usually feed on sugar to gain energy to fly and to cope with metabolic requirements; however, anautogenous mosquitoes require the energy resulting from blood digestion in order to produce eggs, and it is during blood sucking that *Plasmodium vivax* (the parasite from infected females) enters into the vertebrate host to produce Malaria, a major health problem around the world (Das et al., 2010).

Large energetic demands during external work are observed throughout the life of several invertebrate species, and a clear example may be found in insect flight, which is considered to be one of the most energetically demanding processes of animal locomotion (Harrison & Roberts, 2000). Besides this, being an aerobic process that requires a permanent oxygen supply and depends upon ATP cellular production, the high energetic cost of flying is related to the frequency of the flight muscles' contraction (Vishnudas & Vigoreaux, 2006). In vertebrate species, the existence of high-energetic molecules in the muscle (phosphocreatine) during its exercise has been well documented (Jubrias et al., 2001); however, in invertebrate species, the presence of phosphagen-kinases that catalyse the synthesis of these high-energetic phosphorylated molecules has not been widely distributed (Ellington & Hines, 1991). The insect flight muscle seems to lack such molecules, but some flying species are able to surpass such energy needs by the proximity of mitochondria to muscle myofibrils, thus facilitating the export of energy rich nucleotides – such as ATP – to myofibrils (Vishnudas & Vigoreaux, 2006).

Some other invertebrate phyla – such as crustaceans – are able to synthesise phosphagens differently from that of vertebrates, like phosphocreatine. Phosphoarginine – a phosphagen of L-arginine found in the tail muscle of shrimp and crabs as well as in the flight muscle of flying insects – is the chemical energy storage system of these tissues, and thus animals are able to rapidly produce ATP when it is required (Wegener, 1996; Kotlyar et al., 2000). The enzyme responsible for the synthesis of phosphoarginine from ATP and L-arginine in invertebrates is named 'arginine kinase' and it is also considered to be a major allergen protein for shrimp-allergic individuals (Garcia-Orozco et al., 2007).

Since energetics is considered to be a key factor in limiting organisms' adaptation to extreme temperatures, several invertebrate species inhabiting marine polar environments are known to show a remarkable plasticity as regards their cellular system. Such adaptations may include an increasing number of mitochondria per cell as the temperature decreases as well as differences in the mitochondrial characteristics relating to the species' lifestyle, from motile species to sedentary ones (Peck, 2002). Studies in the mitochondrial function of the eurythermal polychaete *Arenicola marina* have concluded that invertebrates inhabiting higher latitudes – and consequently exposed to cold temperatures – showed higher oxygen consumption, mitochondrial densities and mitochondrial capacities when compared with those appraised of organisms living at lower latitudes with higher temperatures (Sommer & Portner, 1999; Peck, 2002). This adaptation of cold-acclimatised organisms is thought to occur in order to equate the level of metabolic activity present at warmer temperatures.

Among other the important environmental factors affecting the bioenergetic state of organisms, marine invertebrates face large daily fluctuations in the dissolved oxygen concentrations of water, as well as wide salinity changes between open ocean and coastal waters (where many species live at least during one specific stage of their life cycle) (Dall et al., 1990). Such variations can adversely affect some species whose physiological mechanisms usually do not allow them to cope with low oxygen levels (as oxyregulators) or to handle salinity changes (as osmoregulators). However, several species are able to swim or move from one place to other, searching for a suitable site to grow, reproduce and survive (Hochachka & Somero, 2002; Abele et al., 2007). Nevertheless, other invertebrate species are highly adapted to live in extreme conditions such as those living in hypoxic or even anoxic environments, like the brine shrimp *Artemia franciscana* (Eads & Hand, 1999; 2003).

As has previously been stated, this chapter reviews the current state of knowledge of the mitochondrial function of invertebrate species. It asks two central questions: 1) How are invertebrates able to adapt to such diverse environmental conditions by using a common set of structures and mechanisms – their mitochondrial machinery – to fulfil their energy requirements along their entire life cycle? 2) Is it really important to understand the role of mitochondria in the life history of invertebrates? This chapter also includes original data on crustacean responses to the external factors affecting such mitochondrial functions as hypoxia, starvation and the energetically expensive molt cycle.

2. The Highly Conserved Mitochondrial Machinery of Invertebrates: Same Functions, Different Challenges.

Following its endosymbiotic origin from primitive bacteria – at least 2 billion years ago – when atmospheric oxygen levels rose and subsequently remained relatively steady, mitochondria have experienced large changes among species, from α-proteobacteria to mammals. During the adaptation process of organisms to their new dynamic environment, some mitochondrial characteristics have remained highly conserved even among distantly related species, such as their rod shape - the overall structure including two phospholipid membranes – and, with some exceptions, their conserved characteristic genome content of 22 tRNAs, 2 rRNAs, and 13 genes encoding protein subunits of the enzymes from the oxidative phosphorylation system (OXPHOS) (Boore, 1999; Gray et al., 1999).

Besides mitochondrial encoded proteins, a significant fraction of the original mitochondrial genes have moved to the nucleus. Thus, in the mammalian mitochondria, approximately 76 subunits – which are part of the respiratory chain – are encoded by nuclear genes, and all of them must be imported into the mitochondria. The complete protein machinery involved in mtDNA replication, transcription and translation (including all of the ribosomal protein subunits) is encoded by nuclear genes (St. John et al., 2005; Falkenberg et al., 2005). Furthermore, several of these imported proteins are highly conserved among species, some of them accomplishing key roles as subunits alpha and beta of the ATP-synthase, which are part of the catalytic sites of the enzyme (Martinez-Cruz et al., 2011).

In addition to those key proteins that maintain a conserved function, hundreds of new proteins have been described among invertebrate species as being imported to mitochondria, each presumed to participate in at least one of the large number of metabolic pathways occurring in this organelle. However, its major conserved function allows mitochondria to produce – from food assimilated compounds via oxidation – the proton motive force that drives ATP synthesis (Rich & Marechal, 2010). This complex process produces 95% of the cellular ATP that cells need for biosynthesis, transport and motility (Wilson et al., 1988; Dudkina et al., 2008; Diaz, 2010), and any significant change in the system could result in deleterious consequences for the whole cell metabolism and – consequently – reduce its efficiency or provoke its death (Mayevsky & Rogatsky, 2007).

Throughout the years (and mostly based in the study of human pathologies) researchers have found that mitochondria are involved in various critical functions – such as thermoregulation – in the synthesis of essential molecules – such as phospholipids and heme – in the programmed cell death or apoptosis of mediating multiple cellular signalling pathways (Ryan & Hoogenraad, 2007). Mitochondria are also essential in the cholesterol metabolism and the detoxification of ammonia in the urea cycle. In addition, there is a close relationship between mitochondria and different cell types. It is well known that the number of mitochondria in individual cell types varies according to their function and energy requirements (St. John et al., 2005; Chen & Chan, 2009). Thus, highly energetic tissues as the flight muscle of flying insects and the midgut gland of crustaceans are known to contain a large number of mitochondria, just as occurs in the skeletal muscles of vertebrates during endurance training (Harrison & Roberts, 2000).

Mitochondria are known as dynamic organelles that cannot be made *de novo*, and instead they divide through a highly regulated process called mitochondrial fission, mediated by a defined set of new proteins recruited from the cytoplasm, which are added to pre-existing sub-compartments and protein complexes. to a point whereby the organelle grows and divides (Ryan & Hoogenraad, 2007). Furthermore, mitochondria are now seen as a set of organelles that are able to migrate throughout the cell, to fuse and divide through the regulating mitochondrial function (Chen & Chan, 2009).

Recent findings have also confirmed the existence of dynamic mitochondrial supercomplexes – defined as the association of protein complexes distributed along the inner mitochondrial membrane – on mammals, plants, yeasts (*Yarrowia lipolytica)*, and bacteria (Nübel et al., 2009; Wittig & Schagger, 2009; Dudkina et al., 2010). Complexes I, III and IV are able to associate in order to promote electron transport as single OXPHOS complexes or else as a supercomplex called respirasome ($I + III_2 + IV_{1-2}$) both of which can autonomously carry out respiration (Wittig et al., 2006). Furthermore, complex V – the mitochondrial F_1F_OATP-synthase – is associated to form dimeric, trimeric and tetrameric organisations (Dudkina et al., 2008). Unfortunately, to our knowledge, there are no reports confirming the existence of these mitochondrial protein associations from invertebrate species.

A general description of the most recent advances covering mitochondrial enzymes participating in the electron transport chain and the OXPHOS, including some particular findings on the enzymes of some invertebrate species, is presented below:

2.1. Complex I, NADH: Ubiquinone-oxidoreductase (EC. 1.6.5.3) is an enzyme which provides the input to the respiratory chain by catalysing the transfer of two electrons from NADH (from glycolysis) to ubiquinone, and which utilises the free energy released in this redox reaction for the translocation of four protons across the membrane, from the matrix to the intermembrane space. The proton translocation from the mitochondrial matrix generates the proton-motive force required for ATP synthesis at the end of the respiratory chain during oxidative phosphorylation (Friedrich & Weiss, 1997; Dudkina et al., 2008). However, this proton-pumping enzyme is the largest, most complicated and least-well understood of the respiratory chain (Zickermann et al., 2008). Another unconventional function of complex I is the generation of reactive oxygen species (ROS) – such as the superoxide ion (O_2^-) – and, even if it is not a strong oxidant, it is a precursor of most other ROS and, consequently, contributes significantly to cellular oxidative stress. In mammalian mitochondria, the superoxide production is predominantly produced by complex I (Turrens, 2003).

The scarce information available concerning mitochondrial complex I from invertebrates includes basic descriptive reports of the nucleotide sequences of the NADH subunits (most of them from the mitochondrial genome), their proteins, and an interesting study of site-directed mutagenesis aiming to understand the subunits' function in model insect species such as *Drosophila spp*. (Tovoinen et al., 2001; Sanz et al., 2010).

In addition, the existence of an alternative oxidase (AOX) in the animal mitochondria has been confirmed. Previously, this enzyme – which catalyses the O_2-dependent oxidation of ubiquinol, producing ubiquinone and H_2O – was thought to be limited to plants, some fungi and protists. The major difference between complex I and AOX is that the electron flow from ubiquinol to AOX is not coupled to the generation of a proton motive force, decreasing energy conservation in oxidative phosphorylation. The complementary DNA sequence that encodes AOX in invertebrate species from the phyla Porifera, Cnidaria, Nematoda, Anellida, Mollusca, and Echinodermata, has been characterised and it has been suggested that it may contribute on the acclimation of animals to stress conditions, mainly when the cytochrome pathway is inhibited (McDonald et al., 2009).

2.2. Complex II, Succinate: Ubiquinone- Oxidoreductase (EC 1.3.99.1), also called Succinate Dehydrogenase (SDH), is a functional member of the Krebs cycle and the aerobic respiratory chain, and it couples the oxidation of succinate to fumarate with the reduction of quinone to quinol (QH_2). Most probably, this enzyme presents the most striking differences among the mitochondrial complexes in the electron transport chain and OXPHOS (Rich & Marechal, 2010). It must be noticed that the oxidation of succinate to fumarate is the only Krebs reaction that takes place in the mitochondrial inner membrane itself; this reaction does not participate in proton translocation from one side to the other of the inner mitochondrial membrane. The energy carrier flavin adenine dinucleotide (FAD) forms a part of complex II, and succinate oxidation begins after the binding of succinate to the enzyme. This covalent binding of FAD to the enzyme increases the redox potential to a level that allows succinate oxidation (Rich & Marechall, 2010).

Contrary to the four human and yeast mitochondrial complexes, which include subunits that are encoded by the mitochondrial genome, the four subunits of SDH are encoded in the nuclear genome (SDH1 to SDH4; Figueroa et al., 2002).

Early studies of complex II (SDH) from invertebrates reported the isolation of mitochondrial fractions from the body muscles of the worm *Nereis virens* and from the tail muscle of the lobster *Homarus gammarus*, and reported high activity in both enzymes (Mattisson, 1965). Unfortunately, there is scarce new information available concerning complex II in invertebrates. However, the study of mitochondria from parasite species – used as animal models – can be considered a framework that has guided our knowledge in the understanding of such critical endogenous processes as aging, mitochondrial dysfunction and the role of the organelle in apoptosis (Grad et al., 2008; Wang & Youle, 2009). Thus, it has been suggested that mitochondria may influence the longevity of the nematode *Caenorhabditis elegans* through the rate of ROS production and by the stress-evoked signals that are known to act in a cell-non-autonomous manner during mitochondrial protein regulation (Durieux et al., 2011). Furthermore, *C. elegans* has been used as a model to investigate the mitochondrial mechanisms of human aging and tumourigenesis by studying the catalytic effects of mutation in the genes encoding the SDH iron-sulphur subunit. Promising results suggest that the SDH ubiquinone-binding site can become a source of superoxide and that the pathological consequences of SDH mutations can be diminished with antioxidants, such as ascorbate and N-acetyl-l-cysteine (Huang & Lemire, 2009).

