Centro de Investigación en Alimentación y Desarrollo, A.C

REGULACIÓN DE LA EXPRESIÓN DE FOSFOFRUCTOCINASA Y FRUCTOSA 1,6-BISFOSFATASA EN CAMARÓN *Litopenaeus vannamei* POR HIF-1 EN HIPOXIA

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Los miembros del comité designado para la revisión de la tesis de Abdiel Keni Cota Ruiz, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

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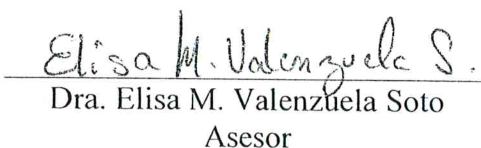

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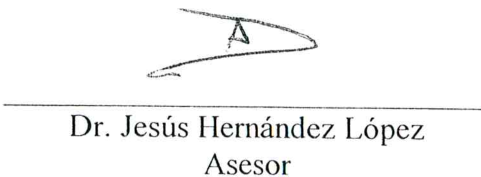

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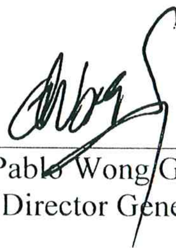

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CONTENIDO

APROBACIÓN…………………………………………………………………… ii
DECLARACIÓN INSTITUCIONAL…………………………………………….. iii
AGRADECIMIENTOS………………...……………………………………… iv
DEDICATORIA……………………………………………………………….. vii
CONTENIDO………………………………………………………………….. ix
RESUMEN……………………………………………………………………... x
ABSTRACT…………………………………………………………............ xi
SINOPSIS……………………………………………………………………… 1
LITERATURA CITADA………………………………………………………. 9
CAPÍTULO I. Expression of fructose 1, 6-bisphosphatase and phosphofructokinase is induced in hepatopancreas of the white shrimp *Litopenaeus vannamei* by hypoxia……………………………………………… 12
CAPÍTULO II. Role of HIF-1 on phosphofructokinase and fructose 1, 6-bisphosphatase expression during hypoxia in the white shrimp *Litopenaeus vannamei*……………………………..…………………………………….. 22
CAPÍTULO III. Revisiting the hypoxia and anoxia resilience in marine animals: from physiological to molecular survival strategies……………………………… 30

RESUMEN

La hipoxia es un fenómeno que enfrenta el camarón blanco *Litopenaeus vannamei* tanto en hábitats naturales como en cultivo. En estas condiciones, emplea diversas estrategias de supervivencia, entre ellas, la activación del metabolismo anaerobio para obtener energía. En esta tesis se estudió la regulación de dos enzimas del metabolismo de la glucosa. En mamíferos se ha reportado que la fosfofructocinasa (PFK) y la fructosa-bisfosfatasa (FBP) se inducen en hipoxia. Por ello, posterior a la caracterización del cDNA de FBP, se evaluó el efecto de la hipoxia en la expresión de PFK y FBP en *L. vannamei*, enzimas clave de glucólisis y gluconeogénesis, respectivamente. Los resultados experimentales demostraron que la expresión de PFK y FBP, es inducida por efecto de la hipoxia en hepatopáncreas pero no en branquias. Partiendo de esto, se evaluó la participación de HIF-1 (Hypoxia Inducible Factor, por sus siglas en inglés) en este proceso. Para ello, se inyectó intramuscularmente RNA de doble cadena (dsRNA) para silenciar HIF-1 y tras un período de 24 h, los camarones fueron sometidos a 3 y 48 h de hipoxia (1.57 ±0.2 mg DOL^{-1}). Los resultados indicaron que HIF-1 participa en la regulación de PFK en hepatopáncreas en ambos tiempos de hipoxia debido a que la inducción de este gen (~1.6 y ~4.2-veces a 3 y 48 h, respectivamente) es afectada significativamente cuando HIF-1 está silenciado. Además, a las 3 h de hipoxia aumentó la actividad de PFK y la concentración de lactato (comparado con sus respectivos controles de normoxia). No obstante, cuando HIF-1 fue silenciado, esta respuesta se restringió. Por otra parte, a las 48 h de hipoxia los transcritos de FBP aumentaron (~5.3 veces) y se demostró que HIF-1 interviene en esta inducción ya que esta respuesta fue parcialmente disminuida en los camarones tratados con dsRNA. Sin embargo, la actividad de FBP no se afectó ni por la hipoxia ni por efecto del silenciamiento, lo cual sugiere cambios post-traduccionales o estabilización de la proteína. Por lo tanto, estos resultados sugieren que la glucólisis se está acelerando en camarón en condiciones de hipoxia y que HIF-1 está involucrado en este proceso. Además, la inducción de FBP sugiere que la gluconeogénesis o la ruta de las pentosas fosfato se activa bajo condiciones de hipoxia. Estos hallazgos posicionan a HIF-1 como un importante factor de transcripción que regula el metabolismo de la glucosa en camarón en condiciones de hipoxia.

Palabras Clave: Hipoxia, HIF-1, PFK, FBP

ABSTRACT

The shrimp *L. vannamei* is exposed to hypoxia either in their natural environments or under farming. In these conditions, they can employ several survival strategies; one of them is the activation of anaerobic metabolism. In this thesis, the regulation of two enzymes of glucose metabolism in shrimp by hypoxia was investigated. In mammals, phosphofructokinase (PFK) and fructose bisphosphatase (FBP) are induced under hypoxia. Taking this into consideration, we evaluated the effect of hypoxia on the expression of PFK and FBP in shrimp. PFK and FBP are key enzymes of glycolysis and gluconeogenesis, respectively. We found that PFK and FBP are both induced in shrimp hepatopancreas but not in gills. Additionally, the cDNA sequence and the expression of FBP in hepatopancreas of the shrimp were analyzed. Also, we evaluated the role of HIF-1 on PFK and FBP expression by intramuscularly injecting double stranded RNA (dsRNA) to silence HIF-1. After 24 hours of injection, shrimps were subjected to 3 and 48 h of hypoxia (1.57 mg ±0.2 DOL^{-1}). The results indicated that HIF-1 is involved in the regulation of PFK in hepatopancreas since the hypoxic induction of this gene (~1.6 y ~4.2-fold at 3 y 48 h, respectively) was significantly reversed when HIF-1 was silenced. Moreover, PFK activity and lactate increased after 3 h of hypoxia, but they were affected by HIF-1 silencing. On the other hand, the FBP transcripts augmented (~5.3-fold) after 48 h of hypoxia and it was shown that HIF-1 regulates this process since the induction response was partially diminished in dsRNA treated shrimp. However, FBP activity was unaffected by the hypoxia-silencing treatments, suggesting post-translational modifications and protein stabilization. These results demonstrate that glycolysis is accelerated to meet the shrimp energy requirements under hypoxia and HIF-1 is involved in this process. Furthermore, FBP hypoxic induction could indicate that the gluconeogenesis or the pentose phosphate pathways are activated. Taken together, these results position HIF-1 as an important transcription factor regulating glucose metabolism in shrimp under hypoxia.

Keywords: Hypoxia, HIF-1, PFK, FBP

SINOPSIS

REGULACIÓN DE PFK Y FBP POR HIF-1 EN HIPOXIA EN *L. vannamei*

Introducción

El camarón *L. vannamei*, conocido comúnmente como camarón del pacífico o camarón blanco, se distribuye desde la costa de Sonora, México, a través de Centroamérica y hasta el norte de Perú, en zonas donde la temperatura del agua, a lo largo del año, es superior a los 20°C. Estos animales habitan ambientes tanto de aguas salobres como de aguas dulces y se exponen a múltiples estresores como la eutroficación, estratificación de la columna de agua, (Li et al., 2016) y fluctuaciones en la disponibilidad de oxígeno (Hardy et al., 2013). Esta especie fue inicialmente cultivada en Estados Unidos de Norteamérica en la década de los 70s, posteriormente, esta práctica se fue esparciendo por Centroamérica, Sudamérica y Asia, llegando a este último continente apenas en los albores de este siglo. El camarón blanco no solo es la especie de camarón más cultivada, sino el crustáceo más cultivado en el mundo de la acuacultura (Ghaffari et al., 2014).

La concentración de oxígeno es una variable clave para los organismos acuáticos. Una baja concentración de oxígeno disuelto (DO, dissolved oxygen, por sus siglas en inglés) en el medio, fenómeno conocido como hipoxia, afecta no solo el crecimiento, comportamiento y tasas reproductivas de los animales, sino también compromete su supervivencia. Desafortunadamente, los sitios costeros con hipoxia se han venido incrementando de forma acelerada durante las últimas décadas, trayendo consigo severas consecuencias para la vida marina (Vaquer-Sunyer y Duarte, 2008). Por su parte, en condiciones de cultivo, especialmente en modalidades intensivas, el camarón se expone a niveles de hipoxia, ocasionada por las bacterias que se “alimentan” de los sedimentos,

descomponiendo la materia orgánica y consumiendo una porción significativa del oxígeno disuelto en el agua (Vinatea et al., 2009).

Muchos animales marinos, entre ellos crustáceos, insectos, mamíferos, reptiles, anfibios y peces, pueden sobrevivir horas o incluso meses en condiciones de hipoxia (Hermes-Lima y Zenteno-Savín, 2002). Esta habilidad de supervivencia es raramente encontrada en los vertebrados endotérmicos (por ejemplo aves y mamíferos terrestres) (Gorr et al., 2006). Por lo anterior, al primer grupo de organismos se les conoce como hipoxia-tolerantes, mientras que los últimos son conocidos como hipoxia-sensibles (Gorr et al., 2010). El camarón *L. vannamei*, es capaz de tolerar condiciones de hipoxia moderadas por semanas (2-2.6 mg de DOL^{-1}) (Racotta et al., 2002) llegando a resistir condiciones severas de hipoxia ya que su dosis letal es de 0.2 mg de DOL^{-1} (Pérez-Rostro et al., 2004). Para ello *L. vannamei* se vale entre otros mecanismos, de la activación de la glucólisis anaerobia (Soñanez-Organis et al., 2012).

Para aportar al conocimiento de los mecanismos bioquímicos y fisiológicos que le permiten al camarón blanco sobrevivir bajo condiciones de hipoxia, esta tesis se enfocó a estudiar parte del metabolismo energético del camarón en condiciones de hipoxia. Se midió la expresión y evaluó la respuesta de dos genes clave de vías primigenias del metabolismo de la glucosa: la fosfofructocinasa (PFK, E.C. 2.7.1.11) de la glucólisis y la fructosa 1,6-bisfosfatasa (FBP, E. C. 3.1.3.11) de la gluconeogénesis.

Glucólisis e hipoxia

La glucosa es la molécula más importante para producir energía en organismos superiores. Existen tres procesos metabólicos clave en los que interviene la glucosa: 1) La glucólisis bajo condiciones anaerobias, 2) La completa oxidación de la glucosa bajo condiciones aeróbicas y 3) La ruta de las pentosas fosfato (Li et al., 2015). En la glucólisis se genera energía en forma de dos moléculas de ATP y representa la primer ruta del metabolismo energético de carbohidratos. Consta de 10 pasos, cada uno catalizado por diez enzimas diferentes. Existen tres enzimas clave en esta ruta: la hexocinasa (HK), la PFK y la piruvato cinasa (PK) (Al Hasawi et al., 2014). Por su parte, la glucólisis anaerobia es la

ruta metabólica donde se convierte la glucosa en lactato con la participación de la enzima lactato deshidrogenasa, que convierte el piruvato (producto final de la glucólisis) en lactato. Evidentemente, la cantidad de ATP que se produce en condiciones anaeróbicas es menor comparada con la que se produce en el proceso aeróbico (hasta 38 moléculas de ATP por molécula de glucosa). No obstante, este proceso de generación de ATP es muy importante bajo condiciones de hipoxia y se sigue manteniendo gracias a la regeneración de NAD^+ por medio de la lactato deshidrogenasa. El NAD^+ es indispensable para la actividad de la enzima glucolítica gliceraldehído-3-fosfato deshidrogenasa (Rose y Rose, 2014).

Mientras tanto, la gluconeogénesis es el proceso mediante el cual se genera glucosa *de novo* a través de precursores diferentes a los carbohidratos (Yoon et al., 2001). La FBP convierte la fructosa 1,6-bisfosfato en fructosa 6-fosfato. La FBP es reconocida como la enzima clave de la gluconeogénesis. Por otra parte, la PFK, que cataliza la reacción contraria a la FBP, es una enzima considerada como el punto clave de regulación de glucólisis (Jenkins et al., 2011), cataliza la fosforilación irreversible de la fructosa 6-fosfato (F6P) para generar fructosa 1,6-bisfosfato (F, 1-6P) (Al Hasawi et al., 2014). Tanto la PFK y la FBP son enzimas alostéricas y son finamente reguladas por varios activadores e inhibidores. En eucariotas, la PFK está compuesta de cuatro subunidades y la proteína funcional tiene un peso molecular aproximado de 320 kDa (Uyeda, 2006). Tanto en vertebrados como invertebrados se han reportado 2 tipos de PFK (PFK-1 y PFK-2) (Havula et al., 2013). PFK-1 es la que interviene directamente en la glucólisis mientras que la PFK-2 está involucrada en la síntesis de la fructosa 2, 6-fosfato, un importante regulador alostérico de la PFK-1 (Pegoraro et al., 2013). Por otra parte, todas las FBP forman tetrámeros, cuyas subunidades tienen un peso molecular de 36-40 kDa (Tillmann et al., 2002).

Debido al alto potencial energético del oxígeno, los organismos aerobios dependen de este gas para llevar a cabo sus funciones vitales. No obstante, muchos animales se exponen a fluctuaciones en los niveles de oxígeno, lo que los obliga a enfrentar la hipoxia. En líneas celulares de mamíferos, se ha demostrado que la expresión de genes glucolíticos aumenta en hipoxia (Denko, 2008), involucrando un cambio del metabolismo aerobio al

metabolismo anaerobio con el concomitante aumento en la producción de ATP (Aragonés et al., 2009). En muchas especies de animales marinos, también se presenta este cambio de metabolismo y se genera ATP de manera independiente al oxígeno (Richards, 2011). Por su parte, en condiciones de hipoxia el camarón *L. vannamei* también activa el metabolismo anaerobio (Martínez-Quintana et al., 2015) para producir energía, registrando un aumento en la expresión de importantes genes como los de la hexocinasa (Soñanez-Organis et al., 2011) y lactato deshidrogenasa (Soñanez-Organis et al., 2012), con la subsecuente acumulación de lactato (Martínez-Quintana et al., 2016).

Por otra parte, en el pez *Gillichthys mirabilis*, el gen de la glucosa 6- fosfatasa (G6-Pasa) es inducido en hipoxia (Gracey et al., 2001), sugiriendo la activación de la gluconeogénesis. En *L. vannamei*, la capacidad gluconeogénica del hepatopáncreas ha sido previamente demostrada (Rosas et al., 2001). En crustáceos, varios estudios reportan la ocurrencia de este proceso metabólico en otros tejidos como hemocitos (Johnston et al., 1973), branquias (Thabrew et al., 1971) y músculo (Schein et al., 2004). A pesar que la gluconeogénesis ha sido analizada en términos enzimáticos y de metabolitos, en invertebrados marinos escasos estudios han evaluado la inducción de genes gluconeogénicos bajo diversos estresores. De esta forma, basado en: (i) que no hay reportes sobre el efecto de la hipoxia en la expresión de FBP en crustáceos y (ii) que poco se sabe sobre el efecto de la hipoxia en la expresión de PFK en animales marinos, no obstante que las propiedades bioquímicas de la PFK y su regulación en hipoxia han sido ampliamente estudiadas en vertebrados terrestres; en el **capítulo I** de esta tesis, se reporta el cDNA de FBP obtenido a partir de hepatopáncreas, así como la expresión de PFK y FBP en normoxia y en respuesta a la hipoxia.

El cDNA de la FBP es de 1140 pb con codón de inicio y de terminación en las posiciones 2 y 1004, respectivamente. La proteína deducida es de 334 animoácidos y su secuencia primaria muestra alta homología con otras FBP de crustáceos e invertebrados marinos, indicando que esta proteína no ha tenido cambios "significativos" durante la evolución. Asimismo, la FBP se expresó en todos los tejidos analizados: hepatopáncreas, branquias, músculo, hemocitos, intestino y pleópodos, lo cual sugiere síntesis de glucosa. También se evaluó el efecto de la hipoxia en la expresión de FBP en hepatopáncreas, branquias y

músculo. En hepatopáncreas, los transcritos disminuyeron significativamente a las 3 h de hipoxia, no obstante, aumentaron significativamente (~1.4), comparados con su control de normoxia, a las 48 h de hipoxia. Por su parte, la hipoxia causó una disminución significativa en los transcritos de FBP a las 3, 24 y 48 h en branquias y músculo.

La inducción de FBP en hepatopáncreas en hipoxia se puede explicar al considerar que: 1) la glucosa producida a través de la activación de la gluconeogénesis puede ser exportada a órganos que la requieren primordialmente (*e.g.* cerebro) o 2) que la activación de la gluconeogénesis puede alimentar la ruta de las pentosas fosfato para la posterior generación de precursores nucleotídicos o poder reductor. Por otra parte, en camarón no hay reportes sobre el efecto de la hipoxia en la expreisón de PFK. Anteriormente Sánchez-Paz et al., (2008) reportaron la secuencia parcial del cDNA de PFK de *L. vannamei* la cual se compone de 489 pares de bases y evaluaron el efecto de ayuno en la expresión de este gen. Sus resultados mostraron que la PFK se induce en hepatopáncreas a las 96 h de ayuno sugiriendo que la PFK responde al glucógeno almacenado. En este capítulo, se evaluó el efecto de la hipoxia en la expresión de PFK en hepatopáncreas y se encontró que éstos aumentan significativamente a partir de las 24 h de hipoxia (~90 veces). Esto sugiere que la PFK se activa para secundar la aceleración de la glucólisis para producir energía.

Regulación de PFK y FBP en hipoxia por HIF-1

En mamíferos, se ha demostrado que algunos genes de la glucólisis (Semenza, 2000) y la gluconeogénesis (Choi et al., 2005) son regulados por HIF-1 (Hypoxia Inducible Factor) en condiciones de hipoxia. HIF-1 es un factor de transcripción heterodimérico que consiste en una subunidad α (sensible al oxígeno) y una subunidad β (expresada constitutivamente) (Gorr, 2004). En condiciones de normoxia, HIF-1 alfa es hidroxilada en residuos específicos de prolina marcándola para que sea ubiquitinada y capturada por la proteína supresora de tumores VHL, completando el proceso para que finalmente sea "rápidamente" degradada en el proteasoma (Bishop y Ratcliffe, 2014). En hipoxia, HIF-1 alfa escapa a la degradación y se transloca al núcleo donde se dimeriza con HIF-1 beta. De esta forma HIF-1 se activa y subsecuentemente regula la transcripción de sus genes blanco (Kaelin y Ratcliffe, 2008).

Experimentos de genética clásica han permitido crear mutantes carentes de HIF-1 y de esta manera se ha podido evaluar su papel en la regulación de muchos genes (Tomita et al., 2003). En camarón no ha sido posible establecer esta clase de experimentos. No obstante, hace aproximadamente una década, en crustáceos se reportó que la inyección intramuscular de RNA de doble cadena, o RNA interferente, es efectiva para evaluar la función de genes en un sistema de tiempo-dosis específicos (Lugo et al., 2006). El RNA de doble cadena es un mecanismo de silenciamiento post-transcripcional altamente específico que degrada el RNA mensajero o inhibe la entrada de la maquinaria responsable de la síntesis proteica (Meister y Tuschl, 2004).

Investigaciones recientes han descrito que HIF-1 interviene en la regulación de genes de glucólisis (*e.g.* hexocinasa y lactato deshidrogenasa) en camarón (Soñanez-Organis et al., 2011; Soñanez-Organis et al., 2012). Además, como parte de esta tesis, se encontró que la PFK de *L. vannamei* se induce a nivel de transcritos por efecto de la hipoxia (Cota-Ruiz et al., 2015). Otros estudios han demostrado la activación de genes gluconeogénicos en animales marinos (Brown-Peterson et al., 2008; Gracey et al., 2001; Le Moullac et al., 2007). Sin embargo, no hay reportes que prueben que HIF-1 interviene en la regulación de dichos genes en condiciones de hipoxia. Por lo anterior, parte de esta investigación que se presenta en el **capítulo II**, se enfocó a evaluar si HIF-1 interviene en la regulación de PFK y FBP, en condiciones de hipoxia, empleando para ello, la estrategia de RNA de interferencia para silenciar HIF-1.

Primeramente, se evaluó la eficiencia del silenciamiento y se encontró que los camarones que fueron tratados con dsRNA y sometidos a 3 h de hipoxia presentaron un 80 y 60 % de silenciamiento para HIF-1 alfa y HIF-1 beta, respectivamente. A las 48 h de hipoxia, el silenciamiento de HIF-1 alfa fue de 82% en tanto que el efecto del silenciamiento en HIF-1 beta desapareció. En los respectivos grupos de normoxia, no se observó silenciamiento para HIF-1 alfa (3 y 48 h) y para HIF-1 beta, se encontró un 34 % de silenciamiento a las 3 h y no hubo efecto a las 48 h.

Consecutivamente, se evaluó el efecto del silenciamiento de HIF-1 en la expresión y actividad enzimática de FBP en hipoxia. Se encontró un aumento significativo en la cantidad de transcritos (~5.3 veces) de FBP a las 48 h de hipoxia. Además, cuando HIF-1

fue silenciado, esta respuesta disminuyó significativamente. A nivel de actividad enzimática, no se encontró efecto de la hipoxia ni del silenciamiento en FBP, lo cual sugiere regulación pos-traduccional.

Finalmente, se evaluó el efecto del silenciamiento de HIF-1 en la expresión y actividad enzimática de PFK en hipoxia. Los resultados mostraron que a las tres horas de hipoxia los transcritos y la actividad enzimática de PFK aumentan significativamente respecto a sus controles de normoxia (~1.6 y ~2.8 veces, respectivamente) y que HIF-1 participa en la regulación en ambos niveles ya que en los camarones tratados con dsRNA la respuesta de PFK disminuyó significativamente. Además, el lactato se acumuló a las 3 h de hipoxia (~1.9 veces), pero los niveles se redujeron significativamente cuando HIF-1 fue silenciado. Estos resultados indican que el camarón activa el metabolismo anaerobio para producir energía y lo hace, al menos en parte, en una manera dependiente de HIF-1.

Estrategias y mecanismos para sobrevivir en hipoxia

El oxígeno es una molécula clave para la vida de animales multicelulares. Específicamente, cuando los animales marinos se exponen a ambientes de hipoxia, son capaces de realizar diversas estrategias de supervivencia enfocadas a mantener un balance adecuado entre la demanda y la generación de energía (Hochachka y Somero, 2014). El **capítulo III** revisa las estrategias fisiológicas, bioquímicas y moleculares empleadas por los animales marinos para contrarrestar la baja disponibilidad de oxígeno. Las respuestas son variadas y sorprendentes en algunos casos, desde la inmediata respuesta fisiológica enfocada en captar y distribuir más oxígeno hacia las células, o incluso la inversión energética para sintetizar más células/moléculas acarreadoras de oxígeno, hasta la depresión del metabolismo para salvaguardar energía. También, en este capítulo se discute, desde el punto de vista molecular, el posible rol de HIF en diversos procesos metabólicos, así como las perspectivas y preguntas de investigación que aún esperan respuesta.

Brevemente, cuando los animales marinos enfrentan la hipoxia, primeramente intentan mantener un adecuado suplemento de oxígeno hacia sus tejidos. Para ello emplean

estrategias como la remodelación de las branquias que consiste en la reorganización celular incluida la apoptosis para incrementar la superficie de exposición. También aceleran la respiración, el pulso cardiaco o modifican la redistribución del flujo sanguíneo. Además, aunque es un proceso energéticamente costoso, diversos crustáceos activan la síntesis de proteínas como la hemocianina/hemoglobina, importantes moléculas en el acarreo y entrega de oxígeno hacia los tejidos. Asimismo, información reciente sugiere que otro proceso que requiere energía como la gluconeogénesis puede ser activado en estas condiciones con el objetivo de: 1) sintetizar glucosa en órganos como hepatopáncreas para enviarla a órganos que "obligadamente" la necesitan (como células nerviosas o cerebro) o 2) para alimentar la ruta de las pentosas fosfato que se deriva en la generación de glutatión, el cual puede funcionar como antioxidante o inhibidor de apoptosis. Adicionalmente, estos organismos activan el metabolismo anaerobio para producir energía con la subsecuente acumulación de diferentes metabolitos. Información obtenida sugiere que el factor de transcripción HIF-1 participa en la regulación de este proceso. Finalmente, si los tejidos aun censan la ausencia de oxígeno, muchos animales entran en un estado de depresión metabólica para salvar energía.

Conclusiones y perspectivas

La hipoxia en camarón induce la expresión en hepatopáncreas, tanto de PFK como de FBP y el factor de transcripción HIF-1 está involucrado en esta respuesta. A nivel de actividad enzimática, HIF-1 participa en la regulación de PFK ya que al silenciar HIF-1, la actividad de PFK en hipoxia no se afecta, mientras que si aumenta en los camarones sin silenciar y expuestos a 3 h de hipoxia. Además, a las 3 h de hipoxia aumentó la concentración de lactato. De esta manera, los datos sugieren que la glucólisis se está acelerando en hipoxia para producir energía. Por otra parte, la actividad de FBP no se afectó ni por la hipoxia ni por el silenciamiento, lo cual sugiere regulaciones pos traduccionales o estabilización de la proteína. En conclusión, en esta tesis se demuestra la participación de HIF en la regulación de la expresión génica de PFK y FBP. Estudios enfocados en evaluar la expresión *in situ* de ambos genes así como la cuantificación de metabolitos darán información oportuna para entender la respuesta metabólica del camarón a la hipoxia.

También, investigaciones orientadas en evaluar la funcionalidad de regiones promotoras brindarán bases moleculares para explicar la participación de HIF-1 en la regulación de genes glucolíticos y gluconeogénicos.

LITERATURA CITADA

Al Hasawi, N., Alkandari, M.F., Luqmani, Y.A., 2014. Phosphofructokinase: a mediator of glycolytic flux in cancer progression. Crit. Rev. Oncol. Hematol. 92, 312-321.

Aragonés, J., Fraisl, P., Baes, M., Carmeliet, P., 2009. Oxygen Sensors at the Crossroad of Metabolism. Cell metabolism 9, 11-22.

Bishop, T., Ratcliffe, P., 2014. Signaling hypoxia by hypoxia-inducible factor protein hydroxylases: a historical overview and future perspectives. Hypoxia 2, 197-213.

Brown-Peterson, N.J., Manning, C.S., Patel, V., Denslow, N.D., Brouwer, M., 2008. Effects of Cyclic Hypoxia on Gene Expression and Reproduction in a Grass Shrimp, *Palaemonetes pugio*. Biol. Bull. 214, 6-16.

Cota-Ruiz, K., Peregrino-Uriarte, A.B., Felix-Portillo, M., Martínez-Quintana, J.A., Yepiz-Plascencia, G., 2015. Expression of fructose 1, 6-bisphosphatase and phosphofructokinase is induced in hepatopancreas of the white shrimp *Litopenaeus vannamei* by hypoxia. Mar. Environ. Res. 106, 1-9.

Choi, J.H., Park, M.J., Kim, K.W., Choi, Y.H., Park, S.H., An, W.G., Yang, U.S., Cheong, J., 2005. Molecular mechanism of hypoxia-mediated hepatic gluconeogenesis by transcriptional regulation. FEBS Lett. 579, 2795-2801.

Denko, N.C., 2008. Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat. Rev. Cancer 8, 705-713.

Ghaffari, N., Sanchez-Flores, A., Doan, R., Garcia-Orozco, K.D., Chen, P.L., Ochoa-Leyva, A., Lopez-Zavala, A.A., Carrasco, J.S., Hong, C., Brieba, L.G., Rudiño-Piñera, E., Blood, P.D., Sawyer, J.E., Johnson, C.D., Dindot, S.V., Sotelo-Mundo, R.R., Criscitiello, M.F., 2014. Novel transcriptome assembly and improved annotation of the whiteleg shrimp (*Litopenaeus vannamei*), a dominant crustacean in global seafood mariculture. Sci. Rep. 4. 1-10.

Gorr, T.A, , Gassmann, M., Wappnerb, P., 2006. Sensing and responding to hypoxia via HIF in model invertebrates. J. Insect Physiol. 52, 349-364.

Gorr, T.A., 2004. Daphnia and Drosophila: two invertebrate models for O2 responsive and HIF-mediated regulation of genes and genomes. Int. Cong. Ser. 1275, 55-62.

Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N.B., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83(5), 733-752.

Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. Proc. Natl. Acad. Sci. U. S. A. 98, 1993-1998.

Hardy, K.M., Burnett, K.G., Burnett, L.E., 2013. Effect of hypercapnic hypoxia and bacterial infection (*Vibrio campbellii*) on protein synthesis rates in the Pacific whiteleg shrimp, *Litopenaeus vannamei*. Am J Physiol-Reg I. 305, R1356-R1366.

Havula, E., Teesalu, M., Hyötyläinen, T., Seppälä, H., Hasygar, K., Auvinen, P., Orešič, M., Sandmann, T., Hietakangas, V., 2013. Mondo/ChREBP-Mlx-regulated transcriptional network is essential for dietary sugar tolerance in *Drosophila*. PLoS Genet. 9(4), 1-13.

Hermes-Lima, M., Zenteno-Savín, T., 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp. Biochem. Phys. C. 133, 537-556.

Hochachka, P.W., Somero, G.N., 2014. Biochemical adaptation. Princeton University Press.

Jenkins, C.M., Yang, J., Sims, H.F., Gross, R.W., 2011. Reversible High Affinity Inhibition of Phosphofructokinase-1 by Acyl-CoA: A Mechanism Integrating Glycolytic Flux With Lipid Metabolism. J. Biol. Chem. 286, 11937-11950.

Johnston, M.A., Elder, H.Y., Davies, P.S., 1973. Cytology of carcinus haemocytes and their function in carbohydrate metabolism. Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol. 46, 569-581.

Kaelin, W.G., Ratcliffe, P.J., 2008. Oxygen Sensing by Metazoans: The Central Role of the HIF Hydroxylase Pathway. Mol. Cell. 30, 393-402.

Le Moullac, G., Bacca, H., Huvet, A., Moal, J., Pouvreau, S., Van Wormhoudt, A., 2007. Transcriptional regulation of pyruvate kinase and phosphoenolpyruvate carboxykinase in the adductor muscle of the oyster *Crassostrea gigas* during prolonged hypoxia. J. Exp. Zool. Part A 307A, 371-382.

Li, X.-b., Gu, J.-d., Zhou, Q.-h., 2015. Review of aerobic glycolysis and its key enzymes – new targets for lung cancer therapy. Thorac. Cancer 6, 17-24.

Li, Y., Wei, L., Cao, J., Qiu, L., Jiang, X., Li, P., Song, Q., Zhou, H., Han, Q., Diao, X., 2016. Oxidative stress, DNA damage and antioxidant enzyme activities in the pacific white shrimp (*Litopenaeus vannamei*) when exposed to hypoxia and reoxygenation. Chemosphere 144, 234-240.

Lugo, J.M., Morera, Y., Rodriguez, T., Huberman, A., Ramos, L., Estrada, M.P., 2006. Molecular cloning and characterization of the crustacean hyperglycemic hormone cDNA from *Litopenaeus schmitti*. Functional analysis by double-stranded RNA interference technique. FEBS J. 273, 5669-5677.

Martínez-Quintana, J.A., Kikuta, S., Felix-Portillo, M., Peregrino-Uriarte, A.B., Yepiz-Plascencia, G., 2015. A novel functional glucose transporter in the white shrimp *Litopenaeus vannamei*-LvGLUT2-is up-regulated during hypoxia in hepatopancreas. Mar. Environ. Res. 112, 61-67.

Martínez-Quintana, J.A., Valenzuela-Soto, E.M., Martínez-Téllez, M.A., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Kikuta, S., Yepiz-Plascencia, G., 2016. Functionality of the white shrimp glucose transporter 1: Expression in *Xenopus oocytes* and gene silencing during hypoxia. J. Exp. Mar. Biol. Ecol. 474, 109-115.

Meister, G., Tuschl, T., 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343-349.

Pegoraro, C., Maczkowiak, F., Monsoro-Burq, A.H., 2013. Pfkfb (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase) isoforms display a tissue-specific and dynamic expression during *Xenopus laevis* development. Gene Expr. Patterns 13, 203-211.

Pérez-Rostro, C.I., Racotta, I.S., Ibarra, A.M., 2004. Decreased genetic variation in metabolic variables of *Litopenaeus vannamei* shrimp after exposure to acute hypoxia. J. Exp. Mar. Biol. Ecol. 302, 189-200.

Racotta, I.S., Palacios, E., Méndez, L., 2002. Metabolic responses to short and long-term exposure to hypoxia in white shrimp (*Penaeus vannamei*),. Mar. Freshwater Behav. Physiol. 35, 269-275.

Richards, J.G., 2011. Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. J. Exp. Biol. 214, 191-199.

Rosas, C., Cuzon, G., Gaxiola, G., Le Priol, Y., Pascual, C., Rossignyol, J., Contreras, F., Sanchez, A., Van Wormhoudt, A., 2001. Metabolism and growth of juveniles of *Litopenaeus*

vannamei: effect of salinity and dietary carbohydrate levels. J. Exp. Mar. Biol. Ecol. 259, 1-22.

Rose, I.A., Rose, Z.B., 2014. Glycolysis: regulation and mechanisms of the enzymes. Comprehensive biochemistry 17, 93-161.

Sánchez-Paz, A., Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Muhlia-Almazan, A., Yepiz-Plascencia, G., 2008. Response of the phosphofructokinase and pyruvate kinase genes expressed in the midgut gland of the Pacific white shrimp *Litopenaeus vannamei* during short-term starvation. J. Exp. Mar. Biol. Ecol. 362, 79–89.

Schein, V., Wache, Y., Etges, R., Kucharski, L.C., van Wormhoudt, A., Da Silva, R.S., 2004. Effect of hyperosmotic shock on phosphoenolpyruvate carboxykinase gene expression and gluconeogenic activity in the crab muscle. FEBS Lett. 561, 202-206.

Semenza, G.L., 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J. Appl. Physiol. 88, 1474–1480.

Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Sotelo-Mundo, R.R., Forman, H.J., Yepiz-Plascencia, G., 2011. Hexokinase from the white shrimp *Litopenaeus vannamei*: cDNA sequence, structural protein model and regulation via HIF-1 in response to hypoxia. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 158, 242-249.

Soñanez-Organis, J.G., Rodriguez-Armenta, M., Leal-Rubio, B., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Yepiz-Plascencia, G., 2012. Alternative splicing generates two lactate dehydrogenase subunits differentially expressed during hypoxia via HIF-1 in the shrimp *Litopenaeus vannamei*. Biochimie, 1-11.

Thabrew, M.I., Poat, P.C., Munday, K.A., 1971. Carbohydrate metabolism in *Carcinus maenas* gill tissue. Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 40, 531-541.

Tomita, S., Ueno, M., Sakamoto, M., Kitahama, Y., Ueki, M., Maekawa, N., Sakamoto, H., Gassmann, M., Kageyama, R., Ueda, N., 2003. Defective brain development in mice lacking the Hif-1α gene in neural cells. Mol. Cell. Biol. 23, 6739-6749.

Tillmann, H., Bernhard, D., Eschrich, K., 2002. Fructose-1,6-bisphosphatase genes in animals. Gene 291, 57-66.

Uyeda, K., 2006. Phosphofructokinase. Advances in Enzymology and Related Areas of Molecular Biology, Volume 48, 193-244.

Vaquer-Sunyer, R., Duarte, C.M., 2008. Thresholds of hypoxia for marine biodiversity. Proc. Natl. Acad. Sci. U. S. A. 105, 15452-15457.

Vinatea, L., Gálvez, A.O., Venero, J., Leffler, J., Browdy, C., 2009. Oxygen consumption of *Litopenaeus vannamei* juveniles in heterotrophic medium with zero water exchange. Pes. Agropec. Bras. 44, 534-538.

Yoon, J.C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., Adelmant, G., Stafford, J., Kahn, C.R., Granner, D.K., Newgard, C.B., Spiegelman, B.M., 2001. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 413, 131-138.

CAPÍTULO I

Expression of fructose 1, 6-bisphosphatase and phosphofructokinase is induced in hepatopancreas of the white shrimp *Litopenaeus vannamei* by hypoxia

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Marine organisms are exposed to hypoxia in natural ecosystems and during farming. In these circumstances marine shrimp survive and synthesize ATP by anaerobic metabolism. Phosphofructokinase (PFK) and fructose 1,6-bisphosphatase (FBP) are key enzymes in carbohydrate metabolism. Here we report the cDNA of FBP from the shrimp *Litopenaeus vannamei* hepatopancreas and expression of PFK and FBP under normoxia and hypoxia. Hypoxia induces PFK and FBP expression in hepatopancreas but not in gills and muscle. Induction in hepatopancreas of the glycolytic and gluconeogenic key enzymes, PFK and FBP, suggests that PFK could be a key factor for increasing anaerobic rate, while FBP is probably involved in the activation of gluconeogenesis or the pentose-phosphates pathway during hypoxia in the highly active metabolism of hepatopancreas.

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1. Introduction

Hypoxia has been rising in marine ecosystems since the 1960ś (Diaz and Rosenberg, 2008) resulting from climate change (e.g. warming and acidification), or anthropogenic distresses (e.g. eutrophication) (Zhang et al., 2013). This can also occur when marine species are cultivated in farming conditions. Hypoxia can have severe consequences for marine organisms, leading even to death (Vaquer-Sunyer and Duarte, 2008). Interestingly, some marine species deal fairly well with a fluctuating supply of ambient oxygen (Gorr et al., 2010). Although some of the mechanisms for this response are known, there are still many unanswered questions. Some marine animals respond to hypoxia attempting to maintain oxygen delivery by increasing respiration rate, oxygen binding capacity of hemoglobin/hemocyanin or by saving energy through metabolic depression, down regulation of protein synthesis and down regulation/modification of certain regulatory enzymes (Wu, 2002). Particularly, some penaeid shrimps, in contrast to other animals, can tolerate low concentrations of dissolved oxygen (DO) (Rosas et al., 1999).

The Pacific white shrimp, *Litopenaeus vannamei*, is native to the Pacific coast from Sonora, Mexico, in the north, through Central and South America and as far South as Tumbes in Peru, in areas where water temperatures are normally >20° throughout the year. It is the major species farmed in the world in shrimp aquaculture. The natural range of this species extends into brackish and freshwaters and are exposed to wide seasonal variations in temperature, salinity (Ponce-Palafox et al., 1997) and fluctuation of oxygen levels (Martínez-Palacios et al., 1996). Normally, the shrimp use aerobic metabolism to obtain energy when the (DO) in the water is above 5 mg/L; however, when DO falls, *L. vannamei* turns to anaerobic metabolism as indicated by the rapid increase of lactate and glucose in the hemolymph (Racotta et al., 2002; Soñanez-Organis et al., 2009). Although the amount of ATP produced during anaerobic glycolysis is much less than in aerobic conditions, the process is maintained by the action of lactate dehydrogenase and the production of ATP via substrate level phosphorylation. Phosphofructokinase (PFK), pyruvate kinase (PK) and hexokinase (HK), are key regulatory enzymes of glycolysis (Fraenkel et al., 1996). PFK, considered by many authors as the main highly regulated enzyme of the pathway, catalyzes the essentially irreversible MgATP-

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dependent phosphorylation of fructose 6-phosphate (F6P) to yield fructose 1, 6-bisphosphate (F, 1-6P) (Hasawi et al., 2014). PFK is a complex enzyme involved in a rate-determining step of glycolysis and thus represents an essential metabolic control point or node for carbohydrate utilization (Jenkins et al., 2011).