2.3. Complex III, Ubiquinol: cytochrome c oxidoreductase or Cytochrome bc_1 (EC 1.10.2.2) is a multimeric enzyme complex involved in the transfer of electrons from ubiquinol to cytochrome C, and it is also coupled to electrons' transfer across the inner mitochondrial membrane. This bovine enzyme is formed by 10 nuclear encoded subunits, with only one encoded in the mtDNA (Xia et al., 1997). The catalytic mechanism of the enzyme includes the complex mechanism of the protonmotive

Q-cycle that provides the additional efficiency of the energy conservation of the electrons transferred (Mitchell, 1976; Rich & Marechal, 2010).

In such species as mammals and yeasts it has been observed that as the rate of electron transfer is reduced, the enzyme may leak electrons to molecular oxygen, promoting the formation of the superoxide ion. This mitochondrial dysfunction has been widely studied, and its role in the O_2 sensing pathway has been investigated because the increasing production of reactive oxygen species (ROS) is the result of organisms in hypoxic/anoxic conditions (Guzy et al., 2007). New evidence suggests that ROS generated by the mitochondrial complex III are required for the hypoxic activation of transcription factors such as HIF (Hypoxia Inducible Factor); however, this topic will be more extensively discussed below.

The mitochondrial complex III from invertebrates has been poorly studied, but recent reports about these species confirms the importance of studying its basis and applications. An interesting example is the study about the control of Chagas disease, which severely affects the health of the human population in Latin America and which is caused by the protozoan parasite *Trypanosoma cruzi*. Genes et al. (2011) reported such bacteria species as *Serratia marcescens* biotype A1a, which is regularly found in the gut of the vector insect *Rhodnius prolixus*, and which demonstrates the trypanolytic activity conferred by prodigiosin. Prodigiosin is a potent bacterial tripyrrolic compound with various biological activities. This study suggests the abnormal mitochondrial function of *T. cruzi* since prodigiosin inhibits the mitochondrial complex III, affecting subsequent oxidative phosphorylation.

2.4. Complex IV, Cytochrome C oxidase (EC 1.9.3.1) is the terminal enzyme of the electron transport chain and it catalyses the reduction of molecular oxygen to water. The reduction of oxygen by this enzyme – which is responsible for biological energy conversion in mitochondria (Belevich et al., 2010) – is also linked to the translocation (pumping) of four protons across the membrane. This movement of electrons is subsequently coupled to ATP synthesis by the ATP-synthase (Khalimonchuk & Rodel, 2005). The cytochrome C oxidase (CO) has been described as one of the electron transport chain elements which is highly affected by changes in oxygen levels – since cytochrome C reduction is oxygen-dependent – and becomes more reduced when oxygen levels increase (Wilson et al., 1988).

The COX from eukaryotes consists of 11-13 subunits, depending on the species. It belongs to the family of heme-cooper enzymes, some of them suggested as hypoxia sensors. The enzyme is highly regulated by transcription factors, hormones, lipid membranes and the second messengers that control its activity (Ripamonti et al., 2006; Semenza, 2007; Fontanesi et al., 2008). As observed in other mitochondrial complexes, COX also includes mitochondrial encoded genes as subunits CO1, CO2, and CO3 which form the functional core of the enzyme; the rest are nuclear-encoded subunits and their functions – even in the most studied animal models – remain unclear, although they are assumed to participate in the assembly, stability and regulation of the enzyme (Rich & Marechal, 2010). Moreover, CO is also regulated by the existence of various isoforms from each nuclear-encoded subunit which is known to be tissue- and specie-specific (e. g. CO5a and CO5b, CO6a, CO6b and CO6c, and CO7a, CO7b, CO7c, etc.; Diaz, 2010).

The CO genes' expression and the activity of the enzyme are known to be affected by external factors. In crustacean species, such as the grass shrimp *Palaemonetes pugio*, the gene expression of subunits CO1 and CO2 is positively or negatively regulated by low dissolved oxygen concentrations in water (Brouwer et al., 2008). In insects, as with the sweet potato hornworm *Agrius convolvuli*, diapause – the delay in development in response to regularly and recurring periods of adverse environmental conditions – is induced by low temperatures. During this physiological state, the neurological activity, oxygen consumption rate and metabolic levels are low compared to undiapause animals; and it has been found that the genetic expression of the CO1 subunit is down-regulated. When the organism terminates diapause, CO1 is up-regulated and the enzyme activity also increases (Uno et al., 2004). Other insect species, such as the cotton boll worm *Helicoverpa*

armigera, show diverse responses during diapause: the levels of CO1 mRNA and enzyme activity are low, suggesting that the diapause state is different in each species (Yang et al., 2010).

In some species, CO participates in organism detoxification, as observed in the polychaetes *Hediste diversicolor* and *Marenzelleria viridis* which inhabit eutrophicated regions with low oxygen levels and high sulphide concentrations (where CO functions as an alternative pathway of oxidation) (Hahlbeck et al., 2000). In addition, when sulphide becomes hydrogen sulphide (HS) – a weak acid that occurs in marine and aquatic environments such as hydrothermal vents, mudflats and marshes – HS is known to reversibly inhibit CO activity, affecting the aerobic metabolism of certain species, such as the worm *Urechis caupo* (Julian et al., 1998).

2.5. Complex V, ATP synthase (EC 3.6.3.14) is a multimeric enzyme that transforms the kinetic energy of the protons' electrochemical gradient to synthesise the high energy phosphate molecule ATP. Nowadays, it is well-known that the enzyme can also hydrolyse ATP, functioning as an ATPase (Boyer, 1997; Tuena de Gomez-Poyou et al., 1999). This mitochondrial enzyme comprises a catalytic sector F_1 (composed by $\alpha_3\beta_3\gamma\delta\varepsilon$ subunits), and a transmembrane hydrophobic sector F_O (composed of at least three subunits: a, b_2 and $c_{10\text{-}12}$), both linked by a central and a peripheral stalk (Mueller et al., 2004). As in other mitochondrial complexes, this enzyme includes subunits encoded in both the nuclear and mitochondrial genomes, in a tightly coordinated process to assemble this multimeric complex (Itoi et al., 2003; Muhlia-Almazan et al., 2008).

During the oxidative phosphorylation process in mitochondria, the electron transport chain generates a proton gradient that is proposed to drive the rotation of Fo, a central rotor located in the inner mitochondrial membrane. This rotation movement is believed to reverse the rotation of the F_1 nanomotor, inducing – via a conformational change – the sequential release of ATP from three identical catalytic sites followed by the sequential synthesis of newly formed ATP from Pi +ADP at these sites (Cardol et al., 2005). Biochemical and structural studies of the F_1 sector from bovine enzymes have demonstrated that catalytic sites are integrated mainly by three β subunits that alternate with three α subunits. The three catalytic sites formed by these three pairs of α/β subunits are grouped in segments forming a sphere, which is connected to the γ subunit which connects F_1 to Fo (Lai-Zhang & Mueller, 2000).

Due to its complex structure and the dual role that the ATP synthase plays in cells, the current state of research concerning this mitochondrial enzyme is both abundant and relevant; however, for the majority of invertebrate taxa, the information regarding this enzyme appears to be almost non-existent, restricted to some insect species for the more studied models. Analyses of the mitochondrial transcriptome and proteome from these species – which have been exposed to different environmental conditions – have shown that the ATP-synthase subunits can be affected in their expression, and that specific subunits of this multimeric complex can also play additional roles in the mitochondrial function. These findings suggest that invertebrates are able to respond by changing their metabolism to maintain cell homeostasis.

In the fruit fly *Drosophila melanogaster* and the California purple sea urchin *Strongylocentrotus purpuratus* the gene expression of the ATP-synthase subunit alpha (atpα) was measured at early developmental stages, and it was found that the amount of mRNA varies throughout development in both species. Contrary results showed that during the larval stage the nuclear and mitochondrially encoded ATP synthase genes appear to be temporally co-regulated in *Drosophila*, although in the sea urchin this development pattern was not observed (Talamillo et al., 1998). In 2005, Kidd et al. analysed null mutants of the *A*TP-synthase subunit ε in *Drosophila* spp., and a dramatic delay in the growth rate of the first instar larvae that finally died was reported. In addition, in fly embryos the ATP-synthase activity had a six-fold reduction.

Most likely, the first two studies concerning the ATP synthase of crustacean species were published in 2001. The authors characterised the enzymatic properties of F_1 and evaluated its sensitivity to specific inhibitors and modulators in the gills of the freshwater crayfish *Orconectes virilis*; they included, as an important contribution, the standardised methods for isolating mitochondria from

crustacean tissues and some results about their enzyme stability at different temperatures and pH conditions (Li & Neufeld, 2001a, 2001b).

Recent reports on the most-studied shrimp species – *Litopenaeus vannamei* – have characterised and studied several mitochondrial and nuclear encoded subunits from tissues such as muscles, gills, pleopods and the midgut gland (Muhlia-Almazan et al., 2008; Martinez-Cruz et al., 2011). The complementary DNA sequences of the atp6 subunit encoded in the mtDNA and the atp9 (a nuclear encoded subunit) were characterised and their deduced proteins, as major components of the F_O sector, were included in a molecular model which predicted that in the shrimp F_OF_1 ATP synthase the atp9 oligomeric ring may contain 9-10 proteins (Figure 1; Muhlia-Almazan et al., 2008).

Over the last decade, the effects of a viral agent which provokes shrimp death have been deeply studied. The white spot syndrome virus (WSSV) is perhaps the most devastating shrimp disease, causing massive mortalities in global aquaculture systems (Sanchez-Paz, 2010). In 2006, Wang et al. analysed the gene expression profile of the fleshy prawn *Fenneropenaeus chinensis* in response to WSSV infection through cDNA microarrays. Genes including the ATP-synthase A chain and arginine kinase were found to be down-regulated during WSSV infection. Additional studies in other shrimp species, reported thirty additional genes which are involved in the antiviral process as part of the shrimp's defence system. One of the most interesting findings of these studies was that the interferon-like protein (IntlP) – known as an antiviral factor – showed increased expression in virus-resistant shrimp (He et al., 2005). Later, Rosa & Barraco (2008) suggested that the shrimp interferon-like protein (IntlP) is rather a region of the insect mitochondrial b subunit of the ATP-synthase, due to the high identity between both proteins (60–73%). Recently, Liang et al. (2010) have suggested the ATP-synthase subunit β (*atp* β) – earlier called BP53 as a protein involved in the WSSV binding to shrimp cells – may play an important role in the antiviral defence system of shrimp against WSSV.

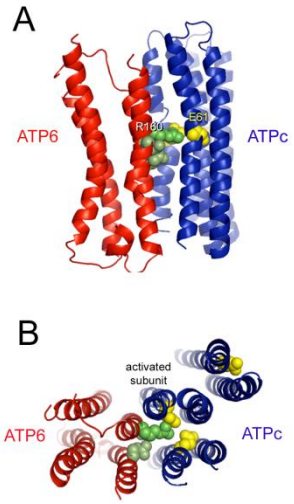

Fig. 1. Molecular Model of the ATP9- ATP6 Subcomplex from the Shrimp *L. vannamei*.
A) Ribbon lateral view, and B) Ribbon front view of the subunit ATP6 complex with three ATP9 subunits. The predicted functional residues are marked in both subunits, R160 from ATP6, and E99 from ATP9. (Taken from Muhlia-Almazan et al., 2008).

Transcriptomes and proteomes have provided a lot of information, not only about the characteristics of specific sequences of nucleotides or amino acids, but also about the proteins' structure and function in invertebrate organisms under diverse environmental conditions (Clavero-Salas et al., 2007). Moreover, novel proteins have been reported as accessories to the mitochondrial

protein complexes in invertebrates species, such as the ticks *Ornithodoros moubata* and *O. erraticus*, where six novel proteins similar to the ATP synthase subunit 6 (*atp6*) were identified in the salivary glands. These proteins are attractive targets for controlling ticks and tick-borne pathogens (Oleaga et al., 2007).

Actually, and based in the mitochondrial highly conserved function, generic models of the electron transport chain in mitochondria have been constructed using bioinformatic tools so as to predict how the rate of oxygen consumption through the system – and the redox states of some intermediates such as NAD/NADH, ubiquinone, and cytochromes – respond to physiological stimuli such varying oxygen levels and other rapid energy demands (Banaji, 2006).

Ultimately, it is remarkable that the mitochondrial function has remained in all animal species through its long and peculiar evolutionary history and under the influence of variable selective pressures. Moreover, structural and biochemical adaptations promoting highly effective mitochondrial functions have allowed organisms to inhabit unusual environments.

3. The Invertebrates Mitochondrial Genome.

The study of the mitochondrial genome has provided enormous amounts of information from which it has become feasible to infer the origins of species by using comparative and evolutionary genomics (Jiang et al., 2009) in order to understand the ancient phylogenetic relationships among species, to comprehend population genetics (Boore et al., 1995; Boore, 1999), and to recognise the mechanisms coordinating the nuclear and mitochondrial genomes so as to synthesise a large number of functional proteins located in this organelle.