On the other hand, glucose is produced *de novo* during gluconeogenesis from non-carbohydrate precursors, such as lactate, pyruvate, glycerol, and amino acids. Gluconeogenesis is not merely the reverse of glycolysis because that would be highly endergonic. The irreversible steps in glycolysis are by-passed in gluconeogenesis by pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1, 6-bisphosphatase (FBP) and glucose 6-phosphatase (GP). FBP is recognized as a key enzyme for gluconeogenesis (Lozinska-Gabska et al., 2003; Kaiser et al., 1996) and catalyzes the production of fructose 6-phosphate from fructose 1,6-bisphosphate. FBP appears to be present in all living organisms with the exception of archaebacteria whose FBP activity resides on a non-homologous protein (Stec et al., 2000). The first mammalian cDNA for FBP known was cloned from rat; it is composed of 1255 bp and corresponds to 362 amino acid residues (El-Maghrabi et al., 1988). Subsequent studies in human showed that FBP is encoded by two genes, FBP1 and FBP2, that are expressed in liver and muscle respectively, generating two isoforms (El-Maghrabi et al., 1995). In contrast to the two genes from mammals, only one gene appears to be present in less complex animals like the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster* (Tillmann et al., 2002).

In crustaceans, the occurrence of gluconeogenesis in hepatopancreas (Oliveira and Da Silva, 1997), hemocytes (Johnston et al., 1973), gills (Thabrew et al., 1971) and muscle (Schein et al., 2004) was reported. Several studies describe gluconeogenic enzymes in crustaceans, albeit with variability between species and tissues (Oliveira and Da Silva, 1997; Phillips et al., 1977; Lallier and Walsh, 1991). In the shrimp *L. vannamei* the gluconeogenic capability of hepatopancreas was demonstrated (Rosas et al., 2001) in response to dietary carbohydrates. Studies of gluconeogenesis in crustaceans have focused on quantification of metabolites and enzyme measurements and, contrary to the advanced studies in vertebrates, the information about the FBP genes is scanty (Nagai et al., 2011). Moreover, there is no information about the effect of hypoxia on FBP expression. Although the biochemical and kinetic characteristic of PFK from some vertebrates have been extensively studied (Hasawi et al., 2014), little information is available about this enzyme and its expression in marine invertebrates, and similarly to FBP, much less is known about the effect of hypoxia on its regulation. Here, we report the cDNA of FBP from shrimp hepatopancreas and the effect of hypoxia on the expression of these two enzymes, PFK and FBP, which catalyze opposite reactions. Also, the deduced FBP amino acid sequence is compared to other FBPs; similarities and differences in conserved regions are discussed in relation to its physiological implications.

2. Materials and methods

2.1. cDNA cloning of Lv FBP

The first cDNA nucleotide sequence was derived from a hepatopancreas clone obtained during construction of an Expressed Sequence Tag (EST) library ((Clavero-Salas et al., 2007) and unpublished data). To thoroughly sequence both strands, the insert was subcloned in two overlapping fragments. A fragment towards the 5′ of the cDNA was amplified using the primers FBPF1 (5′-GATGACTTCCACAGGCCAG-3′) and FBPR2 (5′-CCTATGGAGACGAGGC AATC-3′) and a second fragment towards the 3′ was amplified using the primers FBPF2 (5′-CTGGCCAATGACCTTTTCAT-3′) and the vector primer PT2R1 (5′-CTCTTCGCTATTACGCCAGCTG-3′). Both fragments were separately amplified following these PCR conditions: 94 °C, 3 min (1 cycle); 94 °C, 30 s; 55 °C,1 min; 68 °C, 1 min (36 cycles); and 68 °C, 10 min. Each 25 μL PCR reaction mixture contained 12.5 μL master mix PCR (Fermentas), 1 μL of cDNA, and 1 μL of each primer (20 μM). The PCR products were cloned into the pGEM-T Easy Vector System (Promega, San Luis Obispo, CA, USA) and the purified DNAs were sequenced thoroughly at the Laboratory of Genomic Analysis and Technology Core of the University of Arizona (Tucson, AZ, USA). The full length coding sequence of FBP was obtained by assembling the information from these two overlapping clones derived from a unique EST clone. The nucleotide and deduced protein sequences were compared to non-redundant nucleotide and protein databases in GenBank using the BLAST algorithm (Altschul et al., 1990).

2.2. Hypoxia bioassay

Healthy intermolt shrimp were previously acclimated at laboratory conditions. Shrimp (11.4 ± 0.8 g) were kept in 80 L glass containers with temperature control (28 °C), 35 ppt salinity, water exchange, constant aeration and were fed daily with the feed for shrimp Natural Force® from VIMIFOS™ at 3% of biomass (during all the time). Hypoxia was initiated by bubbling nitrogen gas to the container to give a concentration of 1.45 ± 0.2 mg of dissolved oxygen (DO) per liter and measured using an oxymeter (YSI model 55). Once the hypoxia level was reached, five shrimp were dissected at 3, 24 and 48 h. For the normoxic control tank, 5 shrimps were dissected (5.3 ± 0.3 mg of DO L^{-1}). To obtain hemocytes, hemolymph was extracted with a syringe containing anticoagulant solution (10 mM EDTA, 450 mM NaCl, 10 mM HEPES, pH 7.3) and the hemocytes were separated from plasma by centrifugation at 800 *g* for 10 min at 4 °C. Hemocytes or approximately 100 mg of hepatopancreas, gills, muscle, intestine and pleopods from each animal, were separately placed in 1 mL of TRI REAGENT® and immediately frozen using liquid nitrogen. After that, the samples were stored at −80 °C for further RNA extraction.

2.3. RNA extraction

To analyze gene expression, total RNA was extracted using TRI Reagent (Sigma–Aldrich) from hepatopancreas, gills, muscle, hemocytes, pleopods, and intestine. The RNA was quantitated using a NanoDrop spectrophotometer (Thermo Scientific) and the integrity was confirmed by 1% agarose gel electrophoresis. After that, 12.5 μg of RNA were digested with RNase-free DNAse I (Roche) at 37 °C for 20 min, and analyzed for genomic DNA contamination in a qPCR reaction in the same condition used for FBP amplification (described below). Once verified for no DNA contamination, 500 ng of total RNA were reverse transcribed in duplicates per sample using the Quantitect Reverse Transcription Kit (Qiagen) to give a final concentration of 25 ng/μL of the initial RNA in a final volume of 20 μL.

2.4. qPCR assays

FBP (GenBank accession no. KP057246), PFK (GenBank accession no. EF102107.1), and ribosomal protein L8 used as reference gene (GeneBank accession no. DQ316258.1) transcripts were measured by RT-qPCR on a Cycler iQ5 Real-Time PCR Detection System (Bio-Rad). Two qPCRs for each of the cDNAs duplicates per sample were done (four data for each shrimp per tissue) in a 20 μl final volume. For PFK and L8 the tube reaction contained 10 μL of iQ SYBR Green Supermix (Bio-Rad), 7 μL of nuclease-free water, 1 μL of each primer (20 μM) and 1 μL of cDNA (derived from 25 ng of total RNA). For FBP

the tube reaction contained 10 μL of iQ SYBR Green Supermix (Bio-Rad), 8.5 μL of nuclease-free water, 0.25 μL of each primer (20 μM) and 1 μL of cDNA (derived from 25 ng of total RNA). For FBP and L8 the PCR conditions were as follows: 95 °C for 5 min, 40 cycles at: 95 °C for 30 s, 57 °C for 35 s and a 72 °C for 55 s, with a single fluorescence measurement at the extension step and a final melting curve program with 0.3 °C increments each 20 s from 60 °C to 116 °C. Positive and negative controls were included. For PFK, the same PCR conditions were used, except that the temperature at the annealing step was 63 °C instead of 57 °C. The amplification for FBP was done using the primers FBPF2 and FBPR2 generating a 175 bp fragment and the ribosomal protein L8 was amplified using the L8F2 (5′-TAGGCAATGTCATCCCCATT-3′) and L8R2 (5′-TCCTGAAG-GAAGCTTTACACG-3′) primers which amplify a 166 bp fragment (Sánchez-Paz et al., 2008). Additionally, the amplification for PFK was done with PFKF1 (5′-TTGACCTCTGAGGCTGATTAC-3′) and PFKR1 (5′-GCGAGTGTCAAAACCAAGCTG-3′) producing a 216 bp fragment.

Efficiency of amplification was determined running standard curves each time that the cDNA samples were evaluated: for FBP ten-fold serial dilutions from 2.5×10^{1} to 2.5×10^{-7}, were used and for PFK and L8, five-fold serial dilutions, from 2.5×10^{1} to 6.45×10^{-5}. In both cases, the cDNA was used as a template. Finally, the quantification cycle (Cq) was determined. The data were analyzed with the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008). Ribosomal protein L8 was used as a reference gene (See supplementary data for L8 validation as a reference gene).

2.5. Phylogenetic analyses

FBP amino-acid sequence analysis was done with CLUSTAL W (Thompson et al., 1994). The sequences used for the alignment have the following GenBank accession numbers: *L. vannamei*, KP057246; *Marsupenaeus japonicus*, BAJ23881.1; *Daphnia pulex*, EFX73776.1; *D. melanogaster* (Isoform A), NP_610001.1; *Danaus plexippus*, EHJ73252.1; *Mus musculus* (liver type), NP_062268.1; *M. musculus* (muscle type), CAB65243.1; *Rattus norvegicus* (liver type), NP_036690.2; *R. norvegicus* (muscle type), NP_446168.1; *Oryctolagus cuniculus* (liver type), 1BK4; *O. cuniculus* (muscle type), Q9N0J6.1; *Homo sapiens* (liver type), P09467.5; *H. sapiens* (muscle type): O00757.2; *Escherichia coli*, WP_001390913.1. The alignment of amino acid sequences was used in the construction of the phylogenetic tree using neighbor-joining method Jones-Taylor-Thornton matrix-based method. Bootstrap analysis was done with 1000 replicates. The analyses were done using MEGA version 6 (Tamura et al., 2013; Jones et al., 1992).

2.6. Statistics

Statistical analyses were done using the NCSS software 2007 package (NCSS LLC, Kaysville, Utah, USA). The data for RT-qPCR were subjected to one-way ANOVA. Fisher's least significant difference (LSD) *post-hoc* taste was used to find differences at a significant level of $P < 0.05$.

3. Results

3.1. Cloning of Lv FBP

The complete cDNA sequence for *L. vannamei* FBP is 1140 bp with the start and stop codons in positions 2 and 1004 bp, respectively. The 3′-untranslated region (UTR) is 118 bp long, excluding the poly-A tail (Fig. 1). The predicted protein contains 334 residues and has a calculated molecular weight of 36.26 kDa and pI of 6.0, similar to other FBP deduced proteins: shrimp *M. japonicus* has a MW of 36.64 and a pI of 6.45; in *D. melanogaster* isoform A (GenBank accession no. NP_610001.1), the MW and pI are 36.19 and 6.36 respectively; and, for human (GenBank accession no. AAA35817.1), values of 36.82 for MW and 6.54 for pI are known. These values are also in the same range of other marine cDNA deduced homologous proteins, including the salmon louse *Lepeophtheirus salmonis* (GenBank accession no. ACO12077.1), the sea louses *Caligus rogercresseyi* (GenBank accession no. ACO11320.1) and Caligus clemensi (GenBank accession no. ACO14628.1) whose calculated MW and pI are of 36.76 and 6.01; 36.91 and 6.95; and 37.28 and 6.54, respectively. Although we did not look for FBP isoforms, no other bands were amplified in the multiple PCRs done during the study, which may suggest that there is only one FBP gene in this shrimp, but this remains to be carefully analyzed in the future.

3.2. Amino acid sequence alignment

Sequence analyses of the deduced FBP from *L. vannamei* showed that this protein shares high homology with its FBP counterpart from *M. japonicus* (95%) and with other related taxa FBPs such as the predicted proteins of *D. pulex* (76%) (GenBank accession no. EFX73776.1), *Droshophila persimilis* (73%) (GenBank accession no. XP_002019082.1), *Bombyx mori* (71%) (GenBank accession no. NP_001040381.1), and *D. plexippus* (72%) (GenBank accession no. EHJ73252.1). The homologies found across different taxa indicate that the protein has had no substantial changes during evolution. From the alignment of the amino acid sequences of different FBPs, it is clear that most residues that interact with fructose 1,6-bisphosphate (substrate) are well conserved: Asn 215, Gly 217, Ser 245, Met 246 and Tyr 262 are conserved in *L. vannamei*, but there is an absence of an arginine and tyrosine (between amino acid 242 and 243) compared to the other species analyzed. FBP requires divalent cations such magnesium for activity and the shrimp FBP possesses four conserved amino acids in this binding region: Ser 99, two Asp at position 121 and 124 and a Glu 278. On the other hand, fructose 2,6-bisphosphate, which binds with high affinity to the active site, inhibits FBP synergistically with AMP. In addition to the well conserved amino acids that interact with fructose 1,6-bisphosphate, FBP from *L. vannamei* has the conserved Gly and Asn in positions 125 and 128 that correspond to the residues that bind fructose 2,6-bisphosphate. Furthermore and contrary to the highly conserved amino acid that interact with fructose 2,6-bisphosphate, several amino acids involved with AMP interaction, vary between shrimp and other species analyzed. From the eleven amino acids that interact with AMP, only Thr 30, Lys 33, Thr 34, Arg 143 and Thr 180 are identical, compared to mammalian FBPs, the rest of the residues are conserved (Asp 32 and Arg 112) or non-conserved amino acids (Thr 19, Glu 22, Gln 23 and Gln 113) (Fig. 2).

3.3. Phylogenetic analysis

Fig. 3 depicts the phylogenetic tree that shows two major clades. In one of them the FBPs from insects are associated. In the other one, FBPs from crustaceans form a distinctive group with vertebrates. Clearly, FBP from *L. vannamei* is clustered with other known crustacean FBP. Crustacean FBPs have 64% bootstrap support and are differentiated from vertebrates that hold 100% bootstrap. Interestingly, both liver and muscle FBPs are matched in their respective groups together with 100% bootstrap. Insects have a 62% bootstrap and are associated in a separate group from vertebrate and crustaceans.

```
                10        20        30        40        50        60        70        80
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  GATGACTTCCACAGGCCAGGCAATTGACACGGACTCCATGACGTTGACGCGGTTCACGTTGGGGGAGCAGAAGAGAGTGC
          M  T  S  T  G  Q  A  I  D  T  D  S  M  T  L  T  R  F  T  L  G  E  Q  K  R  V

                90       100       110       120       130       140       150       160
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  CTCATGCCACCGGCGATCTGACACAGCTCTTGGTTAGCATTCAAACTGCTGTCAAGGCCATCTCCTCGGCTGTCAGGAAG
        P  H  A  T  G  D  L  T  Q  L  L  V  S  I  Q  T  A  V  K  A  I  S  S  A  V  R  K

               170       180       190       200       210       220       230       240
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  GCCGGCATTGCCAAGCTGTATGGCATGGCAGGAGACGTGAACGTTCAAGGAGAGGAGGTGAAGAAGCTGGATGTGCTGGC
          A  G  I  A  K  L  Y  G  M  A  G  D  V  N  V  Q  G  E  E  V  K  K  L  D  V  L  A

               250       260       270       280       290       300       310       320
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  CAATGACCTTTTCATCAACATGCTGTCGTCGTCTTACACAACCTGCTTACTTGTGTCGGAAGAAAACAAAACTGTTATTG
          N  D  L  F  I  N  M  L  S  S  S  Y  T  T  C  L  L  V  S  E  E  N  K  T  V  I

               330       340       350       360       370       380       390       400
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  AGGTAGAACAGGAGCGACAAGGAAAGTATGTGGTGTGTTTTGATCCACTCGATGGTTCTTCCAATATTGATTGCCTCGTC
        E  V  E  Q  E  R  Q  G  K  Y  V  V  C  F  D  P  L  D  G  S  S  N  I  D  C  L  V

               410       420       430       440       450       460       470       480
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  TCCATAGGATCTATTTTCAGCATTTATAGAAAGAGCACTGAAGGAGTTCCAACTGTATCAGATGCCTTACAGCCTGGTAA
          S  I  G  S  I  F  S  I  Y  R  K  S  T  E  G  V  P  T  V  S  D  A  L  Q  P  G  N

               490       500       510       520       530       540       550       560
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  CCAAATAGTTGCAGCAGGGTATGCCTTGTATGGGTCAGCTACTATGATGGTCATATCGACTGGCAATGGAGTTAATGGCT
          Q  I  V  A  A  G  Y  A  L  Y  G  S  A  T  M  M  V  I  S  T  G  N  G  V  N  G

               570       580       590       600       610       620       630       640
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  TTATGTTGGATCCGAGTATTGGAGAGTTTGTGTTGACTGACCCCAACATGAGAGTGAAGGAAAAGGGCAAAATCTACAGT
        F  M  L  D  P  S  I  G  E  F  V  L  T  D  P  N  M  R  V  K  E  K  G  K  I  Y  S

               650       660       670       680       690       700       710       720
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  CTTAATGAAGGTTATGCCAACCTCTGGGATCCAGCAGTATCTGAATATGTGCATGGAAAGAAGGCCAAAAAAGCTGGTGC
          L  N  E  G  Y  A  N  L  W  D  P  A  V  S  E  Y  V  H  G  K  K  A  K  K  A  G  A

               730       740       750       760       770       780       790       800
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  ACGTTACATTGGATCTATGGTTGCTGATGTCCATCGTACACTAAAGTATGGGGGTATTTTCATGTACCCTGCCACCTCTG
          R  Y  I  G  S  M  V  A  D  V  H  R  T  L  K  Y  G  G  I  F  M  Y  P  A  T  S

               810       820       830       840       850       860       870       880
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  ATGCTCCTAAGGGCAAGCTTCGTTTACTGTATGAATGTAACCCTATGGCATTCCTAATGGAACTGGCTGGAGGTCAGGCA
        D  A  P  K  G  K  L  R  L  L  Y  E  C  N  P  M  A  F  L  M  E  L  A  G  G  Q  A

               890       900       910       920       930       940       950       960
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  ACAACTGGAAAGATGCGCATCCTTGACATCGTCCCATCTGATATTCATCAGCGCACTCCCATTTTCCTTGGATCAACTGA
          T  T  G  K  M  R  I  L  D  I  V  P  S  D  I  H  Q  R  T  P  I  F  L  G  S  T  D

               970       980       990      1000      1010      1020      1030      1040
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  TGATGTACAAGAAATCATTGACTTGTACAAAAAGCATAATCTGTAGAACCCAATTTCAATAATCTGTGTGAAATAATCCC
          D  V  Q  E  I  I  D  L  Y  K  K  H  N  L  *

              1050      1060      1070      1080      1090      1100      1110      1120
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FBP LV  CTTGAAATGGTACAGGACCTACTGTGTTGCATCTGAGAAGAATTATCATATGTTACATAATGAATAACCCTGGCGTTACC

              1130      1140
        ....|....|....|....|
FBP LV  CAACAAAAAAAAAAAAAAAA
```

Fig. 1. Nucleotide and predicted protein for *L. vannamei* FBP. The start methionine is in bold and the stop codon is represented by an asterisk. The polyadenylation signal is in italics. The primers used to obtain the cDNA are underlined.