To date, the mtDNA of several invertebrates has been sequenced and characterised, including ascidians (Yokobori et al., 1999), echinoderms (Jacobs et al., 1988; Asakawa et al., 1995), insects (Clary & Wolstenholme, 1985), nematodes (Okimoto et al., 1992), molluscs (Yu & Li, 2011; Cheng et al., 2011), and various crustacean species such as shrimp and crabs (Staton et al., 1997; Shen et al., 2007; Peregrino-Uriarte et al., 2009). Several reports have shown that the mitochondrial genome of invertebrate species varies, and ranges between 12 and 20 kbp. This may be due to contrasting ecological habitats or it may be a response to different selective pressures (Table I).

Phylum	**Species**	**mtDNA size (bp)**	**GenBank Acc. No.**	**References**
Porifera	*Plakinastrella sp.*	19,790	NC_010217	Lavrov et al., 2008
	Negombata magnifica	20,088	NC_010171	Belinky et al., 2008
	Aphrocalliste s vastus	17,427	NC_010769	Rosengarten et al., 2008
Cnidaria	*Hydra oligactis*	16,314	NC_010214	Kayal & Lavrov, 2008
	Aurelia aurita	16,937	NC_008446	Shao et al., 2006
	Fungiacyathus stephanus	19,381	NC_015640	---
Platyhelminthes	*Symsagittifera roscoffensis*	14,803	NC_014578	Mwinyi et al., 2010
	Clonorchis	13,877	JF729304	Cai et al.,

	sinensis			2011
	Taenia taeniaeformis	13,647	NC_014768	Liu et al., 2011
Rotifera	*Brachionus plicatilis*	12,672	NC_010484	Suga et al., 2008
Acanthocephala	*Leptorhynchoides thecatus*	13,888	NC_006892	Steinauer et al., 2005
Nematoda	*Caenorhabditis elegans*	13,794	NC_001328	Wolstenholme et al., 1994
	Necator americanus	13,605	AJ417719	Hu et al., 2002
Onychophora	*Oroperipatus sp.*	14,493	NC_015890	Segovia et al., 2011
Brachiopoda	*Laqueus rubellus*	14,017	AB035869	Noguchi et al., 2000
Echinodermata	*Acanthaster planci*	16,234	NC_007788	Yasuda et al., 2006
	Strongylocentrotus purpuratus	15,650	NC_001453	Qureshi & Jacobs, 1993
	Cucumaria miniata	17,538	NC_005929	Arndt & Smith, 1998
Mollusca	*Crassostrea gigas*	18,225	EU672831	Ren et al., 2010
	Cepaea nemoralis	14,100	NC_001816	Terrett et al., 1996
	Octopus minor	15,974	HQ638215	Cheng et al., 2011
Annelida	*Platynereis dumerilii*	15,619	AF178678	Boore & Brown, 2000
	Lumbricus terrestris	14,998	NC_001673	Boore & Brown, 1995
Arthropoda				
Subphylum Chelicerata	*Centruroides limpidus*	14,519	NC_006896	Davila et al., 2005
Subphylum Crustacea	*Litopenaeus vannamei*	15,989	DQ534543	Shen et al., 2007
Subphylum Myriapoda	*Scutigera coleoptrata*	14,922	NC_005870	Negrisolo et al., 2004
Subphylum Hexapoda	*Apis mellifera*	16,343	NC_001566	Crozier & Crozier, 1993

Table 1. Invertebrates' mitochondrial genome size of the species of different phyla.

Because of the wide variability of environmental conditions in which a large number of invertebrate species are distributed, several specific mtDNA-rearrangements have been found when compared with those observed in the mtDNA of mammals. Such novel arrangements include the mitogenome from the blue mussel *Mytilus edulis* (Hoffman et al., 1992), and that of the fruit fly *Drosophila melanogaster* (Clary & Wolstenholme, 1985; Garesse, 1988) and the horseshoe crab *Limulus polyphemus* (Staton et al., 1997).

Also, some species – or groups of species – may lack some genes, such as nematodes whose mtDNA lacks a gene for ATP8 (Keddie et al., 1998), or cnidarians like the coral *Sarcophyton glaucum* which includes an unusual gene encoding an extra tRNA (Beaton et al., 1998). Moreover, major changes

have been found in invertebrates' mtDNA, such as the mitochondrial genes of *Lumbricus terrestris*, which are all known to be encoded in the same strand and, unlike others, the genes coding A8 and A6 are separated by a long 2700 nucleotides fragment (Boore & Brown, 1995).

In 2006, the description of the mtDNA of the moon jellyfish (*Aurelia aurita*) was reported. It was surprising to find that mitochondria of this organism contain a linear genome, which became the first non-circular genome described in a Metazoan. Besides its linearity, its organisation involves two additional sequences of 324 and 969 nucleotides, the last (ORF969) encodes a putative family B-DNA polymerase, tentatively identified as *dnab,* which was previously only reported in algae mtDNAs (Shao et al., 2006). Subsequently, the linear mitogenome of Cnidarians of the genus *Hydra* was also reported, although it was found that it is fragmented as two linear mitochondrial "chromosomes" (mt1 and mt2) where all genes are unidirectionally-oriented (Voigt et al., 2008).

In addition, the invertebrate's mitochondrial genetic code differs from the universal/standard genetic code, and it is suggested that this is species-specific since several studies have identified some changes in animal mitochondrial code, as shown by Table II (taken from Watanabe, 2010). As observed in this table, invertebrate mtDNAs are largely represented by different changeable codons – depending upon the species. This is the case for the AUA codon which usually codes Ile in the standard genetic code but in the mitochondria of some species of Nematoda, Mollusca, Platyhelminthes and Vertebrata it encodes a Met (Himeno et al., 1987; Bessho et al., 1992). Also, in several species, the start codon differs from the AUG but still codifies a methionine, and in most of the species the stop codon is an incomplete codon, such as UA or U (Watanabe, 2010).

Codon (Universal code)	**AUA (Ile)**	**AAA (Lys)**	**AGA (Arg)**	**AGG (Arg)**
Vertebrates (human, bovine, rat, mouse, chicken, frog)	**Met**	Lys	**Ter m**	**Term**
Prochordates (ascidian, asymmetron)	**Met**	Lys	**Gly**	**Gly**
Echinoderms (sea urchin, starfish)	Ile	**Asn**	**Ser**	**Ser**
Arthropods	**Met**	Lys	**Ser**	**Ser**
Most (shrimp, daphnia)	**Met**	Lys	**Ser**	**Ser**
Insect (Drosophila)	**Met**	Lys	**Ser**	-
Molluscs (squid, octopus, Liolophura, Mesogastropoda)	**Met**	Lys	**Ser**	**Ser**
Nematodes (nematodes, ascaris)	**Met**	Lys	**Ser**	**Ser**
Platyhelminthes	**Met**	**Asn**	**Ser**	**Ser**
Most (Echinostomida, Trematoda)	Ile	**Asn**	**Ser**	**Ser**
Rhabditophora (Planaria)	Ile	Lys	Arg	Arg
Coelenterates (jellyfish, coral, sea anemone, hydrozoa)	Ile	Lys	Arg	Arg

Table 2. The relationships between the genetic codes of animal mitochondria. Modified from: Watanabe, 2010. Bold letter: non-universal codon; Term: termination codon

Although, to date, the mitochondrial gene expression mechanisms are not fully understood, and the evolutionary processes by which the mitogenome suffers a rearrangement are not clear. It is proposed that a new order in genes' arrangements must preserve or facilitate those signals or mechanisms required for the transcription and processing of RNAs to accomplish the mitochondrial function in animal species (Boore, 1999).

The mitochondrial DNA from animal cells is known to be easily affected, since it is not protected by DNA-binding proteins or histones such as nuclear DNA. Several studies have found that mtDNA can be affected by aging, hypoxia and random events of the mutation or insertion/deletion (rates of mutation for mitochondrial genomes are known to be much higher than those in the nuclear DNA) that can produce increased oxidative stress and high levels of ROS in this organelle. Defective proteins which result from altered mtDNA molecules cause defective mitochondrial function, as an impaired respiratory chain and increased electron leaks so as to finally generate larger amounts of ROS (Wei et al., 1998).

Insects' mitogenomes are known to be affected at the transcriptional level by chemicals, since the mtDNA copy number has been shown to increase to meet the bioenergetic demands of the organism, as observed in the fly *D. melanogaster* when exposed to tetracycline. Treatment with this antibiotic causes an energetic deficiency, promoting an up-regulation of the mtDNA copy number (Moraes, 2001; Ballard & Melvin, 2007).

4. Invertebrate Challenges and How Marine Species Spend Energy.

In most animal species, high energy levels in their bodies reveal fast growth, adequate energy storage, effective reproduction strategies and viable descendants with characteristic short life spans; however, reduced energy levels in a biological system results in affected gene expression, low survival rates and reduced metabolic rates and, therefore, a need on the part of physiological mechanisms to slow the ageing rate until environmental conditions are enhanced and higher energy levels are again reached (Stuart & Brown, 2006). In their natural habitat, many invertebrate species must undergo endogenous physiological processes during their life cycle, such as molting, starvation, quiescence and metamorphosis, among others. Many of these processes imply high energetic expense, causing a low energy status that reduces their ability to reach the adult stage (Hochachka & Somero, 2002).

The role of metamorphosis – one of the most amazing physiological endogenous processes in nature – becomes strikingly important when considering the large number of animal species that undergo metamorphic changes. Frequently, the energetic balance of holometabolous insects during metamorphosis is negative, because there is no energy gain and species must face all these changes by using any energetic reserves previously stored (Nestel et al., 2003).

During their larval stages, insects – such as Lepidopterans – show fast growth rates, as observed in the tobacco worm larvae of *Manduca sexta* which increases its mass 10,000-fold in just 16 days at the final larval instar (Goodman et al., 1985). The midgut epithelium of this species is a highly aerobic tissue that digests and absorbs nutrients, and transports ions at high rates. During metamorphic changes, the midgut epithelium is programmed to die and the larval midgut should maintain structural and functional integrity until the pupal epithelium is formed. During this process, ATP synthesis and mitochondrial function must be obligatorily maintained. Thus, organisms resolve this by reducing mitochondrial substrate oxidation, a clear indication that the electron transport chain may be a site of modulation during metamorphosis (Chamberlin, 2004).

Quiescence and estivation are also two responses that some species may display during unfavourable environmental conditions in which insufficient energy is available to grow and breed. These dormant states allow species to survive by reversibly down-regulating their metabolism to

low levels for up to several years. Among invertebrates, many species show quiescent states at stress conditions, including nematodes, crustaceans such as the brine shrimp *Artemia franciscana* (Hand, 1998), the estivating pulmonate snail *Helix aspersa* (Pedler et al., 1996), and various insect species entering in diapause, such as *Helicoverpa armigera.* Studies have proposed that a coordination mechanism is required when animals enter into the dormant state so as to maintain cellular homeostasis by both energy-consuming and energy-producing pathways. During quiescence, *A. franciscana* can reduce its metabolism essentially to zero – this metabolic-rate suppression affects the mitochondrial respiratory capacity and the rates of ATP-consuming processes (Barger et al., 2003). In the embryos of *Artemia franciscana*, anoxia provokes the organism to enter into a quiescent state. During experimental gradual oxygen removal, various biochemical responses are observed, such as a pH decrease, the reduction of heat production and the depression of ATP levels. Also, genetic responses, such as the down-regulation of RNA transcription, are observed during quiescence (Hand, 1998).

Often, metabolic rates have been inversely related to the life span of mammals. Moreover, when mitochondrial respiration has been inhibited by RNAi techniques, the life span extends in *C. elegans* (Lee et al., 2003), and long-lived mutants of this nematode concomitantly show decreased metabolic rates (Stuart & Brown, 2006).

The process by which mitochondrial respiration affects or extends life span has been studied in several organisms, including yeasts, worms, flies and mice (Lee et al., 2010). Electron transport in mitochondria is the main producer of superoxide anion ($O^{\cdot-}$), which in turn generates several types of reactive oxygen species (ROS), as has been mentioned (mitochondrial Complex III). In fact, according to various studies, ROS are not only undesirable toxic metabolites promoting organism oxidative stress, but they are also molecules that participate in the mitochondria-nucleus's signalling pathways (Storz, 2006). Emerging data on *C. elegans* suggests a new described pathway where superoxide serves as an intracellular messenger, whereby with increasing superoxide concentration a signal transduction pathway is triggered, resulting in changes in the pattern of the gene expression of nuclear proteins and which finally results in an increased life span (Yang & Hekimi, 2010). However, different mechanisms have also been proposed as being implicated in the aging process, such as diet restriction, ubiquinone deficiency and the hypoxic response (Klimova & Chandel, 2008).

At this point, this chapter would not be complete if the energetic costs of flying for insect species were to be omitted. This activity is probably the most expensive process recorded in nature. It is by now a well-known and remarked-upon fact that the metabolic rate during insect flight increases over 50-100 fold above the resting rate (Ellington, 1985). Thus, it is clear that the flight muscle of insects is the model tissue that many researchers have adopted in order to understand mitochondrial function since it is capable of effectively producing and hydrolysing large amounts of ATP (Sherwood et al., 2005). Insect flight is a highly oxygen-dependent process, and the flight muscle metabolism is fully aerobic; thus, it has been suggested that the amazing aerobic capabilities of insects are based on a highly efficient mode of oxygen delivery that includes their oxygen transport system in a well distributed system of tracheae and tracheoles (Wegener, 1996).

In addition, several studies have demonstrated that the function and energy needs of certain tissues are highly correlated with the number of mitochondria per cell (Robin & Wong, 1988). This agrees with the large quantities of mitochondria with pronounced cristae and large surface areas that are found in the flight muscle cells of the honey bee *Apis mellifera* (Suarez et al., 2000). To date, it is well-known that oxygen uptake rates in mitochondria cristae are much higher in the flying muscle of *A. mellifera* than that observed in mammals' mitochondria – this can explain the higher electron transport rates observed in such enzymes as cytochrome c oxidase, whose maximum catalytic capacity was recorded in this species during flight (Suarez et al., 2000).

Besides the increase on the ATP hydrolysis rate during flight, other mitochondrial adaptations to the highly and continuous energy requirements of flying species have been reported, such as the remarkable dependence on the synthesis of energy-rich phosphate compounds like

phosphoarginine. Phosphoarginine, as mentioned above, constitutes a usable pool of high energy phosphate (Hird, 1986) so as to maintain the high rate of ATP turnover in flying insects (Wegener, 1996).

In addition to the various metamorphic changes in their life, crustaceans undergo a frequent and cyclic process: molting. During the molt cycle, crustaceans are exposed to a temporary scarcity of food since they lack the ability to handle food until their new exoskeleton is synthesised. Several adaptive strategies have been recognised as being employed by these organisms so as to avoid the adverse effects of starvation, such as the storage of fuel compounds in their midgut gland (Sanchez-Paz et al., 2007), changes in locomotor activity (Hervant & Renault 2002), and a decrease in oxygen consumption (Morris et al., 2005). However, little attention has been paid to the bioenergetic consequences of starvation in shrimp; since the composition of food plays an important role in oxidative phosphorylation, the nutritional status of shrimp species, such as *Litopenaeus vannamei*, may affect its major bioenergetic functions.

In our lab, we have hypothesised that, due of its central role in the cell energy metabolism, the expression of genes encoding the different polypeptide subunits that compose ATP synthase during unpredictable episodes of food shortage may ultimately be modulated. Thus, we experimentally evaluate the effect of starvation in the gene expression of subunits ATPα, ATPβ and ATP9 in the shrimp midgut gland, during a period of short-term food deprivation (5 days). Our results (Figure 2) show that the mRNA amounts from subunits ATPα and ATPβ which directly participate during ATP synthesis decreased as starvation time increased; however, no significant changes were observed in the mRNA amounts of ATP9, which forms the oligomeric ring from Fo in the shrimp ATP-synthase.

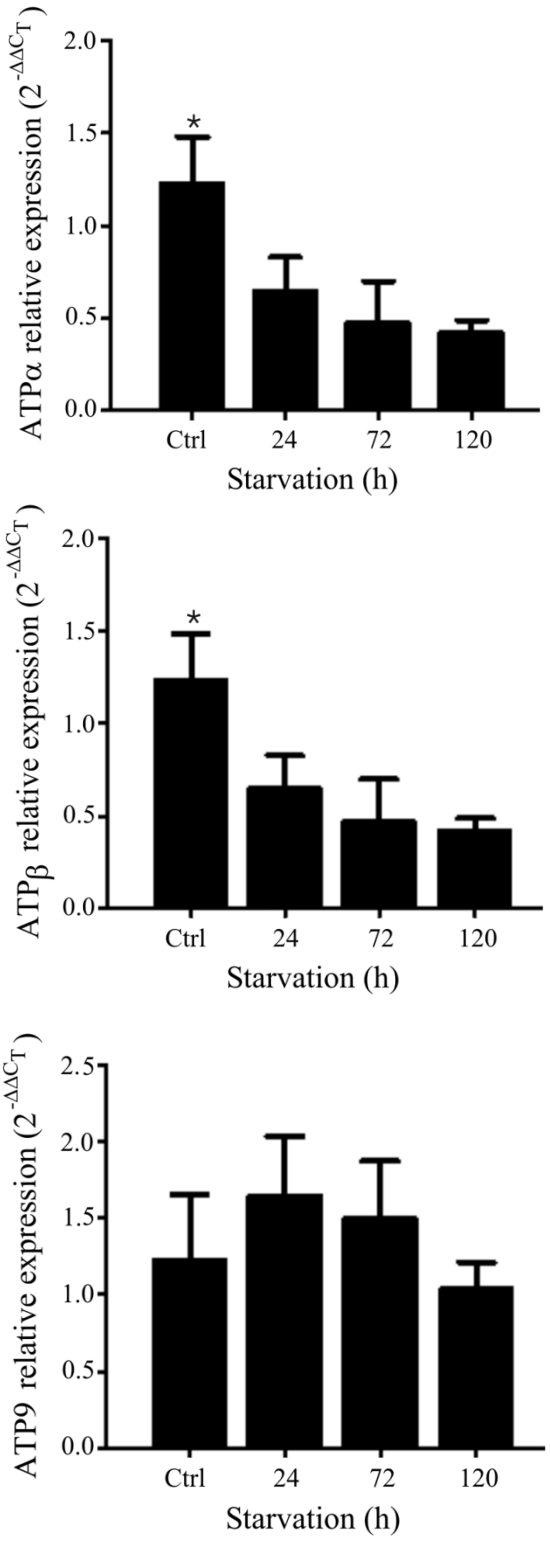

Fig. 2. The relative expression of A) ATPα, ATPβ and C) ATP9 mRNA in the midgut gland of the white shrimp *Litopenaeus vannamei* in response to a short-term starvation period. Expression values are given based on normalisation to L8. The data is represented as the mean and standard deviation of triplicate determinations. (*) Statistical significance was considered at $P < 0.05$.

Sanchez-Paz et al., (2007) reported a gradual decrease of glycogen in the midgut gland of the white shrimp as starvation progressed. After a 24 h starvation period, the glycogen content dropped by about 50%, which correlates with an increase of the *atp*9 subunit after 24 h of starvation, suggesting that glycogen may be used as fuel to generate ATP and pyruvic acid. As glycogen stores become depleted, the organism must increasingly rely on fatty acid catabolism as a source for ATP synthesis. In general, starved shrimp showed a sharp decrease in their midgut gland lipidic constituents for up to 120 h (more noticeable in acylglycerides).

Various studies have shown that during starvation-induced lipolysis there is a decrease in the amount of ATP, which was accompanied by a fall in some subunits of the FoF_1-ATP synthase (Vendemiale et al., 2001). It is well-known that starvation tampers with cellular detoxification systems and may expose cells to oxidative injury (Di Simplicio et al., 1997; Vendemiale et al., 2001), leading to an impaired production of ATP and a reduced uptake of substrates for mitochondrial metabolism. The results from our study, together with results from previous studies, prompt us to suggest that shrimp are capable of satisfying their energy demands through a complex combination of mechanisms that enables them to survive the adverse effects of food scarcity.

Due to its density, viscosity (800 times more dense and 50 times more viscous than air) and low oxygen solubility, water – as a respiratory medium – imposes difficulties for aquatic breathers in obtaining the necessary supply of oxygen from their surrounding environment so as to keep breathing and bringing oxygen into their systems. This process becomes more complicated when considering additional parameters (such as temperature, salinity and depth) affecting the dissolved oxygen concentration of seawater, causing additional constraints on marine species' development (Sherwood et al., 2005). All the species inhabiting marine environments should face these dynamic environmental conditions, which in over the last few decades have been seriously affected by a wide variety of anthropogenic activities, such as industrial and agricultural runoffs (Wu, 2002).

Several studies have found that marine invertebrates may respond to stress conditions by changes at the transcriptional level of the expression of specific genes. In crustacean species such as the crab *Eriocheir sinensis*, different gene expression profiles from gills were characterised during acclimation to high cadmium concentrations in water. Analyses have revealed over-expressed genes, such as disulphide isomerase, thioredoxin peroxidase and glutathione S-transferase. Under the same conditions, ATP synthase beta, alpha tubulin, arginine kinase, glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase were down-regulated. The results demonstrated that acute and chronic exposure to waterborne cadmium induced a decreased abundance of the transcript-encoding enzymes involved in energy transfer; this suggests that chronic metal exposure induced an important metabolic reorganisation (Silvestre et al., 2006).

Some other species which face high cadmium concentrations are marine intertidal molluscs, such as oysters, which live in estuaries were fluctuating temperatures and levels of trace metals are known to directly affect mitochondrial function. Isolated mitochondria from the oyster *Crassostrea virginica* which were exposed to low cadmium concentrations (1 $\mu mol \cdot L^{-1}$) resulted in a progressive uncoupling that increased with the increasing dose of cadmium; this response agrees with that observed in mammals. However, unlike mammals, molluscs are ectotherms and the exposure to the combined effects of high temperatures and cadmium concentrations severely affected mitochondrial function since elevated temperatures increased the sensitivity of this organelle to cadmium and promoted an increase in the rate of ROS production (Sokolova, 2004). These results highlight the key role of temperature in the mitochondrial system of ectotherm species.

Most invertebrates are described as ectotherm species because their body temperatures vary with the environment. At very low temperatures, polar marine invertebrates were expected to show low metabolic rates, as previously observed in Antarctic fish; however, in 1999 Sommer & Portner found important intraespecific differences in the mitochondrial function of the polychaete *Arenicola marina* from the North Sea and the colder White Sea. Their results concluded that invertebrate life is more

costly at higher latitudes, where oxygen uptake, tissues mitochondrial densities and mitochondrial capacities were higher.

Remarkable abilities have been recorded in invertebrate species inhabiting extreme environments. The term "metabolic plasticity" perfectly describes such organisms as the intertidal periwinkle snail *Littorina littorea*, which has the ability to deal with very low temperatures and also to tolerate the changing environmental conditions imposed by the tidal cycle, implying continuous oxygen deprivation (Storey, 1993). Besides the biochemical and physiological mechanisms previously identified in this species, the over-expressed gene encoding a metallothionein (MT) was recently found during the exposure to low temperature and anoxic conditions of the tissues of *L. littorea*. Since thermogenesis is a process that requires high oxygen consumption and since it is also accompanied by a sharp rise in reactive oxygen species (ROS) generation, the authors describe the ability of MT to function as an antioxidant and as a reservoir of essential metals that contributes to survival under these conditions (English & Storey, 2003).

The deep sea hydrothermal vents are a different type of extreme environment which thermophilic species such as the Pompeii worm *Alvinella pompejana* inhabit. Shin et al. (2009) studied the structure and biochemical characteristics of the Cu,Zn-superoxide dismutase (SOD) of this species and found striking similarities between this enzyme and that of humans, but with an enhanced stability and catalysis – characteristics that may mean that this enzyme is potentially suitable for scientific and medical application. Other mitochondrial proteins have been proposed as a part of gene therapy for devastating human diseases by preventing the cell damage caused by oxidative stress. AOX – the mitochondrial alternative oxidase previously mentioned – is suggested to work in any cell, becoming chemically active only when it is required. AOX is provided to the cell by engineering a gene from a marine invertebrate snail *Ciona intestinalis*; this protein is under analysis as a therapeutic tool tested in mammalian disease models (Hakkaart et al., 2006).

5. How Do Invertebrates Face Hypoxia?

Hypoxia is probably one of the most studied factors affecting the central metabolic pathways of living organisms, including invertebrates. Aquatic species usually face hypoxic events in freshwater or marine environments as a daily cyclic routine in the shallow waters of lagoons, estuaries and mangroves during the dark hours, when plants and algae do not produce oxygen and organic matter is continuously oxidised (Dall et al., 1990). However, nowadays the frequency, abundance and severity of hypoxic events in coastal waters have increased due to anthropogenic activities resulting in deteriorating environments affecting marine organisms (Diaz, 2001). It is well known that hypoxia depresses the growth rate of marine animals, as it disturbs metabolic pathways and promotes the reallocation of energy resources (Wei et al., 2008; Wang et al., 2009).

Several studies have examined the physiological responses of invertebrate species to hypoxia, such as growth, stress resistance and even behaviour patterns in aquatic species able to vertically and horizontally migrate through the water column to reach more oxygenated zones (Eads & Hand, 2003; Burgents et al., 2005; Abe et al., 2007; Seibel, 2011). In fact, among invertebrates there are hypoxia-tolerant species, such as bivalve molluscs and annelids, with highly adapted structures and mechanisms to deal with hypoxia, and some others, such as crustaceans, whose tolerance to hypoxia depends on their habitat, food, and energy needs. Unfortunately, the responses to hypoxic conditions – at the molecular and biochemical levels – of the mitochondrial proteins and enzymes that participate in the respiration process are still poorly studied for most invertebrate species.