3.4. FBP and PFK expression under normoxic and hypoxic conditions

Expression of FBP in normoxia was evaluated in hepatopancreas, gills, muscle, hemocytes, intestine, and pleopods. The FBP transcripts were present in all the tissues examined, indicating probably glucose synthesis. The hepatopancreas of crustaceans is a center for carbohydrate metabolism as well as a site for gluconeogenesis (Rosas et al., 2001). Therefore, the expression of FBP in hepatopancreas was used as reference for comparison (Table 1). Surprisingly, this tissue presented almost the lowest expression of FBP, except for hemocytes, where the value is 25 fold-less.

```
                                    a  aa      a aaa
L. vannamei       MTSTGQAIDTDSMTLTRFTLGEQKRVPHATGDLTQLLVSIQTAVKAISSAVRKAGIAKLY 60
M. japonicus      MTSTGQAIDTDSMTLTRFTLGEQKKVPHATGDLTQLLVSIQTAVKAISSAVRKAGIAKLY 60
D. melanogaster   MTQQRPAFDSNAMTLTRFVLQEQRKFKSATGDLSQLLNSIQTAIKATSSAVRKAGIAKLH 60
D. pulex          MSNS--PIDTNCMTLTRFVLAEQRRIPGASGDLSQLLNSLQSAIKAVSSAVRRAGIANLY 58
Human liver       MADQ-APFDTDVNTLTRFVMEEGRKARG-TGELTQLLNSLCTAVKAISSAVRKAGIAHLY 58
Mouse liver       MANH-APFETDISTLTRFVMEQGRKAQG-TGELTQLLNSLCTAIKAISSAVRQAGIAQLY 58
Human muscle      MTDR-SPFETDMLTLTRYVMEKGRQAKG-TGELTQLLNSMLTAIKAISSAVRKAGLAHLY 58
Mouse muscle      MTDR-SPFETDMLTLTRYVMEKGRQAKG-TGELTQLLNSMLTAIKAISSAVRKAGLANLY 58
                  *:.   .::::  ****:.: : ::    :*:*:*** *: :*:** *****:**:*:*:
                                                        m            aa
L. vannamei       GMAGDVNVQGEEVKKLDVLANDLFINMLSSSYTTCLLVSEENKTVIEVEQERQGKYVVCF 120
M. japonicus      GMAGDVNVQGEEVKKLDVLANDLFINMLSSSYTTCLLVSEENKTVIEVEQERQGKYVVCF 120
D. melanogaster   GFAGDVNVQGEEVKKLDVLSNELFINMLKSSYTTCLMVSEENENVIEVEVEKQGKYIVCF 120
D. pulex          GIAGQVNVQGEEVKKLDVLANELFINLLRSSFTTCLLVSEEDETVIEVETEKQGKYIVCF 118
Human liver       GIAGSTNVTGDQVKKLDVLSNDLVMNMLKSSFATCVLVSEEDKHAIIVEPEKRGKYVVCF 118
Mouse liver       GIAGSTNVTGDQVKKLDILSNDLVINMLKSSYATCVLVSEENTNAIIIEPEKRGKYVVCF 118
Human muscle      GIAGSVNVTGDEVKKLDVLSNSLVINMVQSSYSTCVLVSEENKDAIITAKEKRGKYVVCF 118
Mouse muscle      GISGSVNVTGDEVKKLDVLSNSLVINMLQSSYSTCVLVSEENKEAVITAQERRGKYVVCF 118
                  *::*..** *::*****:*:*.*.:*:: **::**::****:  .:    *: ***:***
                  m  m2  2              a                                    a
L. vannamei       DPLDGSSNIDCLVSIGSIFSIYRKSTEGVPTVSDALQPGNQIVAAGYALYGSATMMVIST 180
M. japonicus      DPLDGSSNIDCLVSIGSIFSIYRKNNEAVPSVSDALQPGNQIVAAGYALYGSATMMVIST 180
D. melanogaster   DPLDGSSNIDCLVSIGSIFAIYRKKSDGPPTVEDALQPGNQLVAAGYALYGSATAIVLGL 180
D. pulex          DPLDGSSNIDCLVSIGSIFSIFKKTTEGVPSVKDALQPGRQCVAAGYSLYGSATMMVLST 178
Human liver       DPLDGSSNIDCLVSVGTIFGIYRKKSTDEPSEKDALQPGRNLVAAGYALYGSATMLVLAM 178
Mouse liver       DPLDGSSNIDCLVSIGTIFGIYRKKSTDEPSEKDALQPGRDLVAAGYALYGSATMLVLAM 178
Human muscle      DPLDGSSNIDCLASIGTIFAIYRKTSEDEPSEKDALQCGRNIVAAGYALYGSATLVALST 178
Mouse muscle      DPLDGSSNIDCLASIGTIFAIYRKTTEDEPSEKDALQPGRNIVAAGYALYGSATLVALST 178
                  ************.*:*:**.*::*..   *: .**** *.: *****:****** :.:.
                                                    1 1
L. vannamei       GNGVNGFMLDPSIGEFVLTDPNMRVKEKGKIYSLNEGYANLWDPAVSEYVHGKKAKKAG- 239
M. japonicus      GNGVNGFMLDPSIGEFVLTDPNMRVKEKGKIYSLNEGYANLWDPAVSEYVQGKKAKKAG- 239
D. melanogaster   GSGVNGFTYDPAIGEFVLTDPNMRVPEKGKIYSINEGYAADWEDGVFNYIAAKKDPAKG- 239
D. pulex          GNGVNGFLLDPAIGEFVLTDPDMKVKPRGKIYSINEGYTNNWDKAIQEYVASKKEGP--- 235
Human liver       DCGVNCFMLDPAIGEFILVDKDVKIKKKGKIYSLNEGYARDFDPAVTEYIQRKKFPPDNS 238
Mouse liver       DCGVNCFMLDPSIGEFIMVDRDVKMKKKGNIYSLNEGYAKDFDPAINEYLQRKKFPPDGS 238
Human muscle      GQGVDLFMLDPALGEFVLVEKDVKIKKKGKIYSLNEGYAKYFDAATTEYVQKKKFPEDGS 238
Mouse muscle      GQGVDLFMLDPALGEFVLVEKDVRIKKKGKIFSLNEGYAKYFDAATAEYVQKKKFPEDGS 238
                  . **: *  **::***::.: ::::  :*:*:*:****:  :: .  :*:  **
                       11  11               1   1   1       m
L. vannamei       ARY----IGSMVADVHRTLKYGGIFMYPATSDAPKGKLRLLYECNPMAFLMELAGGQATT 295
M. japonicus      ARYGARYIGSMVADVHRTLKYGGIFMYPATADAPKGKLRLLYECNPMAYLMEQAGGQATA 299
D. melanogaster   KPYGARYVGSMVADVHRTIKYGGIFIYPATKSAPSGKLRLLYECVPMAYLMIQAGGLASD 299
D. pulex          KPYGARYVGSMVADVHRTLKYGGIFMYPATTDAPRGKLRLLYESIPMAFIMEQAGGAATT 295
Human liver       APYGARYVGSMVADVHRTLVYGGIFLYPANKKSPNGKLRLLYECNPMAYVMEKAGGMATT 298
Mouse liver       APYGARYVGSMVADIHRTLVYGGIFLYPANKKSPSGKLRLLYECNPIAYVMEKAGGLATT 298
Human muscle      APYGARYVGSMVADVHRTLVYGGIFLYPANQKSPKGKLRLLYECNPVAYIIEQAGGLATT 298
Mouse muscle      EPYGARYVGSMVADVHRTLVYGGIFMYPANQKSPNGKLRLLYECNPVAYIIEQAGGMATT 298
                    *    :******:***: *****:***. .:* ********. *:*:::  *** *:

L. vannamei       GKMRILDIVPSDIHQRTPIFLGSTDDVQEIIDLYKKHNL----- 334
M. japonicus      GKMRILDIVPTDIHQRTPIFLGSSGDVQEIIDLYKKHNL----- 338
D. melanogaster   GKISILDIVPKKIHERSPIFLGSKSDVEEALSYLK--------- 334
D. pulex          GSIPILDIQPEKIHERCPVILGSKDDVQDVLDIIKRHADQ---- 335
Human liver       GKEAVLDVIPTDIHQRAPVILGSPDDVLEFLKVYEKHSAQ---- 338
Mouse liver       GDKDILDIVPTEIHQKAPVVMGSSEDVQEFLEIYRKHKAK---- 338
Human muscle      GTQPVLDVKPEAIHQRVPLILGSPEDVQEYLTCVQKNQAGS--- 339
Mouse muscle      GTQPVLDVKPESIHQRVPLILGSPEDVQEYLSCVQRNQAGR--- 339
                  *   :**: *  **:: *:.:**  ** : :   .
```

Fig. 2. Amino acid sequence comparisons between selected FBPs. Letters above the sequences indicates residues that bind: a, AMP; m, magnesium; 1, fructose 1,6-bisphosphate; 2, fructose 2,6-bisphosphate. Symbols below the sequences mean: (*), identical residues; (:), conservative substitutions; and (.), semi-conservative substitutions. The GenBank accession numbers for each species are the same as used for phylogenetic tree.

Interestingly, the higher levels of FBP transcripts were found in muscle, where there is 5.11 fold-more transcripts compared to hepatopancreas. Intestine was the second major tissue for FBP transcripts abundance with a 2.51 fold-more compared to hepatopancreas. In gills the expression of FBP doubles the one detected in hepatopancreas. Finally, approximately 1.5 fold-more transcripts were found in pleopods compared to hepatopancreas.

The effect of the hypoxia on FBP expression was also evaluated in hepatopancreas, gills and muscle (Fig. 4). In hepatopancreas, short-time hypoxia (3 h) resulted in significantly lower mRNAs abundance, but a recovery was detected after 24 h of hypoxia. A statistically significant induction of the transcripts was detected after 48 h of hypoxia, corresponding to 40% more compared to normoxic conditions. In contrast, a down-regulation occurred in

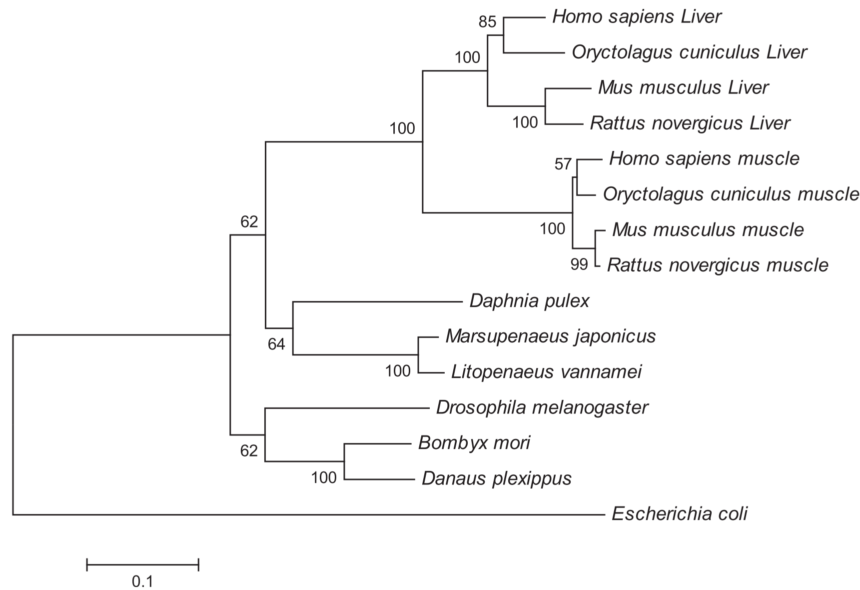

Fig. 3. FBP phylogenetic tree. A neighbor joined method Jones-Taylor-Thornton matrix based was used to obtain an inferred tree from comparison of FBPs (See Materials and Methods for GenBank accession number for each FBP). Numbers indicate the percentages of support based on 1000 bootstraps steps. The scale bars equals 0.1 substitutions per site.

gills and muscle from shrimp subjected to hypoxia. In gills, hypoxia caused a significantly four fold-decrease at three hours and no recovery was seen after 48 h hypoxia. Similarly, in muscle a decrease of 25 fold-change occurred after 48 h of hypoxia stress.

We previously reported a partial cDNA sequence of PFK corresponding to 198 amino acid residues of the predicted protein that contains the highly conserved fructose 6-P, ATP and Mg^{2+} binding sites from *L. vannamei* and also detected its expression using conventional RT-PCR in all the tissues mentioned above (Sánchez-Paz et al., 2008). Therefore, we evaluated the effect of the hypoxia on PFK transcripts. To establish a reference point for comparison and according to their metabolic and functional importance to respond to hypoxia, gills and hepatopancreas were chosen. Hypoxia induces up to 90 fold-change the expression of PFK in hepatopancreas compared to normoxia (Fig. 5). However, PFK expression in hepatopancreas and gills under normoxic condition is lower compared to the expression of FBP in the same normoxic conditions, around three orders of magnitude different in hepatopancreas. In gills, the transcripts are maintained during the first 24 h of hypoxia compared to normoxia and a ~1.5 fold-change in transcripts is detected after 48 h of hypoxia, although this increase is not statistically significant different.

4. Discussion

Some organisms can survive many weeks in oxygen-deprived environments (Holman and Hand, 2009; Hermes-Lima and Zenteno-Savín, 2002). Oxygen is essential for most life forms and many marine organisms have developed diverse strategies to deal with low oxygen concentrations. It is known, for example, that *Droshophila* experiments hypometabolic defense mode (Gorr et al., 2010), while others animals are able to redirect blood flow to tissues requiring higher levels of oxygen (Reiber and McMahon, 1998). Despite the great deal of knowledge on metabolic strategies, little is known about the cellular and molecular mechanisms in invertebrate marine species. Here, we demonstrated that PFK expression, the most important enzyme in glycolysis, is regulated in hypoxia. The oxygen-depriving condition promotes its expression in hepatopancreas, although in gills there is a tendency to increase after 48 h of hypoxia, it is not statistically significant and requires, perhaps, longer hypoxia time to have a substantial change in transcript abundance (Fig. 5). Both hepatopancreas and gills are key metabolic organs; hepatopancreas is a multifunctional organ that participates in carbohydrate metabolism (Yepiz-Plascencia et al., 2000) and gills are involved in respiration and detoxification (Soñanez-Organis et al., 2009). In penaeid shrimp, hypoxia induces anaerobic carbohydrate metabolism (Racotta et al., 2002). In our previous work, we have shown that in *L. vannamei* hypoxia induces the expression of hexokinase (Soñanez-Organis et al., 2009) and lactate dehydrogenase (Soñanez-Organis et al., 2012). Herein we give evidence of PFK induction and, accompanied by the previous findings of lactate accumulation reported for shrimp subjected to hypoxia (Racotta et al., 2002), this suggests that PFK could be a key factor to accelerate the rate of glycolysis to produce ATP anaerobically. Alternative routes of anaerobic carbohydrate catabolism are less efficient in ATP production and do not provide enough energy to maintain aerobic consumption (Sun et al., 2014); however, regulation of PFK at the protein level still needs to be demonstrated in shrimp.

It was interesting to analyze the expression of FBP in the same conditions than those tested for PFK. FBP catalyzes the opposite reaction of PFK, and becomes a rate limiting step in gluconeogenesis. We found that FBP is expressed in all tissues analyzed (Table 1) and, moreover, hypoxia causes a tissue specific induction (Fig. 4), that to our knowledge, becomes the first report in marine invertebrate species. Interestingly, here we found a significant FBP

Table 1
Comparison of FBP relative expression in different tissues[a].

Tissue	Normalized Relative to L8 ($\times 10^{-3}$)	FBP expression relative to hepatopancreas
Hepatopancreas	95.5	1.00
Muscle	487.7	5.11
Intestine	240.2	2.51
Gills	204.0	2.14
Pleopods	140.1	1.47
Hemocytes	4.0	0.04

[a] Total RNA was extracted from shrimp tissues at normoxic conditions.

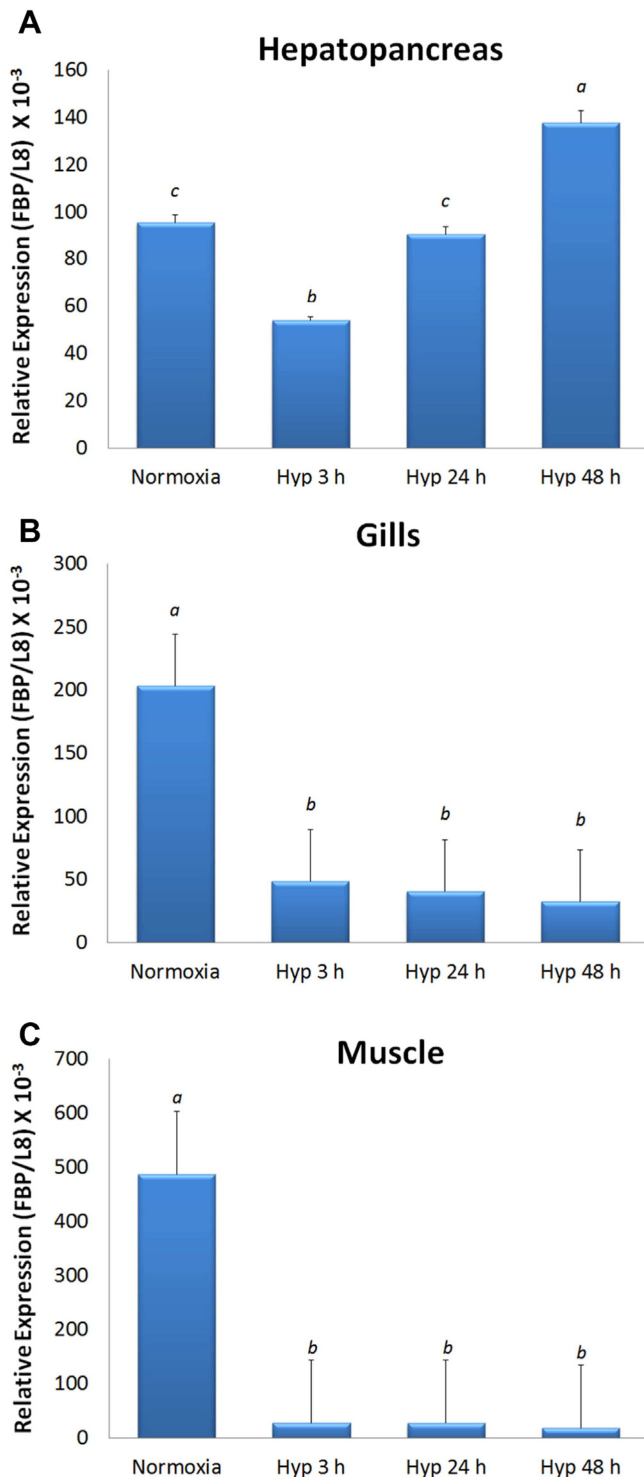

Fig. 4. Effect of hypoxia in FBP gene expression: A) Hepatopancreas, B) Gills and C) Muscle of shrimps in normoxic and hypoxic conditions. RT-qPCR was performed as specified and Materials and Methods. The expression levels were normalized to the ribosomal protein L8 transcript levels. Data are presented as means (n = 3–5). Different letters indicate significant differences from each other ($P < 0.05$; Fisher's LSD Multiple–Comparison Test). Bars represent the standard error.