The main physiological responses from invertebrates to hypoxia are somewhat similar to those from vertebrates since in the reduction or absence of oxygen, animal cells are not able to produce enough energy to survive. Such general responses are clearly a legacy of the evolutionary past from ancestral forms and they serve adaptive ends. In marine species, such as crustaceans and molluscs, reduced oxygen consumption and metabolic rates have been confirmed during hypoxia; in addition, glucose utilisation and lactate accumulation as indicators of a switch to anaerobic metabolism have been detected at low oxygen concentrations in water (Racotta et al., 2002; Martinez-Cruz, 2007;

Soldatov et al., 2010). In the brine shrimp *A. Franciscana*, the intracellular pH falls at anoxia, heat production is reduced and ATP concentrations are also depressed to low levels (Hand, 1998; Eads & Hand, 2003).

A large amount of information is now available about the changes at the transcriptional level promoted by hypoxia in invertebrates, most of it concerning aquatic species. In our lab, we have evaluated the effects of hypoxia in the gene expression of F_OF_1 ATP synthase subunits, such as atp9, atp6, atpα, atpβ, atpγ, atpδ, and atpε, in different tissues of the white shrimp *L. vannamei*. Results show a general trend towards increase the amount of mRNA as oxygen concentrations decrease (Martinez-Cruz, 2007; Martinez-Cruz et al., 2011; Martinez-Cruz et al. in preparation). Also, significant changes in the amount of mRNA from the mitochondrial- and nuclear- encoded subunits of the ATP synthase were detected at different molt stages and tissues, according to the energy requirements of each stage and the specific requirements of the function of each tissue (Muhlia-Almazan et al., 2008). Chronic exposure to severe hypoxia (1.5 mg/mL during 7 days) also causes the increased transcription of mitochondrial-encoded genes, such as the 16S, CO1, and CO2 subunits from the cytochrome C oxidase in the grass shrimp *Palaemonetes pugio* (Brouwer et al., 2008). To date, microarray technologies have revealed a set of genes that are up- and down-regulated in *P. pugio* during chronic, acute and moderate hypoxia; the results revealed that various genes encoding mitochondrial proteins were affected (Li & Brouwer, 2009).

In the absence of oxygen, animal cells activate transcription factors – such as the well-studied vertebrates hypoxia-inducible factor (HIF) – which has been reported in invertebrates from worms to flies (Semenza, 2007). When activated, HIF leads the organism to exhibit metabolic adaptation to hypoxia by regulating the genetic expression of some proteins and enzymes involved in central biological processes such as glycolysis, erythropoiesis, breathing and angiogenesis so as to maintain cell homeostasis (Klimova & Chandel, 2008). In the shrimp *P. pugio*, a homolog protein to HIF-α called gsHIF was found in this hypoxia-tolerant species. It includes all the conserved domains of vertebrates' HIF proteins, and an additional polypeptide sequence of 130 residues that has not been found in databases, and its participation in the functional properties of the protein has not yet been determined (Li & Brouwer, 2007). In the white shrimp *L. vannamei*, HIF-1 is a heterodimer formed by two subunits: HIF-1β, which is constitutively expressed in shrimp cells and HIF-1α, which is differentially expressed in hypoxic conditions. HIF-1 is suggested in crustaceans to be the master regulator that senses decreased oxygen availability and transmits signals promoting the physiological responses mentioned above (Soñanez-Organis et al., 2009). Additional functions have been attributed to HIF in coral species, such as *Acropora millepora*, where the diel cycle in the central metabolism appear to be governed by the circadian clock and regulated by the HIF system operating in parallel (Levy et al., 2011).

As a part of the HIF-regulated metabolic responses to hypoxia in invertebrates, the activities of specific enzymes – most of them part of the central metabolism – are known to increase. In bivalves such as *Anadara inaequivalvis*, the increased activities of enzymes – such as malate and lactate dehydrogenases – were detected at hypoxia (Soldatov et al., 2010). Also, increases in the catalase and GST activities during anoxia in the estuarine crab *Chasmagnathus granulate* have been observed. It has been suggested that such responses may be a strategy to prepare the organisms for oxidative stress in an effort to protect tissues against oxidative damage during re-oxygenation. An important decrease in SOD activity (which occurred after aerobic recuperation) was also detected; and it could have been caused by the accumulation of hydrogen peroxide production during re-oxygenation (de Oliveira et al., 2005).

At normoxia, the small levels of ROS produced by the metabolism in normal animal mitochondria come from carrying electrons along the mitochondrial complexes I, II, and III (Turrens, 2003). However, when oxygen levels are reduced, the presence of the final electron acceptor in the mitochondrial respiratory chain fails, producing a reduction in the rate of electron transport and a

decrease in oxygen consumption. Under these conditions, the membrane potential increases as does ROS production (Guerrero-Castillo et al., 2011).

It has been reported that in invertebrate species considered to be hypoxia-tolerant, the absolute rate of H_2O_2 production is at least an order of magnitude less per mg of mitochondrial protein than that measured on mammalian species (Abele & Puntarulo, 2004). However, some other species which are not tolerant to hypoxia tend to produce higher levels of ROS at low oxygen levels; thus, it is suggested that they display alternate pathways in order to maintain the mitochondrial respiratory rate and avoid an over-production of ROS (Guerrero-Castillo et al., 2011).

Nowadays, the alternative mechanism of proton sinks has been evidenced in invertebrates since uncoupling proteins (UCPs) have been identified in these species (Abele et al., 2007). Such proteins have been involved in various functions, including thermoregulation, body composition, antioxidant defence and apoptosis. UCPS are thought to dissipate the proton gradient across the inner mitochondrial membrane and may help in controlling ROS production (Yu et al., 2000).

In *Drosophila*, an UCP5 protein over-expressed in a heterologous system has shown to have similar functional abilities to an uncoupling protein (Fridell et al., 2004), while in the marine eastern oyster, *Crassostrea virginica*, UCP5 is represented by two transcript forms: UCP5S (small) and UCP5L (large). However, their function has not been determined since its gene expression is not affected by hypoxia, cadmium exposure or different temperatures (Kern et al., 2009). In addition, a novel protein (UCP6) in invertebrates is considered to be an ancestral form of the vertebrates UCP1, UCP2, and UCP3 (Sokolova & Sokolov, 2004).

In mammals, it is known that less-severe hypoxia induces protective mechanisms. This phenomenon – called hypoxic preconditioning (HP) – appears in two forms: immediate preconditioning (which occurs only a few minutes after a sub-lethal hypoxic episode and declines after 4 h) and delayed preconditioning (which requires gene expression changes and takes place 12 to 24 h later and can last for days) (Dirnagl et al., 2009). In the nematode *C. elegans*, the delayed form of HP has been found to induce unfolded protein response pathways – at this point, misfolded proteins serve as early hypoxic sensors that trigger signalling pathways to induce a hypoxia protective response (Mao & Crowder, 2010).

6. The Role of Mitochondria in Invertebrate Programmed Cell Death (Apoptosis).

Besides the various functions just described, mitochondria also acts as the arsenal of the cell. Numerous and complex processes, still poorly understood, can trigger the release of mitochondrial components into the cytoplasm and subsequently induce cellular apoptosis of the organelle (Hengarter, 2000). It is not our intent here to provide exhaustive coverage of all the issues relating to apoptosis in great detail, but rather to give the reader a basic description of the process – to highlight its importance and to show the challenges that those interested in this topic will face.

As has been mentioned, studies in invertebrate biology are paramount to an understanding of biodiversity and to the search for potential uses for their metabolic capabilities and products for biotechnologies. Besides, comparative sciences may facilitate the use of invertebrate models in understanding the biology and pathology of farmed animals and humans. This is due – in spite of differences in the biochemical, physiological, and cellular characteristics that make invertebrates and vertebrates so obviously different – to the fact that most parts of such grades of their biology have remained similar in both groups through their evolution. For example, invertebrate cells – whether wounded by harsh environments or by the expression of abnormal proteins – die as do vertebrate cells, indicating that the powerful advantages of invertebrate molecular genetics might be

successfully used for testing specific hypotheses about human diseases, for the discovery of drugs and for non-biased screens for suppressors and enhancers of maladies (Driscoll & Gerstbrein, 2003). The same criteria apply for all cellular functioning, as for apoptosis.

Apoptosis (from the Greek: "falling off") – or programmed and regulated cell death and elimination – is a pivotal process in embryogenesis, the orderly elimination of wounded or infected cells, and the maintenance of tissue homeostasis. The process is so important that it is estimated that on a daily basis the human body must get rid of approximately 10^{10} cells. Through apoptosis, cells die quietly in a controlled, regulated fashion; while in another forms of cell death – such as in necrosis – a series of uncontrolled events occur leading to serious and irreversible damage. Given the proper conditions, apoptosis destroys the cell swiftly and neatly. In contrast, necrosis causes the rupture of the cell, releasing its content into the surrounding tissue. Tampering with apoptosis may result in devastating health problems, such as cancers, immune diseases, neurodegenerative disorders and the proliferation of viruses. Apoptosis is executed by a variety of membrane, organelle, cytoplasmic and nucleus signalling, and initiator and effector molecules, including a subfamily of cysteine proteases known as caspases (Jiang & Wang, 2004).

In mammals, the active role of mitochondria in apoptosis induction has been well-established. In invertebrate models of apoptosis, such as the fly *Drosophila melanogaster* and the worm *C. elegans*, the role that mitochondria play during apoptosis and, in particular, during apoptosis initiation is less clear (Rolland & Conradt, 2006). While key regulators of apoptosis in *Drosophila* and *C. elegans* have been found in association with mitochondria, the significance of these associations has not been rigorously tested.

The regulated destruction of a cell is a basic process in Metazoa, as multicellular animals are obligated to remove damaged or harmful cells. During apoptosis, cells die in an orderly, regulated sequence of molecular, biochemical, and cellular processes. According to the endosymbiotic theory, the origin of apoptosis is currently regarded as the result of molecular interactions in which some components of a signal transduction pathway affects other pathways through interaction of some initiator and effector proteins. Accordingly, apoptosis could have arisen simultaneously with – and as a by-product of – endosymbiosis (Kroemer, 1997). However, it has also been proposed that apoptosis may be the result of the acquisition of the aerobic metabolism by early eukaryotes (Frade & Michaelidis, 1997).

Apoptosis is a unique phenomenon of tissue kinetics as it can be said that life is critically controlled by the operational centre of cell, the nucleus. Instead, death is a process controlled by the power house of the cell, the mitochondria. Thus, even cells lacking nucleus commit apoptosis. In general, the two-step membrane depolarisation and free radical release taking place in the mitochondria may trigger apoptosis. This in fact is not so peculiar if we understand that mitochondria were once free-living bacteria which did not need an external gene control for achieving their functions. Once each came into symbiosis forming a eukaryotic cell, it retained some capacity to operate partially independently.

There are several major apoptotic pathways, but the most well-known and studied are the extrinsic and the intrinsic pathways, which respond to different environmental and cellular challenges in vertebrates. The intrinsic pathway is also called the mitochondrial pathway because of the involvement of mitochondria. There are mitochondrial proteins that induce this process (proapoptotic) and others that limit cell death (antiapoptotic). Both proteins interact so as to cooperate and govern the cell's fate. Also, the origin of the activation signals of apoptosis taking place on the mitochondria is a clue molecule, cytochrome C (Cyt C), which is released from the mitochondria to form the apoptosome complex. The intrinsic pathway – with some differences – is a mostly conserved pathway among metazoans (for a comprehensive review look at Wang & Youle, 2009). Cyt C is a key component of the apoptosome complex for activating the initiator caspase-9 after its release from mitochondria. Under non-apoptotic conditions, Cyt C is kept inside the respiratory chain. Against some cellular challenges, like the alteration of the DNA in the mitochondria or the nucleus, Cyt C is released from its membrane, crossing the external membrane

and initiating the formation of the apoptosome complex. In essence, mitochondrial proteins – like Cyt C and caspases – are not hired guns and during non-apoptotic conditions they are responsible for various basic mitochondrial roles for normal cell functioning. The compartmentalisation of such mitochondrial proteins isolates them from interacting with partners or targets, a mechanism to prevent the unwanted activation of apoptosis in normal cells. Only after their appropriate release into the cytoplasm do such proteins play the role of triggers to initiate the cell's suicide.

The classical invertebrate model organisms for the study of apoptosis are *C. elegans* and *Drosophila*. In spite of the fact that the regulators of apoptosis have been found in such model organisms, the involvement of mitochondria in apoptosis is not conclusive. So far, no irrefutable evidence of the release of Cyt C from the intermembrane space has been found. Also, the involvement of Cyt C in the apoptosome formation in *Drosophila* is controversial, and some evidence suggests that Cyt C is not necessary (Rolland & Conradt, 2006).

The current evidence indicates that the whole process of apoptosis (including the involved proteins and the regulation mechanisms) in crustaceans is far more diverse than has been assumed from the studies with model organisms. Recent studies have shown that several proteins in the apoptotic network are quite conserved between mammals and arthropods; however, it is clear that the integration of such homologous proteins in the physiology and pathophysiology of crustaceans needs further experimental assessment. Some unresolved questions regarding this topic are: how does the regulation of the process occur? Is crustacean apoptosis transcriptionally regulated, as in *Drosophila* (RHG ´killer´ proteins)? Or is it controlled by pro- and anti-apoptotic Bcl-2 family proteins, as in vertebrates? The issues that should be investigated in the short-term are whether the calcium-induced opening of the mitochondrion permeability transition pore (MPTP), commonly found on vertebrate species, also occurs in crustaceans. Furthermore, the study of the differences in the regulation of the intrinsic pathway of crustacean apoptosis will lead to an understanding of their adaptation to challenging environments; this is because marine organisms have to deal with seasonal as well as circadian changes in environmental variables. Some examples are UV radiation, temperature and dissolved oxygen, and even some biological stresses such as toxins that may vary over time. But this is not all: other variables that may inhibit apoptosis must be considered. "Characterisation of the players, pathways, and their significance in the core machinery of crustacean apoptosis is revealing new insights for the field of cell death"(Menze et al., 2010).

Apoptosis is a key host response to viral infection. Viruses that can modulate a host's apoptotic responses are likely to gain important opportunities for transmission. Here, we review recent studies that demonstrate that the particles of Invertebrate Iridescent Virus6 (IIV-6) (Iridoviridae, genus Iridovirus), or an IIV-6 virion protein extract, are capable of inducing apoptosis in lepidopteran and coleopteran cells, at concentrations 1000-fold lower than that required to shut-off the host's macromolecular synthesis (Williams et al., 2009). Throughout the process of pathogen–host coevolution, viruses have developed a battery of distinct strategies to overcome the biochemical and immunological defences of the host. Thus, viruses have acquired the capacity to subvert host cell apoptosis, control inflammatory responses, and evade immune reactions. Since the elimination of infected cells via programmed cell death is one of the oldest defence mechanisms against infection, disabling host cell apoptosis might represent an almost obligatory step in the viral life cycle. Conversely, viruses may take advantage of stimulating apoptosis, either to kill uninfected cells from the immune system or else to induce the breakdown of infected cells, thereby favouring viral dissemination (Galluzzi et al., 2008).

7. Conclusion and Future Perspectives.

As stated by Van der Giezen in 2009 "over the last 5–10 years, it has become apparent that the organelle known as the mitochondrion is a much more fluid entity than generally believed," so "why should mitochondrion be the same in all eukaryotes while other cellular structures show such great evolutionary malleability?"

It is our belief that since natural selection has given invertebrates the opportunity to evolve in quick steps, a large window is opening in the field of mitochondrial research among these species, giving an outstanding opportunity to researchers to contribute to an increase in knowledge, not only because there is scarce information, but also because many species have shown special and unique characteristics that need to be explained.

At this point, the information reviewed clearly shows that invertebrates display remarkable physiological capabilities, including highly specialised mechanisms for adjusting mitochondrial functions to solve their energetic demands under the stressful conditions they usually face. These species also include within their systems ancient and novel molecules and structures acting to reach an adaptive state, from the increasing number of mitochondria per cell to the highly complex function of the HIF system.

It is also remarkable that the number of invertebrate species considered as potential models in the study of mitochondrial function has increased. New data on marine invertebrates, such as molluscs and crustaceans and non-*Drosophila* species, are emerging. Since there is still an immense lack of knowledge about invertebrates, important efforts in new animal models should focus on i) the description of mitochondrial systems in species inhabiting extreme environments, ii) the recognition and understanding of the causes and effects of mitochondrial disorders, and iii) the development of unsolved phylogenetic relationships among species and phyla. This may also open important opportunities for new biotechnological applications to better face the effects of global changes such as warming, hypoxic conditions and chronic stressors that specifically affect the central metabolic pathways in such species.

If the regulation of apoptosis in crustaceans is as varied as their diversity as a species, or at least their Families, then the potential for discovering novel biomolecules is immense. Such molecules may find uses in biotechnologies across diverse industries, including pharmacology. We endorse the hypothesis that an advanced knowledge in apoptosis will provide some clues about how crustaceans deal with viral infections and enable the proposal of feasible strategies to protect farmed crustaceans.

8. References

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ANEXO III. Martinez-Cruz, O., Calderon de la Barca, A. M., Uribe-Carbajal, S. & Muhlia-Almazan, A. (**2011**). The mitochondrial F_OF_1 ATP-synthase from the whiteleg shrimp *Litopenaeus vannamei*: isolation, immunodetection and enzymatic activity during hypoxia. Manuscrito enviado a *Comparative Biochemistry and Physiology-Part A: Molecular & Integrative Physiology*.

The mitochondrial F_OF_1 ATP-synthase function from the whiteleg shrimp *Litopenaeus vannamei* during hypoxia

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ABSTRACT

The F_OF_1-ATP synthase participates during the mitochondrial respiration process, it produces ATP in a reaction coupled to an electrochemical gradient of protons generated by the electron transfer chain. Since this enzyme has been scarcely studied in crustaceans, and the effects of hypoxia in the mitochondrial respiration process have not been fully understood in these marine species, we evaluated the hypoxia and re-oxygenation effects in the white shrimp *Litopenaeus vannamei* by measuring ATP amounts in the tail muscle and the ATPase activity from isolated mitochondria. Also, we used immunodetection techniques to identify and quantify the ATP-synthase subunit protein in shrimp mitochondria. Results confirmed the hypoxia effect in shrimp by the increasing lactate concentrations in plasma; however, the ATP amounts and the ATPβ protein concentration remained unaffected. Nevertheless, the ATPase activity significantly increased at hypoxia suggesting that the enzyme may be regulated at a post-translational level. Our findings suggest that when the oxidative phosphorylation fails during hypoxia and the ATP amount diminishes, shrimp are able to use the stored ATP in muscle to maintain cellular functions. At this time the ATP-synthase changes from an ATP producer into a consumer to preserve the mitochondrial function in order to maintain the cellular homeostasis.

Key words: ATP-synthase, ATPase, hypoxia, mitochondria, shrimp.

1. Introduction

The dissolved oxygen concentration (OC) in water is a limiting factor in aquaculture practices. In the bottom layer of water ponds, where shrimp spend most of their time, OC may decrease and become hypoxic or even anoxic due to organisms respiration and decomposition of accumulated organic matter of feed remains and feces, particularly at nighttime. These hypoxic conditions can certainly threaten shrimp life affecting their growth, survival and culture success (Zhang et al., 2006).

Central metabolic pathways, especially those tightly related to chemical energy production and mitochondrial function, are highly affected by oxygen lack in all aerobic organisms (Wei et al., 2008). Hypoxia affects the mitochondrial respiration since oxygen is the last electron acceptor in the electron transport chain, which is not completed during hypoxia, promoting that oxidative phosphorylation fails and the ATP synthesis ceases (Hochachka and Somero, 2002).

The mitochondrial F_OF_1 ATP-synthase, described as a splendid molecular machine, accomplishes a dual role since it is able to synthesize / hydrolyze ATP in eukaryotes and bacteria. The enzyme consists of two nanomotors, the F_1 that is driven by ATP hydrolysis, and the second which is embedded in the mitochondrial membrane, contained within $F_{O,}$ and driven by a proton gradient (Boyer, 1997; Leslie and Walker, 2000). F_1 is the catalytic portion of the enzyme, it is composed by five major subunits α3, β3, γ, δ, and ε, and includes the three catalytic sites formed by three alternating pairs of subunits □/□ forming a sphere that functions as an ATPase by hydrolyzing ATP to ADP + Pi, and as an ATP-producer by

synthesizing ATP molecules from ADP + Pi (Futai et al., 1989; Lai Zhang and Mueller, 2000).

Studies on the structure and function of mitochondrial enzymes as F_OF_1 ATP-synthase from marine invertebrates are scarce, moreover, the effects of hypoxia on this enzyme which plays a central role during respiration and ATP production, have not been evaluated in marine invertebrates. Recently, Martinez-Cruz et al. (2011) analyzed the cDNA sequences of subunits α (*atpα*) and β (*atp*□) from the ATP-synthase of the whiteleg shrimp *Litopenaeus vannamei,* and evaluated the effect of hypoxia by measuring changes in the mRNA amounts of both subunits. The results confirmed highly conserved regions for both deduced proteins and underlined some unique features in shrimp sequences. Hypoxia (1.5 mg/L) caused an increase in *atpβ* transcripts and a subsequent decrease when shrimp were re-oxygenated. The results confirmed that changes in the mRNAs of the ATP-synthase subunits are part of the molecular mechanisms that shrimp display during metabolic adjustment at hypoxia.

Studies in vertebrates have showed in ischemic myocardium an adjustment in cells ATP-demand to decreased O_2 concentrations, where cardiomyocytes initiate a suppression of ATP utilization (Budinger et al., 1998). During oxygen deprivation, the mitochondrial electrochemical gradient collapses and the ATP-synthase changes from an ATP producer to a consumer which hydrolyses ATP in order to maintain the mitochondrial membrane potential by using the still available intracellular ATP molecules (Di Lisa et al., 1998; Grover et al., 2004). The aim of this research was to evaluate the effect of hypoxia and re-oxygenation in the protein expression of the catalytic *atpβ* subunit of the

ATP-synthase. We also evaluated the ATPase activity of the enzyme, and the ATP amounts in the tail muscle of the whiteleg shrimp *L. vannamei*.

2. Materials and methods

2.1. *Experimental animals and hypoxia bioassay*

Adult whiteleg shrimp (*L. vannamei*) weighing 30 ± 1 g each were obtained from aquaculture facilities in La Paz, B.C.S. Mexico. All shrimp were randomly distributed in three 1,000 L tanks filled with 300 L of marine water, and kept 8 days under controlled conditions at 28 °C, 35 ppt salinity, and 6 mg/L dissolved OC (oxygen concentration) at normoxia during acclimatization. Shrimp from each tank (n = 30) were fed twice a day with commercial pelletized food (35% protein), then uneaten food and feces were removed daily and 70% of the total water volume was exchanged daily. After acclimatization, three shrimp were sampled from each tank at normoxia (6.0 mg/L) as controls, then the air stones were removed, tanks were sealed, and nitrogen gas was constantly bubbled instead of oxygen through an air stone to induce hypoxia.

The OC in water was continuously monitored with a digital submersible oxymeter and controlled throughout the experiment in each tank. During the OC decrease, three shrimp samples were taken from each tank after 3h at 2.0 mg/L, and then after 3 h at 1.5 mg/L. Afterwards, tanks were re-oxygenated and shrimp were sampled again at 2.0 and 6.0 mg/L. Each shrimp was weighed and 400 µL of haemolymph were extracted from the base of the fifth pereiopod with a 1 mL-syringe containing two volumes of pre-cooled shrimp anticoagulant solution containing 300 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA at pH 7.3 (Vargas-Albores et al., 1993). Each haemolymph

sample was centrifuged at 7,000 *g* for 10 min at 4 °C. Plasma and haemocytes were separated and stored at –80 °C until use. Experimental shrimp were decapitated and the tail muscle was excised and weighed, each was submerged in liquid nitrogen and kept frozen (-20 ºC) until use.

2.2. *Lactate concentration measurement*

Plasma lactate concentration was measured on each collected sample using commercial kits for medical diagnosis, LACTATE-PAP (RANDOX, UK), according to the manufacturer protocols using a Synergy microplate reader (Bio-Tek Instruments). Briefly, each sample was tested in a final volume reaction of 200 µL, including 10 µL of plasma and 190 µL of reactive solution, the reaction was followed at 550 nm in triplicates. Concentration of lactate in plasma was determined in shrimp at normoxic, hypoxic and re-oxygenated conditions.

2.3. *Shrimp muscle ATP concentration*

The ATP concentration was measured in individual shrimp tail muscle using the method reported by Ryder (1985). Briefly, 5 mL of 0.6 M $HClO_4$ were added to 1g of tail muscle, the mix was homogenized and centrifuged at 3,000 g for 10 min at 0 ± 2° C. The supernatant (aliquot of 2 mL) pH was adjusted with 1M and 0.1 M KOH to 6.5-6.8. Then, solution was incubated 30 min on ice and filtered. Finally, purified water was added to a final volume of 10 mL and stored at -20° C until further analysis.

All samples were analyzed using a previously validated reversed-phase HPLC method for shrimp muscle (Diaz-Tenorio, 2006). Separation was achieved with an analytical scale Beckman Coulter Ultrasphere ODS C18 column (4.6 x 250 mm, 5 µM particle

size, Beckman, Germany). Chromatographic conditions were as follows: the mobile phase used was 0.04 M KH_2PO_4 and 0.06 M K_2HPO_4, the flow rate was constant at 2 mL/min and the column was maintained at 28 °C during the analysis. The detection was at 254 nm using a UV Hewlett Packard 1100 GmbH (Germany) detector. A 20 µL aliquot of standard containing 0.166 mM ATP was injected.