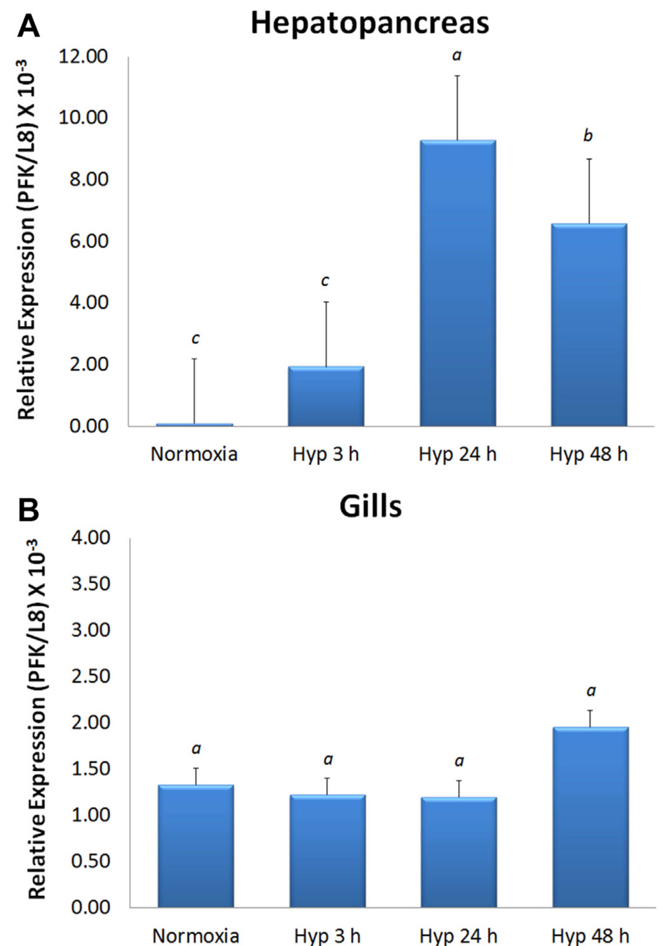

Fig. 5. Effect of hypoxia in PFK gene Expression. A) Hepatopancreas and B) Gills from shrimp exposed to normoxic and hypoxic conditions. RT-qPCR was performed as specified and Materials and Methods. The expression levels were normalized to the ribosomal protein L8 gene transcript levels. Data are presented as means (n = 3–5). Different letters indicate significant differences from each other (P < 0.05; Fisher's LSD Multiple–Comparison Test). Bars represent the standard error.

induction in hepatopancreas after 48 h hypoxia. This finding is somehow in agreement with our previous report since it correlates with higher glucose plasma concentration (Soñanez-Organis et al., 2009), suggesting carbohydrate mobilization from hepatopancreas to other tissues through hemolymph. Conversely, in gills and muscle the expression drops drastically compared to their respectively normoxic expression. It is important to recognize that induction of FBP in *L. vannamei* hepatopancreas is not an immediate response; since it appears to have a lag phase and may indicate a time course for synthesis or stabilization of a transcription factor needed afterwards for regulation. In this context, hypoxia-inducible factors (HIFs) are transcriptional activators that function as master regulators of oxygen homeostasis in all metazoan species (Semenza, 2012). From studies in mammals we now know that HIF-1 directly binds to the specific PEPCK promoter region inducing transcription in hypoxic conditions (Choi et al., 2005). HIF-1 binds a core sequence of the Hypoxia Response Elements (HRE) in the promoters of hypoxia-responsive genes and induces their expressions (Lee et al., 2004). We know that HIF-1 regulates the expression of hexokinase and LDH in shrimp in hypoxia (Soñanez-Organis et al., 2012; Sonanez-Organis et al., 2011). Moreover, there is evidence that hypoxia induces the enzymatic activity of FBP in marine vertebrate species (Martínez et al., 2006). Expression of PEPCK in the grass shrimp hepatopancreas (Brown-Peterson et al., 2008) and glucose 6-phosphatase in fish liver (Gracey et al., 2001), both irreversible enzymes of gluconeogenesis, increases during hypoxia. However, if HIF-1 regulates both PFK and FBP in shrimp, it remains

to be elucidated, since no genomic promoter sequences upstream the coding regions are available yet.

Hepatopancreas functions include production of digestive enzymes, hemolymph proteins and absorption of nutrients (Yepiz-Plascencia et al., 2000). In *L. vannamei* the gluconeogenic capability in this organ was recently demonstrated (Rosas et al., 2001). Paradoxically, in our study the higher expression of both catabolic (glycolytic) and anabolic (gluconeogenic) key transcripts, PFK and FBP, respectively, were detected in hepatopancreas of shrimp during hypoxia, and this would suggest a futile cycle considering that they are promoting at the same time, glycolysis and gluconeogenesis, respectively. From the energetic point of view, gluconeogenesis is an ATP consuming process. When comparing it with glycolysis, the end balance is −2 ATP -2 GTP. Actually, it is sensible to think that in low oxygen concentration ATP becomes precious. Notably, hypoxia induces FBP expression in hepatopancreas, thus, we can think this would trigger the synthesis of glucose. There is some evidence that even in depriving oxygen conditions, hepatopancreas can synthesize proteins like hemocyanin (Sun et al., 2014; Defur et al., 1990), which is an energy demanding process. Therefore, it is possible that the cells use the poor stock of energy in some cost/energy metabolic processes as appear to be the case in shrimp. Indeed, there is evidence in teleost fish of diverse cell types that differ in their glycolytic and gluconeogenic capabilities (Mommsen et al., 1991). Hence, it is feasible to think that glycolysis could be occurring in some type of cells of the hepatopancreas, while in others, gluconeogenesis is taking place. Also, we cannot exclude the possibility that in a single cell both process might occur even against energy imbalance.

Our research group has previously reported a raise of glucose in shrimp hemolymph during hypoxia (Soñanez-Organis et al., 2009). Here, we show evidence that FBP is induced in shrimp at 48 h hypoxia, arguing in favor of gluconeogenesis. Induction of FBP could be supported by the fact that some tissues like brain necessitates obligatory glucose as a main carbohydrate source to maintain their ATP levels, otherwise, a catastrophic event like death could occur. Consequently, shrimp could mimic what the crucian carp and the fresh water turtles do, which also deviates from mammals in one crucial aspect: they maintain ATP levels when exposed to oxygen stingy conditions (Nilsson and Lutz, 2004). Another important fact to consider by FBP induction, is the generation of glucose 6-phosphate that is the substrate for the pentose phosphate pathway; thus, the activation of this metabolic pathway can lead to the increase of total cellular glutathione. In mammals, glutathione is the main intracellular antioxidant and inhibits some forms of apoptosis (Voehringer et al., 2000). Studies in mammals have revealed that tumors cells are exposed to significantly reduced oxygen concentrations and is accepted that the main adaptations that aid the tumor in acquiring tolerance and surviving are related with the induction of expression of multiple genes, among others, those that give resistance to apoptosis (Gilkes and Semenza, 2013). If the shrimp cells have acquired or conserved some type of apoptosis resistance that allow them to increase its survival capabilities is for now unclear and this remains to be investigated.

Regulation of mammalian FBPs has been investigated in detail. Enzyme activity is regulated by fructose 2,6-bisphosphate (F-2, 6-BP), an inhibitor that binds to the substrate site, and adenosine monophosphate (AMP), an inhibitor that binds to the allosteric site (Stec et al., 1996). In shrimp FBP, just around 60% of the residues that are in contact with the inhibitor AMP are conserved (Fig. 2). AMP causes a shift in the mammalian enzyme from high activity R (relaxed) state to and inactive T (tense) state (Zhang et al., 1994). Muscle type and liver type terrestrial vertebrate FBPs have differences in some of the amino acids that bind AMP. The muscle FBP is strongly inhibited by AMP and is indispensable to restrict the enzyme activity, contrary to liver FBP (Tillmann et al., 2002). Our results show that the shrimp FBP has less conservative substitutions in the residues that bind AMP compared to the muscle and liver FBP (Fig. 2). As a result, shrimp FBP could somehow behave as mammalian liver FBP. Thus, weaker inhibition in shrimp FBP may suggest that it keeps its activity even with the increasing concentration of AMP that occurs when glycolysis is accelerated in hypoxia conditions.

In conclusion, hypoxia induces the expression of PFK in hepatopancreas. This cellular response is oriented to ensure the contribution of PFK in glycolysis to finally generate substantial ATP. Likewise, hypoxia induces the expression of FBP in a tissue-specific manner; the mRNA rise in hepatopancreas can be well explained taking into consideration the multi-functionality of this organ where: 1) glucose produced by activation of gluconeogenesis (trough FBP induction) can be exported to be utilized by other tissues as brain or ganglia cells, or, 2) The pentose-phosphate pathway can be activated to generate antioxidant equivalents. However, more studies need to be done to confirm this. Though gluconeogenesis is a highly endergonic process; it can be stimulated in an important organ such as hepatopancreas and annulated in tissues as gills and muscle via FBP down-regulation. If HIF-1 proteins are regulating this process, it remains to be elucidated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2015.02.003.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Brown-Peterson, N.J., Manning, C.S., Patel, V., Denslow, N.D., Brouwer, M., 2008. Effects of cyclic hypoxia on gene expression and reproduction in a grass shrimp. Palaemonetes Pugio. Biol. Bull. 214, 6–16.

Choi, J.H., Park, M.J., Kim, K.W., Choi, Y.H., Park, S.H., An, W.G., Yang, U.S., Cheong, J., 2005. Molecular mechanism of hypoxia-mediated hepatic gluconeogenesis by transcriptional regulation. FEBS Lett. 579, 2795–2801.

Clavero-Salas, A., Sotelo-Mundo, R.R., Gollas-Galvan, T., Hernandez-Lopez, J., Peregrino-Uriarte, A.B., Muhlia-Almazan, A., Yepiz-Plascencia, G., 2007. Transcriptome analysis of gills from the white shrimp *Litopenaeus vannamei* infected with White Spot Syndrome Virus. Fish Shellfish Immunol. 23, 459–472.

Defur, P.L., Mangum, C.P., Reese, J.E., 1990. Respiratory responses of the blue crab *Callinectes sapidus* to long-term hypoxia. Biol. Bull. 178, 46–54.

Diaz, R.J., Rosenberg, R., 2008. Spreading dead zones and consequences for marine ecosystems. Science 321, 926–929.

El-Maghrabi, M.R., Pilkis, J., Marker, A.J., Colosia, A.D., D'Angelo, G., Fraser, B.A., Pilkis, S.J., 1988. cDNA sequence of rat liver fructose-1,6-bisphosphatase and evidence for down-regulation of its mRNA by insulin. Proc. Natl. Acad. Sci. U. S. A. 85, 8430–8434.

El-Maghrabi, M.R., Lange, A.J., Jiang, W., Yamagata, K., Stoffel, M., Takeda, J., Fernald, A.A., Le Beau, M.M., Bell, G.I., Baker, L., Pilkis, S.J., 1995. Human fructose-1,6-bisphosphatase gene (FBP1): Exon-intron Organization, localization to chromosome bands 9q22.2-q22.3, and mutation screening in subjects with fructose-1,6-bisphosphatase deficiency. Genomics 27, 520–525.

Fraenkel, D.G., 1996. Glycolysis. In: Neidhardt, F., Curtiss, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B. (Eds.), Escherichia coli and Salmonella. Cell Mol. Biol. American Society for Microbiology, pp. 1325–1343.

Gilkes, D.M., Semenza, G.L., 2013. Role of hypoxia-inducible factors in breast cancer metastasis. Future Oncol. 9, 1623–1636.

Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N.B., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83 (5), 733–752.

Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression

profiling in the euryoxic fish *Gillichthys mirabilis*. Proc. Natl. Acad. Sci. U. S. A. 98, 1993–1998.
Hasawi, N.A., Khandari, M.A., Luqmani, Y.A., 2014. Phosphofructokinase: a mediator of glycolytic flux in cancer progression. Cr. Rev. Oncol.-Hem 92, 312–321.
Hermes-Lima, M., Zenteno-Savín, T., 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 133, 537–556.
Holman, J.D., Hand, S.C., 2009. Metabolic depression is delayed and mitochondrial impairment averted during prolonged anoxia in the ghost shrimp, *Lepidophthalmus louisianensis* (Schmitt, 1935). J. Exp. Mar. Biol. Ecol. 376, 85–93.
Jenkins, C.M., Yang, J., Sims, H.F., Gross, R.W., 2011. Reversible high affinity inhibition of phosphofructokinase-1 by acyl-CoA: a mechanism integrating glycolytic flux with lipid metabolism. J. Biol. Chem. 286, 11937–11950.
Johnston, M.A., Elder, H.Y., Davies, P.S., 1973. Cytology of carcinus haemocytes and their function in carbohydrate metabolism. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 46, 569–581.
Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.
Kaiser, R., Olsson, H., Erman, M., Weeks, C.M., Hjelmqvist, L., Ghosh, D., Jörnvall, H., 1996. Fructose-1,6-bisphosphatase. Primary structure of the rabbit liver enzyme. 'Intermediate' variability of an oligomeric protein. FEBS Lett. 389, 249–252.
Lallier, F.H., Walsh, P., 1991. Metabolic potential in tissues of the blue crab, *Callinectes sapidus*. Bull. Mar. Sci. 48, 665–669.
Lee, J.-W., Bae, S.-H., Jeong, J.-W., Kim, S.-H., Kim, K.-W., 2004. Hypoxia-inducible factor (HIF-1)[alpha]: its protein stability and biological functions. Exp. Mol. Med. 36, 1–12.
Lozinska-Gabska, M., Dziewulska-Szwajkowska, D., Szalagan, E., 2003. Isolation of fructose-1, 6-bisphosphatase from the foot muscle of a snail *Helix aspersa* maxima. Zool. Pol. 481–4, 81–91.
Martínez, M.L., Landry, C., Boehm, R., Manning, S., Cheek, A.O., Rees, B.B., 2006. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*. J. Exp. Biol. 209, 3851–3861.
Martínez-Palacios, C.A., Ross, L.G., Jiménez-Valenzuela, L., 1996. The effects of temperature and body weight on the oxygen consumption of *Penaeus vannamei*, Boone, 1931. J. Aquac. Trop. 11, 59–65.
Mommsen, T.P., Danulat, E., Gavioli, M.E., Foster, G.D., Moon, T.W., 1991. Separation of enzymatically distinct populations of trout hepatocytes. Can. J. Zool. 69, 420–426.
Nagai, C., Nagata, S., Nagasawa, H., 2011. Effects of crustacean hyperglycemic hormone (CHH) on the transcript expression of carbohydrate metabolism-related enzyme genes in the kuruma prawn, *Marsupenaeus Japonicus*. Gen. Comp. Endocrinol. 172, 293–304.
Nilsson, G.E., Lutz, P.L., 2004. Anoxia tolerant brains. J. Cereb. Blood Flow. Metab. 24, 475–486.
Oliveira, G.T., Da Silva, R.S.M., 1997. Gluconeogenesis in hepatopancreas of *Chasmagnathus granulata* crabs maintained on high-protein or carbohydrate-rich diets. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 118, 1429–1435.
Phillips, J.W., Mckinney, R.J.W., Hird, F.J.R., Macmillan, D.L., 1977. Lactic acid formation in crustaceans and the liver function of the midgut gland questioned. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 56B, 427–433.
Ponce-Palafox, J., Martinez-Palacios, C.A., Ross, L.G., 1997. The effects of salinity and temperature on the growth and survival rates of juvenile white shrimp. Penaeus vannamei, Boone, 1931 157, 107–115 (Elsevier).
Racotta, I.S., Palacios, E., Méndez, L., 2002. Metabolic responses to short and long-term exposure to hypoxia in white shrimp (*Penaeus vannamei*). Mar. Freshw. Behav. Physiol. 35, 269–275.
Reiber, C.L., McMahon, B.R., 1998. The effects of progressive hypoxia on the crustacean cardiovascular system: a comparison of the freshwater crayfish, (*Procambarus clarkii*), and the lobster (*Homarus americanus*). Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 168, 168–176.
Rosas, C., Martinez, E., Gaxiola, G., Brito, R., Sánchez, A., Soto, L.A., 1999. The effect of dissolved oxygen and salinity on oxygen consumption, ammonia excretion and osmotic pressure of *Penaeus setiferus* (Linnaeus) juveniles. J. Exp. Mar. Biol. Ecol. 234, 41–57.
Rosas, C., Cuzon, G., Gaxiola, G., Le Priol, Y., Pascual, C., Rossignyol, J., Contreras, F., Sanchez, A., Van Wormhoudt, A., 2001. Metabolism and growth of juveniles of *Litopenaeus vannamei*: effect of salinity and dietary carbohydrate levels. J. Exp. Mar. Biol. Ecol. 259, 1–22.
Sánchez-Paz, A., Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Muhlia-Almazan, A., Yepiz-Plascencia, G., 2008. Response of the phosphofructokinase and pyruvate kinase genes expressed in the midgut gland of the Pacific white shrimp *Litopenaeus vannamei* during short-term starvation. J. Exp. Mar. Biol. Ecol. 362, 79–89.
Schein, V., Wache, Y., Etges, R., Kucharski, L.C., van Wormhoudt, A., Da Silva, R.S., 2004. Effect of hyperosmotic shock on phosphoenolpyruvate carboxykinase gene expression and gluconeogenic activity in the crab muscle. FEBS Lett. 561, 202–206.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108.
Semenza, Gregg L., 2012. Hypoxia-inducible factors in physiology and medicine. Cell 148, 399–408.
Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Gomez-Jimenez, S., Lopez-Zavala, A., Forman, H.J., Yepiz-Plascencia, G., 2009. Molecular characterization of hypoxia inducible factor-1 (HIF-1) from the white shrimp *Litopenaeus vannamei* and tissue-specific expression under hypoxia. Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol. 150, 395–405.
Sonanez-Organis, J.G., Peregrino-Uriarte, A.B., Sotelo-Mundo, R.R., Forman, H.J., Yepiz-Plascencia, G., 2011. Hexokinase from the white shrimp *Litopenaeus vannamei*: cDNA sequence, structural protein model and regulation via HIF-1 in response to hypoxia. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 158, 242–249.
Soñanez-Organis, J.G., Rodriguez-Armenta, M., Leal-Rubio, B., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Yepiz-Plascencia, G., 2012. Alternative splicing generates two lactate dehydrogenase subunits differentially expressed during hypoxia via HIF-1 in the shrimp. Litopenaeus Vannamei. Biochim. 94, 1250–1260.
Stec, B., Abraham, R., Giroux, E., Kantrowitz, E.R., 1996. Crystal structures of the active site mutant (Arg-243 → Ala) in the T and R allosteric states pig kidney fructose-1,6-bisphosphatase expressed *Escherichia coli*. Protein Sci. 5, 1541–1553.
Stec, B., Yang, H., Johnson, K.A., Chen, L., Roberts, M.F., 2000. MJ0109 is an enzyme that is both an inositol monophosphatase and the 'missing' archaeal fructose-1,6-bisphosphatase. Nat. Struct. Mol. Biol. 7, 1046–1050.
Sun, S., Xuan, F., Ge, X., Fu, H., Zhu, J., Zhang, S., 2014. Identification of differentially expressed genes in hepatopancreas of oriental river prawn, *Macrobrachium nipponense* exposed to environmental hypoxia. Gene 534, 298–306.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular Evolutionary Genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Thabrew, M.I., Poat, P.C., Munday, K.A., 1971. Carbohydrate metabolism in *Carcinus maenas* gill tissue. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 40, 531–541.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Tillmann, H., Bernhard, D., Eschrich, K., 2002. Fructose-1,6-bisphosphatase genes in animals. Gene 291, 57–66.
Vaquer-Sunyer, R., Duarte, C.M., 2008. Thresholds of hypoxia for marine biodiversity. Proc. Natl. Acad. Sci. U. S. A. 105, 15452–15457.
Voehringer, D.W., Hirschberg, D.L., Xiao, J., Lu, Q., Roederer, M., Lock, C.B., Herzenberg, L.A., Steinman, L., 2000. Gene microarray identification of redox and mitochondrial elements that control resistance or sensitivity to apoptosis. Proc. Natl. Acad. Sci. U. S. A. 97, 2680–2685.
Wu, R.S.S., 2002. Hypoxia: from molecular responses to ecosystem responses. Mar. Pollut. Bull. 45, 35–45.
Yepiz-Plascencia, G., Gollas-Galvan, T., Vargas-Albores, F., Garcia-Bañuelos, M., 2000. Synthesis of hemolymph high-Density Lipoprotein beta-Glucan binding protein by *Penaeus vannamei* shrimp hepatopancreas. Mar. Biotechnol. 2, 485–492.
Zhang, Y., Liang, J.-Y., Huang, S., Lipscomb, W.N., 1994. Toward a mechanism for the allosteric transition of Pig Kidney fructose-1,6-bisphosphatase. J. Mol. Biol. 244, 609–624.
Zhang, J., Cowie, G., Naqvi, S.W.A., 2013. Hypoxia in the changing marine environment. Environ. Res. Lett. 8, 015025.

CAPÍTULO II

Role of HIF-1 on phosphofructokinase and fructose 1, 6-bisphosphatase expression during hypoxia in the white shrimp *Litopenaeus vannamei*.

Cota-Ruiz, K., Leyva-Carrillo, L., Peregrino-Uriarte, A.B., Valenzuela-Soto, E.M., Gollas-Galván, T., Gómez-Jiménez, S., Hernández, J., Yepiz-Plascencia, G.

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ABSTRACT

HIF-1 is a transcription factor that controls a widespread range of genes in metazoan organisms in response to hypoxia and is composed of α and β subunits. In shrimp, phosphofructokinase (PFK) and fructose bisphosphatase (FBP) are up-regulated in hypoxia. We hypothesized that HIF-1 is involved in the regulation of PFK and FBP genes in shrimp hepatopancreas under hypoxia. Long double stranded RNA (dsRNA) intramuscular injection was utilized to silence simultaneously both HIF-1 subunits, and then, we measured the relative expression of PFK and FBP, as well as their corresponding enzymatic activities in hypoxic shrimp hepatopancreas. The results indicated that HIF-1 participates in the up-regulation of PFK transcripts under short-term hypoxia since the induction caused by hypoxia (~1.6 and ~4.2-fold after 3 and 48 h, respectively) is significantly reduced in the dsRNA animals treated. Moreover, PFK activity was significantly ~2.8-fold augmented after 3 h in hypoxia alongside to an ~1.9-fold increment in lactate. However, when animals were dsRNA treated, both were significantly reduced. On the other hand, FBP transcripts were ~5.3-fold up-regulated in long-term hypoxic conditions (48 h). HIF-1 is involved in this process since FBP transcripts were not induced by hypoxia when HIF-1 was silenced. Conversely, the FBP activity was not affected by hypoxia, which suggests its possible regulation at post-translational level. Taken together, these results position HIF-1 as a prime transcription factor in coordinating glucose metabolism through the PFK and FBP genes among others, in shrimp under low oxygen environments.

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1. Introduction

Oxygen is essential to maintain many forms of life on earth. Many marine animals are exposed to low oxygen concentration (hypoxia) or to a complete absence of the gas (anoxia). Hypoxia, even for brief periods, can be detrimental or fatal to humans and most mammals and birds. However, many species of crustaceans, fish, amphibians and reptiles, and some diving mammals are adapted to withstand hypoxia or anoxia environments for hours or even for months (Hermes-Lima and Zenteno-Savín, 2002). Particularly, the shrimp *Litopenaeus vannamei* suffer fluctuations of oxygen levels and experience hypoxia (Parrilla-Taylor and Zenteno-Savín, 2011) and under these circumstances, the shrimp uses anaerobic metabolism to produce energy (Soñanez-Organis et al., 2012). It was recently shown that the expression of glycolytic phosphofructokinase (PFK) and gluconeogenic fructose bisphosphatase (FBP) is induced in shrimp hepatopancreas during hypoxia (Cota-Ruiz et al., 2015). Both PFK and FBP enzymes are key regulators of their respective pathways (Al Hasawi et al., 2014; Yánez et al., 2003) and catalyze the non-reciprocal inter-conversion between fructose 6-phosphate and fructose 1,6-bisphosphate. PFK induction supports the hypothesis of acceleration of the rate of glycolysis in hypoxic hepatopancreatic shrimp cells, a fact that has been amply documented in mammalian corresponding systems (Semenza, 2012), while the increase of FBP transcripts could lead to additional glucose and other derived metabolites (*i.e.* glutathione, NADPH) production (Liang et al., 2013).

In mammals, several genes for enzymes of the glycolysis (Semenza, 2000) and gluconeogenesis pathways (Choi et al., 2005) are up-regulated by the Hypoxia Inducible Factor (HIF-1) under low oxygen. In these organisms, HIF-1 is involved in the control of more than 100 genes (Kaelin and Ratcliffe, 2008; Semenza, 2003). HIF is a transcription factor considered a master regulator of oxygen homeostasis in all metazoan species (Semenza, 2012). HIF-1 binds a core sequence of the HRE (Hypoxia Responsive Element) in the promoters of hypoxia-regulated genes and regulates their expression (Lee et al., 2004). Through the metazoan species, from nematodes, insects and crustaceans to mammals, the functional complex is a heterodimer composed of an α and a β subunit (also known as aryl hydrocarbon receptor nuclear translocator or ARNT). HIF proteins are members of the family of basic helix–loop-helix/PAS (Per/ARNT/Sim) transcription factors (Gorr et al., 2004), recognizing the sequence RCGTG (Wang et al., 2015).

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On the other hand, since the report of the ability to silence genes using injection of double stranded RNA (dsRNA) in crustaceans (Lugo et al., 2006), this approach, also named interference RNA (RNAi), has been gaining more attention for its relevance to study gene function in these groups of organisms (García-Triana et al., 2010; Manfrin et al., 2015; Martínez-Quintana et al., 2016; Soñanez-Organis et al., 2010), where there are no mutants obtained by classical genetics available. RNAi is a highly specific mechanism for post-transcriptional silencing of the corresponding mRNA enabling transient knock-down of specific gene expression, with the advantage to avoid the prerequisite to genetically modify the organisms (Sagi et al., 2013). Here, we hypothesized that HIF-1 participates in PFK and FBP gene regulation in hepatopancreas of hypoxic shrimps. Hence, we used long dsRNA intramuscular injection to silence HIF-1 and to assess if HIF-1 participates in the expression of PFK and FBP.

2. Materials and methods

2.1. Hypoxia and silencing bioassay

L. vannamei sub-adult intermolt shrimps (21.6 g ± 1.5) were acclimated for 1 week in six 300 L tanks at 27.7 ± 0.6 °C containing sea water with a salinity of 35 ppt. Water was constantly aerated and recirculated. Shrimps were fed *ad libitum* twice per day with shrimp Natural Force® from VIMIFOS™ and at least once a day uneaten food and excretes were removed. 24 h before starting the hypoxia challenge, all shrimps (normoxic and hypoxic groups) were injected with dsRNA of HIF-1 α and β dissolved in saline solution (SS, 0.9% NaCl) or with SS. Subsequently, the shrimps were returned to the corresponding tanks for 24 h maintaining the acclimation conditions. Two sampling times during hypoxia exposure at 1.57 ± 0.2 mg of dissolved oxygen (DO) per liter were included: 3 and 48 h. Time lapsed normoxic controls were included for each condition. To provoke hypoxia, water recirculation was stopped and nitrogen gas was bubbled to the container. DO was measured using an oxymeter (YSI model 55). After 3 h of hypoxia, four tanks were sampled: 15 normoxic shrimps SS injected, 15 normoxic shrimps injected with dsRNA, 15 hypoxic shrimp SS injected and 15 shrimps dsRNA injected. Finally, after 48 h of hypoxia, the last four groups of animals were sampled similarly as for the 3 h exposure to hypoxia. A portion of 100 mg of hepatopancreas was placed in a microtube containing 1 mL of TRI REAGENT® (Sigma-Aldrich) for RNA extraction and expression analysis, and the rest of the hepatopancreas (approximately 400 mg) was used to determine lactate concentration and enzymatic activity. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until used.

2.2. HIF-1 knockdown

dsRNA production was done as reported by (Soñanez-Organis et al., 2010) with some modifications. First, PCR products for α (580 bp) and for β (625 bp) were generated. The 580 bp fragment of α corresponds to the positons 243–822 of the coding sequence (GenBank accession number FJ807918) and includes ~50% of the bHLH, PAS-A and PAS-B domains, but has less than 70% identity at the nucleotide level with these type of domains that are known in other shrimp genes. For β, the fragment corresponds to positions 152–776 (GenBank accession number FJ807919), also in the coding region, and covers the bHLH domain (47%), the whole PAS-A domain and does not cover the PAS-B domain; the maximum identity found with other proteins containing these domains was less than 70% at the nucleotide level, therefore; the fragments are specific. The PCR products (α and β) were cloned into the pGEM®-T Easy Vector System (Promega) in both directions and used as templates to generate single stranded RNA (ssRNA). The T7 RNA polymerase (RiboMAX™ Large Scale RNA Production System, Promega) was employed to generate a ssRNA of 580 nt for α and a ssRNA of 625 nt for β, (both in sense and anti-sense direction). Quantification was done in a NanoDrop 2000c Spectrophotometer (Thermo Scientific). dsRNA was obtained by annealing the corresponding complementary ssRNAs at 85–90 °C. After incubation for 10 min, the samples were slowly cooled to 29 °C (~2 h at room temperature). Confirmation of dsRNA hybrid formation was done in agarose gels by the differences in migration compared to the ssRNA. dsRNA to silence α and β transcripts were generated. Equivalent amount (~20 μg of RNA) of both were mixed and used simultaneously to silence the HIF-1 transcripts.

2.3. RNA extraction and RT-qPCR analysis

Total RNA was independently extracted from each shrimp hepatopancreas in TRI-Reagent. RNA quantification was done using a NanoDrop 2000c at 260 nm, and the integrity analyzed in 1% agarose gels. To eliminate possible DNA contamination, the samples were treated with DNase I (Roche) at 37 °C for 20 min. Genomic contamination analysis was done as previously reported (Cota-Ruiz et al., 2015). RNA was reverse transcribed in sample duplicates using the Quantitect Reverse Transcription Kit (Qiagen) to get a final cDNA derived from 25 ng/uL of total RNA. qPCRs of five genes PFK, FBP, ribosomal protein L8 (L8) for normalization of expression, HIF-1α and HIF-1β were done in a CFX96™ Real-Time System C 1000 Touch ™ Thermal Cycler. Transcript detection for PFK, FBP and L8 was done exactly as reported before (Cota-Ruiz et al., 2015), except that the final melting curve program was done in the same manner as for HIF-1 (see below). HIF-1 amplifications were prepared in duplicates per each cDNA in a 20 μL final volume containing 10 μL of iQ SYBR Green Supermix (Bio-Rad), 8 μL of nuclease-free water, 0.5 μL of each primer (20 μM) and 1 μL of cDNA (derived from 25 ng of total RNA). HIF-1α was amplified with the primers HIF rtF (5′-GGAG AGCGAGATCTTCACG-3′) and HIFrtR (5′-GCCTCCTTCCGTGATCTTC-3′), giving a product of 157 bp. HIF-1 β was amplified with primers ARNTF (5′-CAAGAGCCAGCCAACCAAG-3′) and ARNTR (5′-GGAATTTCTCTGAC GCAGC-3′), the product generated was of 189 bp. For both HIF-1 amplifications, PCR conditions were as follows: 95 °C for 5 min, 40 cycles at: 95 °C for 30 s, 60 °C for 35 s, and 72 °C for 55 s, with a single fluorescence measurement at the extension step and a final melting curve program with 0.5 °C increments each 5 s from 60 °C to 94.5 °C. Efficiency of amplifications for HIF-1 was determined with standard curves ranging from 2.5×10^{1} to 2.5×10^{-2} of cDNA as template.

2.4. Enzymatic activities and lactate determination

PFK and FBP activities and lactate determination were performed in duplicates from 3 individually prepared samples of each hepatopancreas-treatment. Cellular soluble material was obtained by rapidly homogenizing approximately 20 mg of tissue in 200 μL of ice-cold PFK Assay Buffer (see below). The homogenates were centrifuged at 13,000 ×g for 10 min and the supernatant was used for further measurements. Protein concentration of each sample was used to calculate the specific enzymatic activity and to normalize the concentration of lactate. Protein was determined by the Bradford method (Bradford, 1976).

2.4.1. PFK activity

Fructose-6-phosphate and ATP are converted to fructose 1,6-bisphosphate and ADP by PFK. PFK activity was measured using the Phosphofructokinase (PFK) Activity Colorimetric Assay Kit (Sigma-Aldrich®, catalog number MAK093). It is based on a coupled enzyme assay where the ADP produced by PFK is used as substrate by the enzyme mix to produce AMP and NADH. This later compound reduces a probe and generates a colorimetric product that was continuously monitored at 450 nm for 35 min. The assay was adapted to a final microplate volume of 100 μL containing 50 μL of the sample extract and 50 μL of the appropriate reaction mix. NADH formation was determined by comparison to the standard curve. One unit of PFK is defined as the amount of enzyme that generates 1.0 μmol of NADH per minute at 37 °C and pH 7.4.

Measurements were done in a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific).

2.4.2. FBP activity

FBP catalyzes the production of fructose-6-phosphate and Pi from fructose 1,6-bisphosphate. FBP activity was measured according to Reyes et al. (1987) with some modifications. The assay is based on a coupled enzymatic assay where fructose-6-phosphate produced directly by FBP is converted to glucose-6-phosphate by phosphoglucose isomerase. Then, glucose-6-phosphate dehydrogenase oxidizes this metabolite using NADP as a co-substrate. The rate of NADPH formation at 30 °C is monitored at 340 nm in the presence of an excess of both, glucose-6-phosphate dehydrogenase and phosphoglucose isomerase. The assay was adapted to a final volume of 100 μL. Reaction mixture contained 50 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 5 mM $MgSO_4$, 50 μM fructose-1,6-P_2, 0.3 mM $NADP^+$, 1.2 and 1.3 units of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase, respectively. The reaction was started after adding 20 μL of cellular soluble protein extract. The absorbance change ratio in the linear range and the NADPH absorbance coefficient value of $6.3 \times 10^{-3} L \cdot mol^{-1} \cdot cm^{-1}$ were used to determine the enzyme activity. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate per min.

2.4.3. Lactate determination

Lactate concentration in hepatopancreas was determined with the RANDOX lactate kit that uses lactate oxidase to convert lactate to pyruvate and peroxide. The amount of peroxide produced is measured using peroxidase and following the formation of a purple product that is detected at 546 nm. Cellular soluble extracts obtained for each individual shrimp were analyzed. Volumes were scaled down for microplate (final reaction volume of 202 μL) and readings were done after 10 min of reaction time at 25 °C in a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific).

2.5. Statistical analysis

Two way ANOVA was used to evaluate the effect of hypoxia and silencing of HIF-1 on the transcripts levels, lactate and enzymatic activities. Tukey *post hoc* test was done to determine differences in means at $p < 0.05$. Shapiro–Wilk test to determine normality was used. Box-cox data transformation was applied when necessary. The analyses were done using Minitab 17 software (State College, Pennsylvania).

3. Results

3.1. HIF-1 silencing

Comparison of the transcripts levels of HIF-1α and HIF-1β in shrimp injected with the dsRNA and 24 h later exposed to hypoxia revealed that the silencing was effective. The shrimps that were silencing and had 3 h of hypoxia presented 80 and 60% of the transcript levels for α and β, respectively. Similarly, in the silenced shrimps that were exposed to 48 h of hypoxia, 82% of the original transcript levels were detected for HIF-1α, while no statistical significant effect was detected for HIF-1β transcripts at this hypoxic time. In their respective normoxic groups where silenced shrimps were sampled after 3 h, HIF-1β transcripts were only 34% of the non-silenced animals and no change was found for HIF-1α. Finally, in the normoxic control groups sampled after 48 h, no silencing effect was detected for HIF-1β (Table 1).

3.2. PFK transcripts are induced in hypoxia via HIF-1

We evaluated the effect of silencing HIF-1 on PFK expression in normoxic and hypoxic shrimps. The results showed a significant difference, indicating that PFK expression under hypoxia depends on whether HIF-1 was silenced or not. PFK expression was higher by ~1.6-fold after 3 h of hypoxia compared to its corresponding normoxic group (Fig. 1A). In contrast, when shrimps were injected with dsRNA for HIF-1, PFK expression was lower after 3 h of hypoxia. Moreover, after 48 h of hypoxia, PFK transcripts were ~4.2-fold higher compared to the normoxic control; this hypoxia-effect was reversed in RNAi treated shrimps compared to the corresponding non-silenced shrimps (Fig. 1B).

3.3. PFK enzymatic activity is up-regulated in short-term but not in long-term hypoxia

To assess the effect of hypoxia along with the effect of silencing of HIF-1 on PFK activity, we evaluated the enzymatic activity of PFK in hypoxic and normoxic shrimp dsRNA treated and untreated. There was a significant interaction between hypoxia and dsRNA treatment on the PFK activity. PFK activity was ~2.8-fold higher after 3 h of hypoxia. However, when HIF-1 was silenced, hypoxia exposure did not cause a change on PFK activity, showing that the effect of hypoxia on PFK expression is HIF-1 dependent (Fig. 2A). Contrarily, 48 h of hypoxia did not result in changes in enzymatic activity neither in SS hypoxic group nor in the silenced hypoxic group. No significant interaction was found in the two-way ANOVA analysis (Fig. 2B).

Table 1
HIF-1 normalized expression after 3 and 48 h of hypoxia or normoxia in dsRNA shrimp treated and untreated.

Hypoxic group			Normoxic group		
HIF-1 subunit	Relative expression HIF-1/L8 $\times 10^{-3}$[a]	Silencing percentage	HIF-1 subunit	Relative expression HIF-1/L8 $\times 10^{-3}$[a]	Silencing percentage
Alpha 3 h silenced	0.28 ± 8.5%	80	Alpha 3 h silenced	0.47 ± 25%	–
Alpha 3 h control	1.41 ± 12.9%		Alpha 3 h control	0.44 ± 38%	
Alpha 48 h silenced	0.04 ± 21%	82	Alpha 48 h silenced	0.08 ± 9%	–
Alpha 48 h control	0.22 ± 3.9%		Alpha 48 h control	0.09 ± 8%	
Beta 3 h silenced	0.25 ± 9.7%	60	Beta 3 h silenced	0.58 ± 22%	34
Beta 3 h control	0.62 ± 3.9%		Beta 3 h control	0.88 ± 21%	
Beta 48 h silenced	0.2 ± 6.1%	–	Beta 48 h silenced	0.22 ± 32%	–
Beta 48 h control	0.25 ± 21%		Beta 48 h control	0.22 ± 22%	

[a] Data represent the mean of 5 shrimp measurements and variation coefficient in percentage. Silencing percentage was expressed only when the silencing effect was significant.

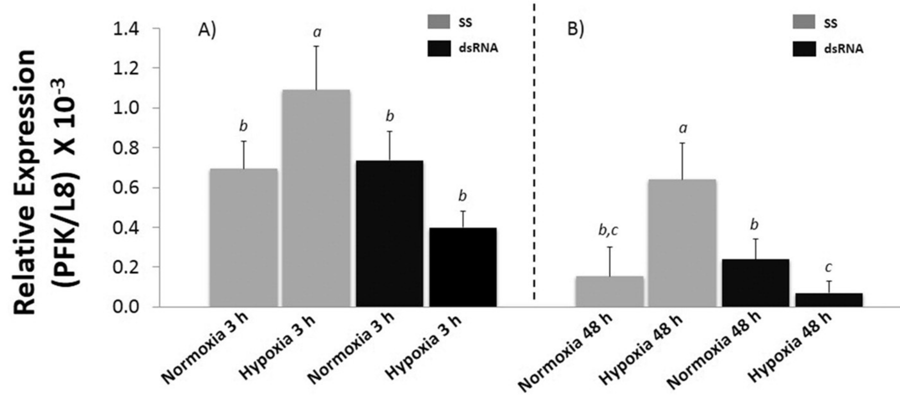

Fig. 1. Effect of hypoxia and HIF-1 silencing on the expression of PFK. Transcript levels were determined in shrimp that were injected with saline solution (SS) or dsRNA after A) 3 h or B) 48 h of hypoxia. Two qPCR for each of the two cDNA per organism were done and normalized to ribosomal protein L8. Bars indicate mean of three individual shrimps ± SD. Different letters indicate significant differences at $p < 0.05$.