2.4. Mitochondria isolation from shrimp muscle

Up to 50 g of tail muscle from shrimp samples at normoxia, hypoxia and the re-oxygenated were dissected and pooled each in 400 mL of cold isolation buffer A (250 mM sucrose, 5 mM Tris, 15 mM EDTA, pH 7.4), all subsequent procedures were carried out at 4 °C. The tissue was homogenized in a blender at medium speed by 1 min. After homogenization, the mixture pH was adjusted pH to 7.4 + 0.1 as rapidly as possible by adding saturated Tris. The neutralized and minced muscle was placed in a double layer cheesecloth and was squeezed free of the sucrose solution. The homogenate was centrifuged at 2,500 rpm for 10 min at 4 °C to pellet the cellular debris. The supernatant was removed and centrifuged at 10,000 rpm for 15 min at 4 °C to pellet the mitochondria fraction. The resulting pellet was then resuspended in 20 mL of ice-cold isolation buffer B (250 mM sucrose, 5 mM Tris, pH 7.4) and centrifuged again at 2,500 rpm for 10 min at 4 °C. The supernatant was removed and centrifuged at 10,000 rpm for 15 min at 4 °C to pellet the mitochondria fraction. The mitochondrial pellet was resuspended in 0.5 mL of isolation buffer B.

2.5. *Mitochondrial proteins separation*

The protein concentration of the mitochondrial suspended pellet was determined using the bicinchoninic acid method using BSA as the standard (Pierce, USA) in triplicates. Measurement was performed in microplate final volume reactions of 200 µL including 10 µL of the mitochondrial fraction, and 190 µL of reactive solution, incubated 30 min at 37°C, and the absorbance was measured at 550 nm on a model 680 plate reader (Bio-Rad, USA).

Mitochondrial protein complexes were separated by blue native polyacrylamide gel electrophoresis (BN-PAGE) on gradient gels 3.5 -11% (Wittig et al., 2006). Eighty µg of mitochondrial protein, previously solubilizated with digitonine (10 mg/mg protein), were centrifuged at 26,000 rpm for 2 h at 4°C to remove insoluble material. Supernatants were mixed with loading buffer (6 µg/µL Serva G, dissolved in 0.5 M 6-aminohexanoic acid) and directly loaded into the native gel. The cathode buffer was prepared with 50 mM Tricine, 15 mM Bis-Tris, 0.01% Serva G, pH 7, and the anode buffer was 50 mM Bis-Tris pH 7. Electrophoresis was run at 35 v for 15 h at 4 °C in a Mini-PROTEAN® Tetra Cell (Bio-Rad, USA) as described by Wittig et al. (2007). The high molecular weight calibration kit (General Electric, USA) was used as a molecular mass marker. Once separated, the protein band identified by its molecular weight (over 660 kDa approximately) as the ATP-synthase complex was excised from gel, and mixed with sample buffer (0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue), then prepared for SDS-PAGE.

The SDS-PAGE was carried out following the method of Laemmli (1970). Gels of 12% polyacrylamide were prepared from a stock solution. The previously excised protein band in sample buffer was immersed in boiling water for 5 min to completely dissociate

proteins and then loaded to the polyacrylamide gel. Electrophoresis was carried out with a current of 15 mA per gel until the bromophenol blue marker reached the bottom of the gel. Gels were stained with 0.1% Coomassie brilliant blue R250 after electrophoresis.

2.6. *Production of rabbit anti-shrimp ATPβ polyclonal antibodies*

Based on the deduced amino acid sequence of atp*β* (GenBank accession no. **ADC55252**; Martinez-Cruz et al., 2011) a synthetic peptide 23 residues long and 90% purity was purchased (PEPTIDE 2.0 company, USA). This peptide matches with the N-terminus residues (FDGELPPILNALEVANRSPRLVL) of the ATP protein.

One mg of the synthetic peptide was first diluted to a final concentration of 3.5 M urea and 0.9% NaCl; then it was diluted 1:1 v/v with Freund's complete adjuvant. A rabbit was first immunized intradermally in the back and proximal limbs with the synthetic peptide solution. After 28 days, the rabbit was boosted intramuscularly with 0.5 mg of synthetic peptide solution diluted 1:1 v/v with Freund´s incomplete adjuvant. Rabbit serum was collected after 7 and 14 days and purified by Protein A affinity chromatography. Briefly, the serum was diluted 1:1 v/v in 0.2 M Tris-HCl, 2 M NaCl, pH 8.6 and loaded into the column at a flow of 1 mL/min. The protein A column was washed with 0.1 M Tris-HCl, 1M NaCl, pH 8.6, and the IgG fraction was eluted with 0.1 M sodium citrate pH 3, and immediately neutralized with 0.2 M K_2HPO_4, pH 9. Collected IgG was ultrafiltrated with 0.1M NaCl using a 10 kDa membrane at 60 psi until a final volume of 1 mL, 100 µL of

5 mM NaN_3 were added and the antibodies were stored at 4 °C.

2.7. *Immunoblotting and ATPβ immunodetection*

Mitochondrial proteins previously separated by SDS-PAGE were transferred onto an Immobilon PVDF membrane using a transfer buffer containing 100 mM CAPS with 10% methanol at pH 11. Transferred dots were blocked with 2% Tween-20 in 20mM Tris, 500 mM NaCl (pH 7.5) for 2 min and then washed 4 times with TTBS buffer (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5). The membrane was incubated overnight at 4 °C with purified rabbit anti-shrimp ATPβ polyclonal antibody diluted 1:500 v/v in TTBS. The blots were washed 4 times, and then incubated for 2 h with goat anti-rabbit IgG alkaline-phosphatase conjugated secondary antibodies (Bio-Rad, USA) diluted 1:3000 in TTBS. Alkaline phosphatase activity was developed according to Immuno-Blot Alkaline Phosphate Assay Kit instructions (Bio-Rad, USA). After color development, the membranes were washed with distilled water and dried. Additionally, two negative controls were included (one sample without primary antibody, and the other without secondary antibody).

An enzyme-linked immunoabsorbent assay (ELISA) was carried out to quantify the ATP-synthase protein from shrimp mitochondrial extract by detecting ATP□ subunit. Briefly, microplates were coated overnight at 4 °C with mitochondrial extract in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Then, coating buffer was removed and plates were washed 4 times with washing buffer (0.10 M Tris-HCl, 0.05% Tween-20, 5 mM NaN_3, 0.05% phenol red, pH 7.4). Plates were blocked with washing buffer plus 3% gelatin for 1h at room temperature, then wells were washed and incubated overnight at 4 °C with 100 μL of anti-shrimp ATPβ polyclonal antibody diluted 1:500 v/v in washing buffer. After 4 washes, the plates were incubated for 2 h at room

temperature with swine anti-rabbit IgG antibody, conjugated with horseradish peroxidase (Bio-Rad, USA; diluted 1:2000 in washing buffer). After 3 washes with washing buffer and one wash with PBS buffer (1 M $K_2HPO_4.3H_2O$, 1 M KH_2PO_4, 0.9% NaCl, pH 7.2), peroxidase activity was developed with 3, 3', 5, 5'-tetramethylbenzidine (TMB) as substrate by adding 100 µL of substrate solution to each well. After color development (2 min), the reaction was stopped by adding 50 µL of 1M H_2SO_4. Absorbance was read at 450 nm in a 550 microplate reader (Bio-Rad, USA). Negative controls were included for each treatment without primary or secondary antibodies. The final concentration of the ATPβ subunit in the sample was calculated using a standard curve which was done with a 1:2 v/v series dilution of the synthetic peptide from 2.5 – 0.156 µg/mL diluted in coating buffer.

2.8. *Measurement of ATPase activity*

ATPase activity was measured as described by Itoi et al. (2003) using a CARY 50 BIO spectrophotometer at a wavelength of 340 nm coupled to a CARY single cell peltier accessory with temperature controller. The F_OF_1-ATPsynthase activity was measured in the direction of ATP hydrolysis. The reaction mixture in 1.5 mL at pH 8.2 contained 100 mM Tris, 300 mM sucrose, 13 mM $MgCl_2$, 133 mM KCl, 20 mM phosphoenolpyruvate, 0.13 mM NADH, 6.7 mM ATP, 2 U of pyruvate kinase (Sigma-Aldrich, USA), and 3 U of lactate dehydrogenase (Sigma-Aldrich, USA). Assays were performed in triplicates at 30°C in the presence of 60 µg of mitochondrial protein.

2.9. *Statistical analysis*

All data were analyzed by one-way ANOVA and post hoc analysis was done using the Tukey test. Statistical significance was considered at $p < 0.05$. Analyzes were performed using Statistica v. 8.0 software.

3. Results and Discussion

Aerobic organisms have evolved to use oxygen for energy production by developing efficient respiratory strategies. When environmental oxygen is reduced (hypoxia) or absent (anoxia), organisms are often not able to satisfy their metabolic demands, thus they have developed adaptive strategies to surpass energy demands (da Silva-Castiglioni et al., 2011; Martinez-Cruz et al., 2011).

Among invertebrates, crustaceans are probably the most studied phylum of marine organisms. It is known that under hypoxia, crustaceans respond by switching to anaerobic metabolism that promotes glucose and lactate accumulation as metabolic indicators (Abe et al., 2007). In this study we detected high plasma lactate concentrations in shrimp exposed to 1.5 mg/L OC, this value was 54% higher than that of shrimp at normoxia ($P< 0.05$), and remained until shrimp were re-oxygenated and water reached an OC of 7 mg/L OC (Fig. 1). These results confirmed that control shrimp showed normal lactate levels in plasma (0.13- 0.15 mg/mL) according to Pascual et al. (2003); and that organisms responded to the effect of hypoxia by increasing lactate concentrations as reported in other crustacean species as *Eriocheir sinensis* (Zou et al., 1996) and *Marsupenaeus japonicus* (Abe et al., 2007).

Hypoxia also is known to produce ATP depletion in animal tissues as muscle. In invertebrates, the ATP concentrations may decline modestly or drastically according to

the ability of the species to respond in a conformer/regulator manner to changes in the environmental oxygen concentrations (Hochachka et al., 1996). We measured the ATP content in shrimp muscle during normoxia, hypoxia and re-oxygenation, but significant changes were only detected during re-oxygenation (Fig. 2). At normoxia and hypoxia, ATP content was around 4.2 μMol/g. This agrees with values reported in other crustacean species as the rock lobster *Jasus lalandii* (Sidhu et al., 1974), the common shrimp *Crangon crangon* (Sartoris and Pörtner, 1997), the Japanese spiny lobster *Panulirus japonicus,* the American lobster *Homarus americanus* (Shimada et al., 2000) and the mud crab *Scylla serrata* (Chiou and Huang, 2004).

We detected a decrease of 25% in the ATP concentration of muscle when organisms were re-oxygenated ($P < 0.05$; Fig. 2), this late reduction can be explained as the result of shrimp exposed to hypoxia for several hours which produced a failure in the respiratory chain, and promoted an energy reduction just as observed in other vertebrate species as mammals and fish (Di Lisa et al., 1995; Itoi et al., 2003). Furthermore, this late answer on ATP depletion which was observed after 19 h (10 h of hypoxia exposure and 3h of re-oxygenation at 2.0 mg/L), suggests that during the early exposure to hypoxia organisms were able to use energy stores as observed in crabs and lobsters (Hill et al., 1991; Speed et al., 2001); and that the ATP synthesis during anaerobic glycolysis would be supplemented by increased ATP availability from the phosphagen phosphoarginine, which is commonly found in the muscle of crustaceans and other invertebrates, thus functioning as a large, long-term, energy reservoir that generates ATP when acute lack of energy occurs (Bailey et al., 2003; Morris et al., 2005).

In this study, for the first time we isolated mitochondria from *L. vannamei* muscle. A 10µg/mL fraction of mitochondrial protein was obtained from 50 g of tissue. Protein recovery was low when compared with mitochondrial preparations from different sources that recovered larger protein quantities (Li and Neufeld, 2001; Smith, 1967); and although larger mitochondrial protein quantities will probably be obtained from using sonication methods, this is known to affect ATPase activity in crayfish tissues as reported by Li and Neufeld (2001).

Shrimp mitochondrial proteins were separated by BN-PAGE, and the ATP synthase complex was first identified as a protein band with a molecular weight over 660 kDa (Fig. 3A), similar to that reported for the ATP-synthase monomer of bovine heart and rat (Wittig and Schägger, 2008). This agrees with the highly conserved molecular weight of individual subunits already reported from the ATP-synthase complex of shrimp (Martinez-Cruz et al., 2011; Muhlia-Almazan et al., 2008).