3.4. FBP transcripts are induced in long-term hypoxia in a HIF-1 dependent manner

Hypoxia for 3 h did not result in a significant effect in FBP transcript levels in animals injected with SS. Contrarily, in the silenced shrimps, a significant reduction in FBP transcripts was presented after 3 h of hypoxia (Fig. 3A). On the other hand, we found a significant ~5.3-fold induction of FBP transcripts in hepatopancreas of the shrimps subjected to 48 h hypoxia. Moreover, a significant interaction between the oxygen conditions (hypoxia/normoxia) and silencing treatments was detected. Essentially, the induction caused by 48 h of hypoxia is dependent on HIF-1; when HIF-1 was silenced, the hypoxic effect on FBP transcripts was abolished (Fig. 3B).

3.5. FBP enzymatic activity is not induced in hypoxia

We found no significant interaction between the oxygen conditions (hypoxia/normoxia) and the silencing treatment on FBP enzymatic activity after 3 h of hypoxia (Fig. 4A). Although there is a 2.14-fold higher FBP enzymatic activity after 3 h of hypoxia compared to its corresponding normoxic group, it is not statistically significant, due to the high variability among the individual shrimps. Additionally, FBP activity was not affected after 48 h of hypoxia. In fact, no statistical differences were detected in each group (Fig. 4B).

3.6. Lactate is accumulated at early lapse-time hypoxia regime

Lactate accumulation is significantly induced after 3 h of hypoxia by 1.91-fold compared to the normoxic group. This induction did not occur in the hypoxic shrimp hepatopancreas that were dsRNA treated (Fig. 5A). These results clearly indicate that lactate accumulation after 3 h of hypoxia is HIF-1 dependent. Contrarily, no significant effect of hypoxia-silencing interaction was observed after 48 h of hypoxia. In fact, lactate concentrations in hepatopancreas from normoxic/hypoxic and dsRNA treated/untreated shrimp at 48 h are not significantly different (Fig. 5B).

4. Discussion

4.1. HIF-1 silencing

HIF-1α and HIF-1β transcripts were detected in very low relative amounts in shrimp hepatopancreas, as previously reported in this same shrimp species (Soñanez-Organis et al., 2009). A particular pattern of HIF-1α expression levels in hypoxia was observed: the transcripts after 3 h of hypoxia were 6.4-fold higher than at 48 h hypoxia. In other words, short-term hypoxia caused a notorious increment in HIF-1α transcripts while in long-term hypoxia, the transcripts levels tended to decrease (Table 1). The immediate transitory rise in messages of α is probably due to the necessity of more mRNAs while the α protein was

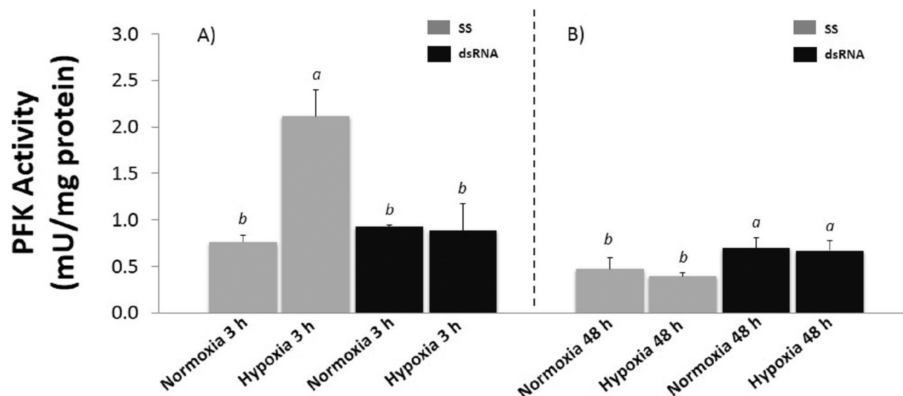

Fig. 2. PFK specific activity in shrimp injected with SS or dsRNA under normoxic or hypoxic conditions after A) 3 h or B) 48 h of hypoxia. Bars indicate mean of 3 independent shrimps per treatment ± SD. Means with different letters are significantly different.

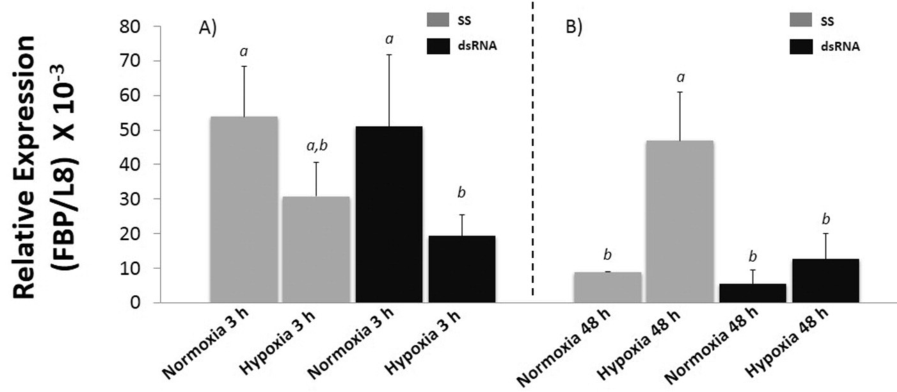

Fig. 3. Effect of silencing HIF-1 and hypoxia on FBP relative expression. The expression of FBP (normalized to L8) was evaluated under hypoxic and normoxic conditions in shrimp treated with SS or dsRNA after A) 3 h and B) 48 h of hypoxia. Different lower case letters indicate that mean ± SD in comparison are significantly different at $p < 0.05$ to n = 5.

still being marked for degradation, as occurs in oxygen presence; likely a time window is required for the accumulation of the α protein and formation of the HIF-1 complex, followed by the induction of the genes involved in the adaptive responses (Giannetto et al., 2015).

It is known that the efficiency of dsRNA to silence target transcripts depends, among others, on the abundance of the target transcripts (Fire et al., 1998). In this sense, dsRNA against "low" abundance target transcripts, as occurs for transcription factors, can be at some degree ineffective (Martínez-Quintana et al., 2016). This is particularly interesting since the HIF-1 transcript levels are quite low in shrimp hepatopancreas. However, it does not seem to be a determinant factor in HIF-1α silencing since transcripts from hypoxic dsRNA treated shrimp were similarly silenced even when the level of the transcripts varied in at least 6-fold through the two hypoxic terms (3 and 48 h). Moreover, no HIF-1α silencing effect was detected in shrimps exposed to any normoxic condition, even when the relative transcript amounts are comparable among hypoxic conditions. These results might be related to HIF-1α stabilization (at the protein level) under hypoxic condition. We found that HIF-1β expression in shrimp is not significantly affected by oxygen availability. In metazoan species HIF-1β is expressed constitutively (Gorr et al., 2010). Here, we were able to silence HIF-1β at short-term for both normoxic and hypoxic conditions. Contrarily, dsRNA to silence HIF-1β had no effect in long-term animals treated.

4.2. PFK induction by hypoxia: the role of HIF-1

When oxygen is poorly available to cells, oxidative phosphorylation in mitochondria becomes limiting and the cells must rely on anaerobic ATP production (Fago and Jensen, 2015). PFK is a critical rate limiting enzyme in glycolysis. In higher organisms as well as in cancer cells, PFK is up-regulated in hypoxia. Here, in the white shrimp *L. vannamei*, we have confirmed our last observation on PFK transcript induction under hypoxic conditions (Cota-Ruiz et al., 2015). Interestingly, herein we show that HIF-1 is involved in this regulation. Furthermore, we also detected that after 3 h of hypoxia, there is a significant increase in enzymatic activity and this behavior depends on HIF-1, since its silencing reduces this activity. Similar results were found for hexokinase activity in this same shrimp species; a tissue specific induction of the enzymatic activity is reduced when HIF-1 was silenced (Soñanez-Organis et al., 2011). Another important element about HIF-1 on PFK activity induction is that the rapid increase of HIF-1α transcripts in hypoxic conditions (Table 1), very likely results in active HIF-1, and thus, more PFK transcripts that ultimately lead to higher PFK activity. This is in agreement to studies in cell lines where the HIF-1α protein is rapidly degraded (<5 min half-life when oxygenated) or stabilized (by its immediate accumulation in hypoxic conditions) (Huang et al., 1998; Jewell et al., 2001).

The higher PFK activity after 3 h of hypoxia, is very important since PFK is a main element in regulation of the glycolytic flux (Banaszak et al., 2011; Sharma, 2011), indicating that ATP production is accelerating to meet the energy needs of the cell. It is evident that PFK is prime to sense the "energy level" in shrimp. Also, PFK as an allosteric enzyme is tightly subjected to metabolite concentrations, hence, more studies focused to evaluate the role of metabolites such as ATP, citrate or fructose 2,6-bisphosphate (F 2,6-P), as well as to determine the expression of PFK-2 (whose product is F 2,6-P) will provide significant elements to understand how PFK is operating and controlling this vital pathway during hypoxia.

Additionally, we detected lactate accumulation after 3 h of hypoxia. As in other animals, lactate accumulates in different crustacean tissues

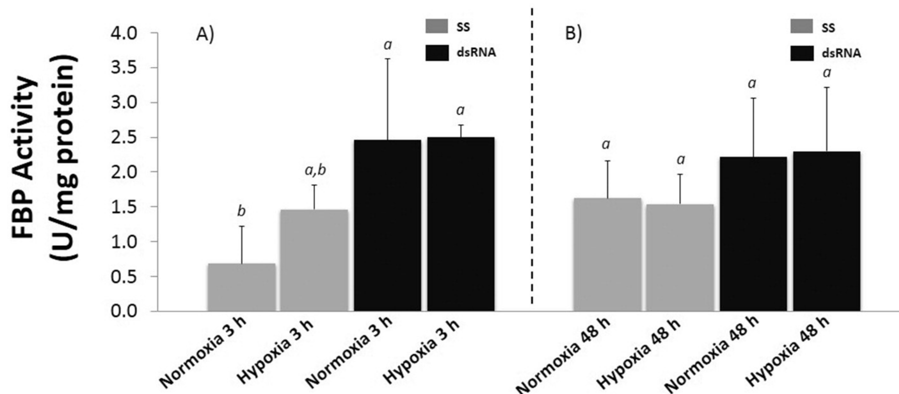

Fig. 4. Specific activity of FBP in hepatopancreas under hypoxia and HIF-1 silencing after A) 3 h and B) 48 h of hypoxia. Three different animals by duplicates for each condition were evaluated and the results are represented as mean values ± SD. Different letters indicate significant differences between means at $p < 0.05$.

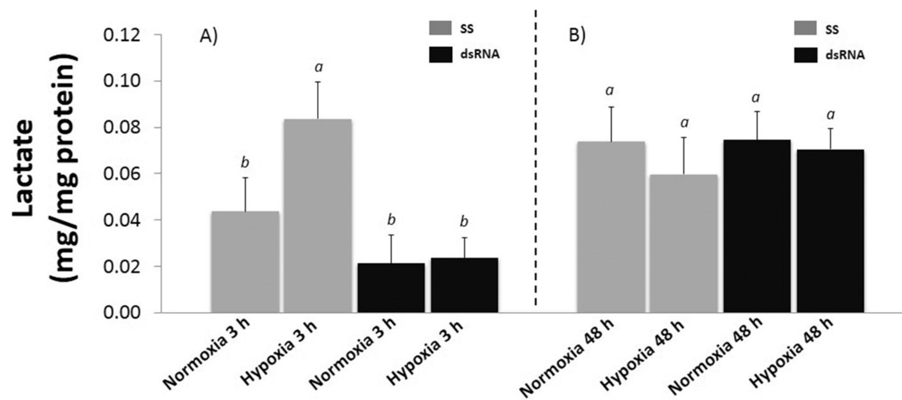

Fig. 5. Lactate concentration in shrimp hepatopancreas SS or dsRNA treated under normoxic and hypoxic conditions. Lactate content (mg/mg protein) was measured after A) 3 h and B) 48 h of hypoxia. Means ± SD represent the values for three independent shrimp samples per treatment and significant differences are indicated by different letters at $p < 0.05$.

as a consequence of anaerobic metabolism (Oliveira et al., 2004). Interestingly, after 3 h of hypoxia, lactate accumulation did not occur in hepatopancreas of shrimp treated with dsRNA (Fig. 5), suggesting that HIF-1 plays a key role on glycolysis regulation under these circumstances. A previous report in this species is in line with our current findings since the induced expression of lactate dehydrogenase (LDH) caused an increase in lactate production; however, when HIF-1 was silenced, lactate, LDH mRNA and activity were down-regulated in hypoxic shrimp (Soñanez-Organis et al., 2010; Soñanez-Organis et al., 2012). Our results provide elements to confirm that the Pasteur Effect is occurring in hepatopancreas cells from shrimp, since an opposite relation between glycolytic flux (which is incremented) and low oxygen available (hypoxia) is presented along with a simultaneous generation of ATP. Additionally, we do know, although not to what extent, that HIF-1 controls this process. Nevertheless, cumulative amounts of lactate as an end-product are generated (Jackson et al., 2001; Nilsson and Lutz, 2004).

Although we found that HIF-1 is involved in PFK transcripts up-regulation in long-term hypoxic conditions, we did not detect a significantly higher activity after 48 h of hypoxia. This finding is somewhat surprising; however, we cannot exclude the possibility that a fine tune regulation at protein level could occur. By studies in mammalian systems, it has been shown that activity level of glycolytic enzymes such as PFK, is controlled by hormones and metabolites (*i.e.* ammonium and citrate) (Minchenko et al., 2003). Additionally, accumulation of protons under anaerobic conditions reversibly inhibits PFK activity (Robin et al., 1984) whose activity can be recuperated after lactate clearance.

4.3. FBP up-regulation in long-term hypoxia and the involvement of HIF-1

Taking into consideration our previous report on FBP induction in long term hypoxic conditions (48 h) (Cota-Ruiz et al., 2015), we were encouraged to see if HIF-1 was involved in FBP up-regulation. We found that HIF-1 participates in its expression since the FBP induction under 48 h of hypoxic conditions is significantly reduced when HIF-1 was silenced (Fig. 3B). Despite reports showing the induction of gluconeogenic genes in fish (Gracey et al., 2001), mollusks (Le Moullac et al., 2007) and crustaceans (Brown-Peterson et al., 2008) by hypoxia, to our knowledge, this is the first report in invertebrate marine animals that demonstrates the direct involvement of HIF-1 in a gluconeogenic gene regulation. In mammalian cells, HIF-1 can bind to the specific phosphoenol pyruvate kinase (PEPCK) gene promoter to control its expression (Choi et al., 2005). In our study, at this hypoxia time, lactate concentration was not higher with respect to the corresponding control (Fig. 5). In fact, lactate accumulation is indicative of anaerobic metabolism activation (Soñanez-Organis et al., 2010). Thus, a lack in lactate accumulation suggests its further conversion or excretion. For instance, in crustaceans it has been suggested that clearance of lactate could be possible by complete oxidation, conversion back into storage products such as glycogen, or excretion. A previous report on crustaceans shows that lactate is metabolized to glucose in anoxic conditions, indicating the glucogenogenic capability of hepatopancreas (Oliveira et al., 2004). We must consider that the importance of the gluconeogenic pathway activation resides not only in glucose or the reducing equivalents' (through pentose phosphates pathway) production, but in maintaining the acid–base balance. These elements, along the fact that FBP transcripts were up-regulated at this point, prompt us to measure FBP at activity level. Our results showed no significant effect of the hypoxia on FBP activity (Fig. 4). However, it is worthwhile to mention that FBP was not down regulated, as occurs in many other processes under low oxygen condition (Hochachka and Somero, 2002), but then remains unaffected. This means that somehow, this pathway is still active. Indeed, bearing in mind the importance of gluconeogenesis and the enzymes involved, other processes as post-translational modifications, allosteric effects of metabolites, and enzyme binding interactions with cellular structural elements may influence the overall gluconeogenic response to hypoxia (Lushchak et al., 1998). Furthermore, we cannot exclude the possibility that some hepatopancreas cell populations could operate in a “gluconeogenic fashion” (Cota-Ruiz et al., 2015), promoting the usage of the anaerobic end-product lactate or certain amino acids in the gluconeogenic pathway. Thus, studies committed to evaluate the expression *in situ* not just of the FBP but the other gluconeogenic important genes such as PEPCK, will contribute to obtain insights about how shrimp exploits glucose metabolism in response to hypoxia.

We also believe that the hypoxia time-frame we explored represents a single small “window” and perhaps longer time would give more evidence to test FBP increased activity. For instance, when fish were subjected to 4 weeks of hypoxic conditions, a higher FBP activity was found (Martínez et al., 2006). In this sense, and according to the “energetic logic” point of view, the accumulation of FBP transcripts we detected in shrimp hepatopancreas after 48 h hypoxia, probably would lead later to produce more FBP protein that will eventually take action.

5. Conclusions

Both PFK and FBP transcripts are up-regulated by hypoxia. This induction occurs in a HIF-1-dependent manner. Interestingly, PFK activity was higher when animals were subjected to short-term hypoxia but significantly lower in hypoxic HIF-1 silenced shrimp. These findings, along with lactate production, argue in favor that glycolysis is accelerated to produce ATP anaerobically in response to the energy requirements of the cells, where HIF-1 becomes a central molecule coordinating this process. Although FBP transcripts were up-regulated in long-term hypoxia in a HIF-1 dependent fashion, FBP activity was unaffected,

which suggests post-translational regulation; nevertheless, the results again show that HIF-1 has a relevant role also in the gluconeogenesis pathway. Immuno-histochemical studies focused to evaluate the expression *in situ* of PFK and FBP as well as the quantification of key metabolites involved in glucose metabolism will provide the basis to better understand the energy metabolism in shrimp during hypoxia. Moreover, further studies characterizing promoter regions as well as investigations oriented to evaluate functional properties of HIF-1 interacting with HRE of the target genes, will give deeper insights about specific details in shrimp species about HIF-1 function.

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References

Al Hasawi, N., Alkandari, M.F., Luqmani, Y.A., 2014. Phosphofructokinase: a mediator of glycolytic flux in cancer progression. Crit. Rev. Oncol. Hematol. 92, 312–321.

Banaszak, K., Mechin, I., Obmolova, G., Oldham, M., Chang, S.H., Ruiz, T., Radermacher, M., Kopperschläger, G., Rypniewski, W., 2011. The crystal structures of eukaryotic phosphofructokinases from baker's yeast and rabbit skeletal muscle. J. Mol. Biol. 407, 284–297.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brown-Peterson, N.J., Manning, C.S., Patel, V., Denslow, N.D., Brouwer, M., 2008. Effects of cyclic hypoxia on gene expression and reproduction in a grass shrimp, *Palaemonetes pugio*. Biol. Bull. 214, 6–16.

Choi, J.H., Park, M.J., Kim, K.W., Choi, Y.H., Park, S.H., An, W.G., Yang, U.S., Cheong, J., 2005. Molecular mechanism of hypoxia-mediated hepatic gluconeogenesis by transcriptional regulation. FEBS Lett. 579, 2795–2801.

Cota-Ruiz, K., Peregrino-Uriarte, A.B., Felix-Portillo, M., Martínez-Quintana, J.A., Yepiz-Plascencia, G., 2015. Expression of fructose 1, 6-bisphosphatase and phosphofructokinase is induced in hepatopancreas of the white shrimp *Litopenaeus vannamei* by hypoxia. Mar. Environ. Res. 106, 1–9.