The over 660 kDa protein identity was confirmed as complex V by evaluating ATP hydrolysis in polyacrylamide gel following the method reported by Wittig et al., 2007 (data no shown), this also supported the presence of a catalytically active F_1. The ATP-synthase complex protein band was loaded in to a SDS-PAGE. Proteins with molecular masses from 40 to 200 kDa were detected (Fig. 3B); and those between 40- 66 kDa corresponded to the previously calculated molecular weight of subunits α and β from shrimp ATP-synthase reported by Martinez-Cruz et al. (2011). The ATPβ subunit was identified from the isolated mitochondrial proteins by using specific polyclonal antibodies. Figure 3C shows a single band that corresponded to the calculated protein mass of ATPβ subunit of 51.45 kDa.

On the other hand, we evaluated the ATPβ subunit protein concentration in the muscle of shrimp at normoxia, hypoxia and re-oxygenation by the ELISA assay, which resulted in no significant differences between treatments (Fig. 4; *P > 0.05)*. These results agree with no changes detected in the ATPβ gene expression previously evaluated (Martinez-Cruz et al., 2011), which may imply a reduction in the energy consumption for the genetic expression and translational mechanisms , and the ability of shrimp to reduce their metabolic rates to a new hypometabolic state during hypoxia, to also reduce ATP demands as reported in various invertebrate species (Storey and Storey, 1990).

We detected a significant increase of 70% in the ATPase activity of the mitochondrial protein extract of shrimp at hypoxia (2.0 mg/L) when compared to shrimp at normoxia (*P > 0.05*). Afterwards, a decrease of 76% was detected in the activity when shrimp were re-oxygenated at 7 mg/L (*P> 0.05*; Fig. 5). This agrees with Di Lisa et al. (1995) results, they stated that during hypoxia, organisms suppress energy production in the mitochondrial apparatus by reducing oxidative phosphorylation and fail to produce ATP. At this point, ATP-synthase becomes a major ATP consumer by hydrolyzing the ATP found in the mitochondrial matrix (Vinogradov, 2000; Grover et al., 2004). During re-oxygenation, when the ATPase activity was significantly reduced, the mitochondrial ATP production is suggested to continue via the electron transport chain and oxidative phosphorylation restoring the membrane potential previously lost (Budinger et al., 1998).

Finally, our results confirmed the ability of this penaeid species to face low oxygen levels during prolonged periods as oxy-regulators, what agrees with Rosas et al. (1999) observations in the species *Penaeus setiferus*. We suggest that shrimp show adaptive

responses to hypoxia as their ability to rapidly produce ATP from stored phosphoarginine buffering the cell ATP amounts to maintain the organism basic biological functions.

Since the amount of expressed proteins as the catalytic ATPβ subunit did not changed during hypoxia, but instead the ATPase activity significantly increased, the detected changes may then suggest a post-translational control of the enzyme activity in shrimp. We believe that since shrimp mitochondrial ATPase activity is promoted during environmental stress conditions, a specific mechanism may exist to regulate this activity of the enzyme, probably through an inhibitor of the so called IF1 group whose existence has not been proved in this species.

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Figure captions

Figure 1. Plasma lactate concentration in the white shrimp *L. vannamei* exposed to normoxia, hypoxia and re-oxygenation. Different letters show significant difference at $p < 0.05$. Bars represent means ± SD.

Figure 2. Muscle ATP concentration in the white shrimp *L. vannamei* exposed to normoxia, hypoxia and re-oxygenation. Different letters show significant difference at $p < 0.05$. Bars represent means ± SD.

Figure 3. Coomassie-stained native gel strips corresponding to **A**) BN-PAGE, A1) High molecular weight marker, A2) Mitochondrial proteins isolated from *L. vannamei*.**B**) SDS-PAGE, B1) Prestained SDS-PAGE standars, broad range, B2) Band extracted from BN-PAGE (~660kDa). **C**) Immunoblotting, C1) Prestained SDS-PAGE standars transferred, C2) Specific polyclonal antibodies recognition against the ATPβ subunit, C3) Negative control (sample without primary antibodies incubation), C4) Negative control (sample without secondary antibodies incubation).

Figure 4. Muscle ATP-synthase β subunit concentration in the white shrimp *L. vannamei* exposed to normoxia, hypoxia and re-oxygenation. Different letters show significant difference at $p < 0.05$. Bars represent means ± SD.

Figure 5. ATPase activity of ATP-synthase in muscle mitochondria of shimp *L. vannamei* during normoxia, hypoxia and re-oxygenation. Enzyme activity was measured at an assay temperature of 30 °C. Different letters show significant difference at $p < 0.05$. Bars represent means ± SD.

Figure 1. Martinez-Cruz et al. (2011).

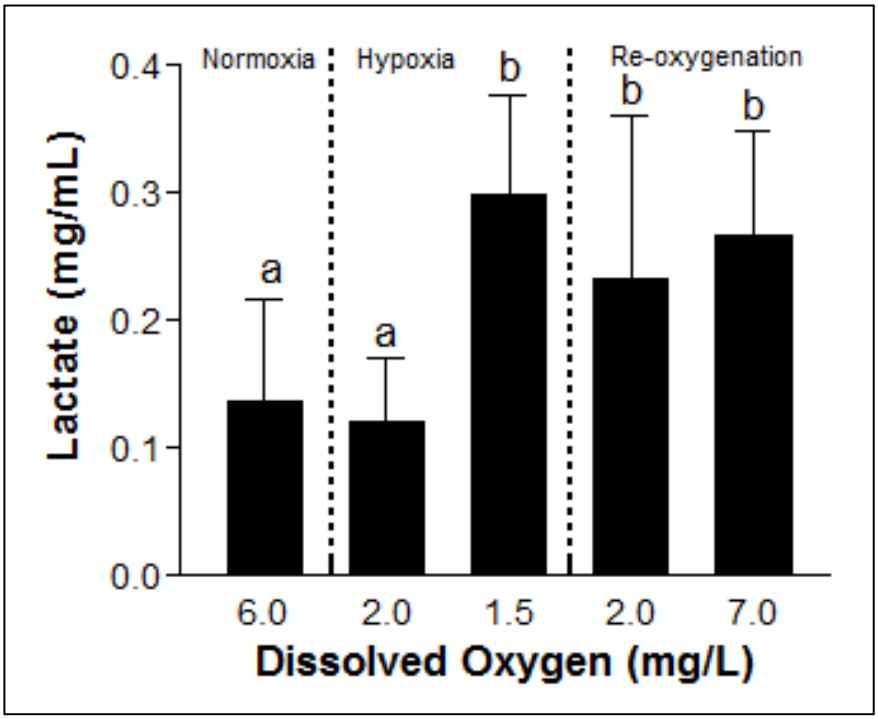
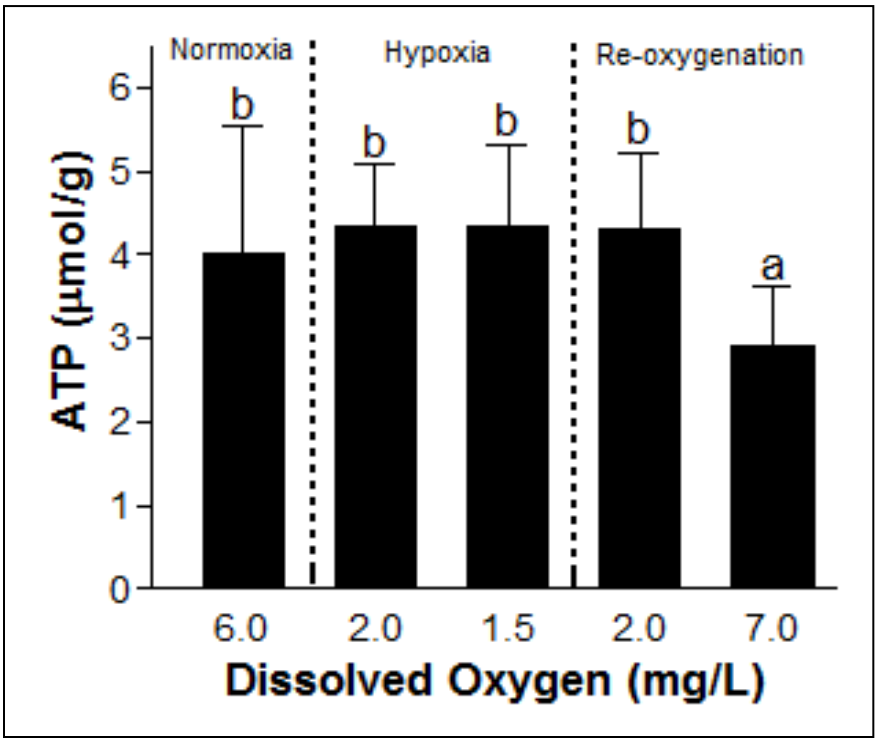


Figure 2. Martinez-Cruz et al. (2011).



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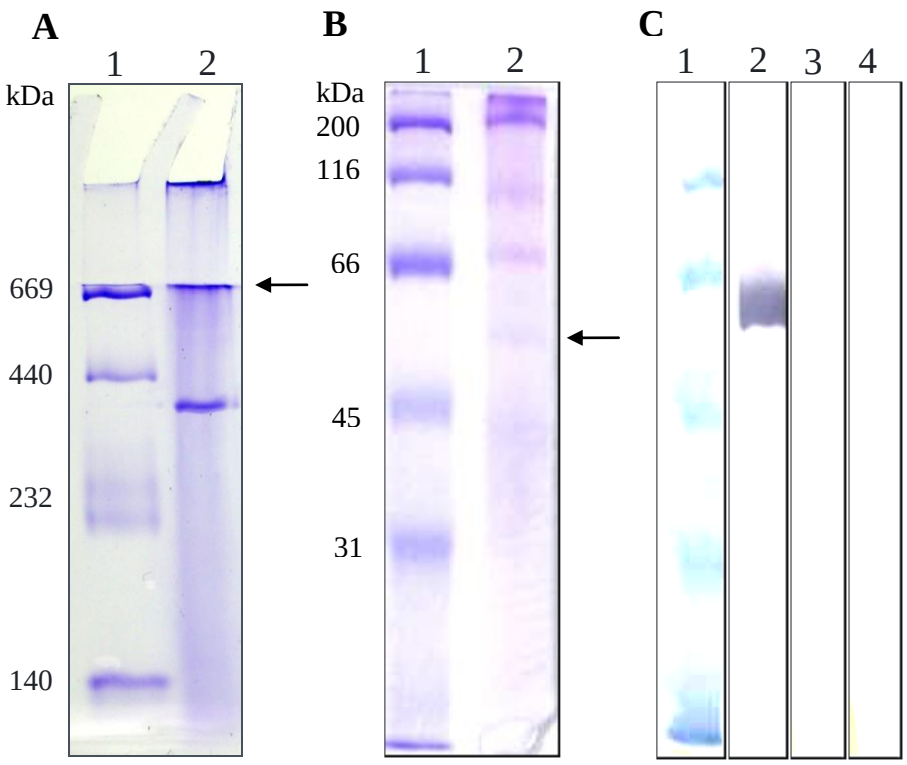

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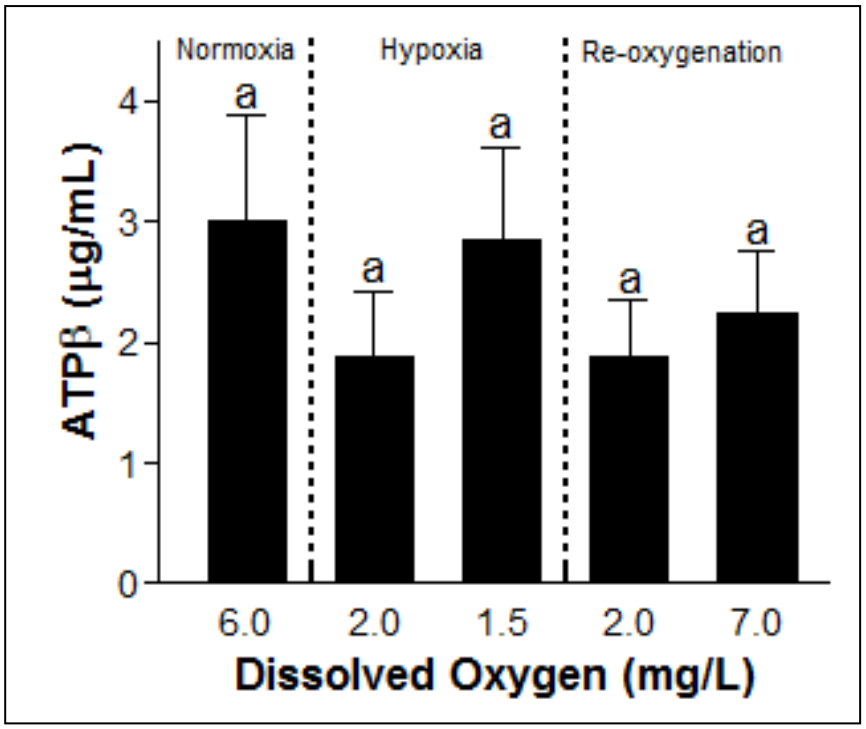

Figure 5. Martinez-Cruz et al. (2011).