Fago, A., Jensen, F.B., 2015. Hypoxia tolerance, nitric oxide, and nitrite: lessons from extreme animals. Physiology 30, 116–126.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

García-Triana, A., Gómez-Jiménez, S., Peregrino-Uriarte, A.B., López-Zavala, A., González-Aguilar, G., Sotelo-Mundo, R.R., Valenzuela-Soto, E.M., Yepiz-Plascencia, G., 2010. Expression and silencing of Selenoprotein M (SelM) from the white shrimp *Litopenaeus vannamei*: effect on peroxidase activity and hydrogen peroxide concentration in gills and hepatopancreas. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 155, 200–204.

Giannetto, A., Maisano, M., Cappello, T., Oliva, S., Parrino, V., Natalotto, A., De Marco, G., Barberi, C., Romeo, O., Mauceri, A., Fasulo, S., 2015. Hypoxia-inducible factor α and hif-prolyl hydroxylase characterization and gene expression in short-time air- exposed *Mytilus galloprovincialis*. Mar. Biotechnol. 17, 768–781.

Gorr, T.A., Cahn, J.D., Yamagata, H., Bunn, H.F., 2004. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor- dependent. J. Biol. Chem. 279, 36038–36047.

Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N.B., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83 (5), 733–752.

Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. Proc. Natl. Acad. Sci. U. S. A. 98, 1993–1998.

Hermes-Lima, M., Zenteno-Savín, T., 2002. Animal reponse to drastic changes in oxygen availability and physiological oxidative stress. Comp. Biochem. Physiol. C 133, 537–556.

Hochachka, P.W., Somero, G.N., 2002. Biochemical adaptation: mechanism and process in physiological evolution. Biochem. Mol. Biol. Educ. 30, 215–216.

Huang, L.E., Gu, J., Schau, M., Bunn, H.F., 1998. Regulation of hypoxia-inducible factor 1α is mediated by n O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. U. S. A. 95, 7987–7992.

Jackson, D.C., Wang, T., Koldkjaer, P., Taylor, E.W., 2001. Lactate sequestration in the carapace of the crayfish *Austropotamobius pallipes* during exposure in air. J. Exp. Biol. 204, 941–946.

Jewell, U.R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R.H., Gassmann, M., 2001. Induction of HIF-1α in response to hypoxia is instantaneous. FASEB J. 15, 1312–1314.

Kaelin, W.G., Ratcliffe, P.J., 2008. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol. Cell 30, 393–402.

Le Moullac, G., Bacca, H., Huvet, A., Moal, J., Pouvreau, S., Van Wormhoudt, A., 2007. Transcriptional regulation of pyruvate kinase and phosphoenolpyruvate carboxykinase in the adductor muscle of the oyster *Crassostrea gigas* during prolonged hypoxia. J. Exp. Zool. A Ecol. Genet. Physiol. 307A, 371–382.

Lee, J.W., Bae, S.H., Jeong, J.W., Kim, S.H., Kim, K.W., 2004. Hypoxia-inducible factor (HIF-1)[alpha]: its protein stability and biological functions. Exp. Mol. Med. 36, 1–12.

Liang, P., Sun, J., Huang, Y., Zhang, F., Zhou, J., Hu, Y., Wang, X., Liang, C., Zheng, M., Xu, Y., Mao, Q., Hu, X., Li, X., Xu, J., Lu, G., Yu, X., 2013. Biochemical characterization and functional analysis of fructose-1,6-bisphosphatase from *Clonorchis sinensis*. Mol. Biol. Rep. 40, 4371–4382.

Lugo, J.M., Morera, Y., Rodriguez, T., Huberman, A., Ramos, L., Estrada, M.P., 2006. Molecular cloning and characterization of the crustacean hyperglycemic hormone cDNA from *Litopenaeus schmitti*. Functional analysis by double-stranded RNA interference technique. FEBS J. 273, 5669–5677.

Lushchak, V.I., Bahnjukova, T.V., Storey, K.B., 1998. Effect of hypoxia on the activity and binding of glycolytic and associated enzymes in sea scorpion tissues. Braz. J. Med. Biol. Res. 31, 1059–1067.

Manfrin, C., Peruzza, L., Bonzi, L., Pallavicini, A., Giulianini, P., 2015. Silencing two main isoforms of crustacean hyperglycemic hormone (CHH) induces compensatory expression of two CHH-like transcripts in the red swamp crayfish *Procambarus clarkii*. ISJ 12, 29–37.

Martínez-Quintana, J.A., Valenzuela-Soto, E.M., Martínez-Téllez, M.A., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Kikuta, S., Yepiz-Plascencia, G., 2016. Functionality of the white shrimp glucose transporter 1: expression in *Xenopus oocytes* and gene silencing during hypoxia. J. Exp. Mar. Biol. Ecol. 474, 109–115.

Martínez, M.L., Landry, C., Boehm, R., Manning, S., Cheek, A.O., Rees, B.B., 2006. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*. J. Exp. Biol. 209, 3851–3861.

Minchenko, O., Opentanova, I., Caro, J., 2003. Hypoxic regulation of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene family (PFKFB-1-4) expression in vivo. FEBS Lett. 554, 264–270.

Nilsson, G.E., Lutz, P.L., 2004. Anoxia tolerant brains. J. Cereb. Blood Flow Metab. 24, 475–486.

Oliveira, G.T., Eichler, P., Rossi, I.C., Da Silva, R.S.M., 2004. Hepatopancreas gluconeogenesis during anoxia and post-anoxia recovery in *Chasmagnathus granulata* crabs maintained on high-protein or carbohydrate-rich diets. J. Exp. Zool. A Comp. Exp. Biol. 301A, 240–248.

Parrilla-Taylor, D.P., Zenteno-Savín, T., 2011. Antioxidant enzyme activities in Pacific white shrimp (*Litopenaeus vannamei*) in response to environmental hypoxia and reoxygenation. Aquaculture 318, 379–383.

Reyes, A., Burgos, M.E., Hubert, E., Slebe, J.C., 1987. Selective thiol group modification renders fructose-1,6-bisphosphatase insensitive to fructose 2,6-bisphosphate inhibition. J. Biol. Chem. 262, 8451–8454.

Robin, E.D., Murphy, B.J., Theodore, J., 1984. Coordinate regulation of glycolysis by hypoxia in mammalian cells. J. Cell. Physiol. 118, 287–290.

Sagi, A., Manor, R., Ventura, T., 2013. Gene silencing in crustaceans: from basic research to biotechnologies. Genes 4, 620–645.

Semenza, G.L., 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J. Appl. Physiol. 88, 1474–1480.

Semenza, G.L., 2003. Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer 3, 721–732.

Semenza, G.L., 2012. Hypoxia-inducible factors in physiology and medicine. Cell 148, 399–408.

Sharma, B., 2011. Kinetic Characterisation of phosphofructokinase purified from *Setaria cervi*: a bovine filarial parasite. Enzym. Res. 2011, 1–10.

Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., López-Zavala, A., Forman, H.J., Yepiz-Plascencia, G., 2009. Molecular characterization of hypoxia inducible factor-1 (HIF-1) from the white shrimp *Litopenaeus vannamei* and tissue- specific expression under hypoxia. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 150, 395–405.

Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Sotelo-Mundo, R.R., Forman, H.J., Yepiz-Plascencia, G., 2011. Hexokinase from the white shrimp *Litopenaeus vannamei*: cDNA sequence, structural protein model and regulation via HIF-1 in response to hypoxia. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 158, 242–249.

Soñanez-Organis, J.G., Racotta, I.S., Yepiz-Plascencia, G., 2010. Silencing of the hypoxia inducible factor 1 -HIF-1- obliterates the effects of hipoxia on glucose and lactate concentrations in a tissue-specific manner in the white shrimp *Litopenaeus vannamei*. J. Exp. Mar. Biol. Ecol. 393, 51–58.

Soñanez-Organis, J.G., Rodriguez-Armenta, M., Leal-Rubio, B., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Yepiz-Plascencia, G., 2012. Alternative splicing generates two lactate dehydrogenase subunits differentially expressed during hypoxia via HIF-1 in the shrimp *Litopenaeus vannamei*. Biochimie. 94, 1250–1260.

Wang, J., Fu, X., Yang, K., Jiang, Q., Chen, Y., Jia, J., Duan, X., Wang, E.W., He, J., Ran, P., Zhong, N., Semenza, G.L., Lu, W., 2015. Hypoxia inducible factor-1-dependent up-regulation of BMP4 mediates hypoxia-induced increase of TRPC expression in PASMCs. Cardiovasc. Res. 107, 108–118.

Yánez, A.J., Nualart, F., Droppelmann, C., Bertinat, R., Brito, M., Concha, I.I., Slebe, J.C., 2003. Broad expression of fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase provide evidence for gluconeogenesis in human tissues other than liver and kidney. J. Cell. Physiol. 197, 189–197.

CAPÍTULO III

MINI REVIEW

Revisiting the hypoxia and anoxia resilience in marine animals: from physiological to molecular survival strategies

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MINI REVIEW

Revisiting the hypoxia and anoxia resilience in marine animals: from physiological to molecular survival strategies

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Abstract

Some marine animals are hypoxia and/or anoxia tolerant and can survive from hours to days or months with very little oxygen environments. In this review, we discuss how they make this possible. A series of physiological and biochemical processes coupled to molecular responses orchestrated by these organisms to maintain a balance between ATP supply and ATP demand are described. Aquatic animals first try to maintain oxygen delivery by employing several strategies such as remodeling gills, modifying their breathing or cardiac pulse rate, or altering blood flow distribution, including vasodilatation, where nitric oxide (NO), plays an important role as a signal molecule. If the animals still sense a lack in oxygen availability, they lower their metabolic rate to save energy. However, not all the energy consuming processes are down regulated. For example, the synthesis of hemoglobin/hemocyanin, necessary to carry oxygen, is maintained to increase the oxygen/carrying capacity of the blood. Additionally, some data suggest that in liver/hepatopancreas of marine animals, gluconeogenesis could be activated for at least two reasons: 1) to make available glucose for those tissues-fueled only by this carbohydrate and/or, 2) to feed the pentose phosphate pathway through the branch-point glucose 6-phosphate metabolite. This latter provides the advantage to produce reducing equivalents for cells. On the other hand, hypoxia-tolerant marine animals may have high glycogen reserves. To sustain ATP energy requirements, glycogen-fueled anaerobiosis is employed by these animals with a variety of end-products. Additional information indicates that the Hypoxia Inducible Factor (HIF) is intrinsically involved in the hypoxic induction of globin and glycolytic enzymes genes of these organisms, positioning HIF as a central molecule coordinating these events.

Keywords

Hypoxia, ATP, gluconeogenesis, reducing equivalents, hypoxia-tolerant, anaerobiosis, glycolysis, HIF

1. Introduction

Oxygen is essential to maintain many forms of life on earth. In their natural environments, animals can be exposed to low concentration (hypoxia) or to a complete absence of oxygen (anoxia). Hypoxia can have life-threatening consequences for humans and most mammals and birds. Opposite, many species including invertebrates, fish, mollusks, amphibians, reptiles, and some diving mammals are adapted to withstand hypoxia or anoxia environments for hours or even for months (Hermes-Lima and Zenteno-Savín, 2002). Mainly in aquatic environments, acquiring oxygen from water has its special concerns since oxygen concentration in air is 30 times higher than in the completely aerated water, and oxygen diffuses roughly 10,000 times faster in air than in water (Nilsson and Lutz, 2004).

Aquatic hypoxia appears when the rate of oxygen consumption exceeds the rate of oxygen supply. It occurs when algae and other organisms die and eventually sink to bottom latitudes providing organic sources for bacteria that uses surrounding oxygen to decompose them. Certainly, the over enrichment of sea waters by nutrients caused by human pollution, process known as eutrophication, remarkably increases hypoxia (Selman et al., 2008). Also, salinity and temperature in water surface drive to stratification, avoiding oxygen restoration and leading to hypoxic water zones (Rabalais et al., 2001) Hypoxia has been increasing exponentially since the 1960´s in the coastal areas and has become a key stressor for marine ecosystems (Diaz and Rosenberg, 2008). The adverse impacts of hypoxia comprise changes in marine organisms populations, including behavioral responses, variation of species distributions, biodiversity and even mortality, as well as physiological stress, and reduction in growth and reproduction (Zhang et al., 2013).

Hypoxia-tolerant animals can deal and survive in oxygen depriving conditions. A fundamental trait of these organisms is their ability to acquire O_2 from its environment (Fu et al., 2011). In this sense, organisms with a greater capacity to take up oxygen have a better potential to survive. Crustaceans normally keep constant oxygen consumption independently of oxygen pressures, acting as oxy-regulators, however, when oxygen lowers to a critical level, ventilation and aerobic metabolism cease (Havird et al., 2014). Essentially, marine animals use energy conservation instead of energy compensation.

They have the ability to reversibly experiment metabolic depression, consequently lowering ATP consumption with an equilibrated balance between ATP supply and ATP depletion (Hochachka et al., 1996; Hochachka and Somero, 2002). To satisfy their energy requirements, these animals eventually turn to anaerobic metabolism (Wu, 2002). Besides, exposure to low oxygen adversely affects growth, reproduction, locomotion and feeding (Levin et al., 2009; Wu, 2002). In this review we focus on physiological and metabolic adaptations as well as molecular responses displayed by marine animals to cope with hypoxia and/or anoxia.

2. Physiological and biochemical strategies to cope with hypoxia

Compared to air breather organisms, marine animals have limited access to oxygen and frequently experience more hypoxia. Oxygen diffuses more slowly through water than it does in air, thus, animals in water are exposed to a larger diffusion boundary. Hypoxia can be a natural phenomenon caused by stratification (Obenour et al., 2012) or can result from accumulation of nutrients and consequent eutrophication (Zhang et al., 2013). Changes in water temperature can also impact the dissolved oxygen amount with harmful effects on marine fauna (Melzner et al., 2013; Vaquer-Sunyer and Duarte, 2011).

There are reports on the physiological and biochemical responses of marine animals to hypoxia, especially in fish (Richards, 2011; Wu, 2002). In table 1, we summarize the crucial responses exhibited by these animals. First, aquatic organisms respond to hypoxia by enhancing oxygen uptake to maintain its delivery to the cells. The first response is clear in teleost fish that reversibly remodels the gill morphology. In the crucian carp *Carassius carassius* the gills are surrounded by a cell mass during normoxic conditions and much of these cells die in hypoxic conditions, exposing a larger respiratory surface area (Mitrovic et al., 2009; Nilsson, 2007) Second, marine animals alter their respiratory and cardiovascular rate. There are two factors that determine the rate of gas transfer in fish: ventilation and perfusion. Thus, fish must be able to increase ventilation volume induced by changes in breathing frequency and/or amplitude and, the capacity of fish to mount appropriate cardiorespiratory responses aimed to promote oxygen delivery to appropriate tissues (Perry et al., 2009). Additionally, these animals have the ability to re-direct blood flow distribution in hypoxic conditions (Hopkins and Powell, 2001). In crustaceans, for example, an active mechanism for redistribution of hemolymph flow

occurs during hypoxic exposure increasing blood supply to aerobic tissues (Airriess and McMahon, 1994).

Another physiological characteristic observed in these organisms when exposed to hypoxia is the capability to lower their metabolic rate (Hochachka and Lutz, 2001) or even to show an absence of metabolism as occurs in normally hydrated in diapaused eggs of the brine-shrimp during anoxia (Podrabsky and Hand, 2015)). Metabolic depression is a common and remarkably consistent pattern for various animals such as mollusks, crustaceans, fishes, amphibians and reptiles (Guppy and Withers, 1999; Hermes-Lima et al., 2015). When animals experience metabolic rate reduction, they gain an extension of survival time going from months or even to years of dormancy. This manner of energy conservation includes a drastic suppression of ATP demanding processes in the cell that includes protein synthesis, protein degradation, and ion pumping systems arrest (Hochachka et al., 1996; Hochachka and Somero, 2002). This kind of regulation maintain ATP levels constant, despite ATP turnover rates considerably tends to falling-off (Hochachka and Lutz, 2001).

Marine mammals and some birds and turtles are well recognized by their breath-holding diving capabilities (Lutz and Storey, 2010; Ponganis et al., 2011). Surprisingly, elephant seal can dive for at least 25 minutes without breathing (López-Cruz et al., 2014). When diving, these animals suddenly experience blood shift to lungs, consequently blood becomes instantly fully oxygenated. Under these circumstances, organisms enter into an energy based oxygen conservation rather than energy compensation strategy; they lower the heart rate (bradycardia), experience peripheral vasoconstriction and re-distribute their blood to vital organs (i.e. brains); consequently, tissues like skeletal muscle rely on anaerobic energy metabolism (Panneton, 2013). Additionally, by experiencing tissue or body cooling, these animals lower their metabolism and reduce oxygen consumption.

There are several steps of the respiratory cascade that can be modified to enhance oxygen uptake during hypoxia in marine animals. However, the oxygen binding characteristic of hemoglobin has grown in attention. Exposure to hypoxia in the rainbow trout *Oncorhynchus mykiss*, caused a raise of hemoglobin concentration through release from erythrocytes and, under prolonged hypoxia, an erythropoietin/mediated synthesis of new erythrocytes rise the oxygen/carrying capacity of the blood (Lai et al., 2006). When

the demand for oxygen exceeds supply, as occurs in hypoxia, the pH of the internal environment cell falls as a result of both, accumulated dissolved carbon dioxide from respiration, and the activation of anaerobic metabolism leading to lactic acid production. This causes a conformational change in hemoglobin resulting in a reduced oxygen affinity and thus more oxygen is released to tissues in response to higher proton loads (Wells, 2009). Interestingly, It is known that estuarine crustaceans are often exposed to hypoxia accompanied by elevated CO_2 (hypercapnia), which lowers water pH. This latter suggest that hypercapnia/low pH increases oxygen delivery to tissues resulting in less harming effects of hypoxia (Rathburn et al., 2013). Remarkably, hypercapnia has also been suggested to be involved in stimulating beneficial metabolic depression by altering ion mechanisms causing channel arrest (Guppy and Withers, 1999).

Marine animals have an additional approach that permits them to deal with hypoxia: crustaceans can sustain high glycogen reserves (Geihs et al., 2013). The crayfish *Parastacus brasiliensis* maintain their glycogen reserves even in hypoxic conditions (Silva-Castiglioni et al., 2011). Hypoxia-tolerant fishes like the crucian carp have normally 300-2000 µmol glycosil units per gram wet mass tissue whereas hypoxia-sensitive fishes, have only around 100 µmol glycosil units per gram wet mass tissue (Vornanen et al., 2009). Glycogen reserves represent an important fuel to provide glucose trough anaerobic metabolism supporting ATP generation. Hence, those animals with larger amount of glycogen will be able to produce more ATP even for longer periods of time. However, if animals would not enter in a metabolic-depression state, even large amounts of stored fuels could be faster consumed (Richards, 2011).

3. Anaerobic Metabolism: an approach to front hypoxia and anoxia environments

Aerobic metabolism may eventually be limited by oxygen delivery to the tissues. In this sense, organisms like daphnia, stimulated hemoglobin synthesis (Gorr et al., 2006), and fishes do the same with respect to myoblobin (Fraser et al., 2006) to satisfy their oxygen tissue requirements when faced to oxygen scariness. There is evidence that even hemocyanin can be synthetized in oxygen lacking conditions in shrimp (Sun et al., 2014). However, below the critical oxygen level, animals cannot maintain the aerobic metabolism in several tissues since an inadequate oxygen supply occurs. Consequently,

a series of other responses can be exhibited by these animals. In this section we will discuss these responses.

Many species of mollusks, fish, amphibians and crustaceans are able to survive periods going from hours to months, in oxygen depriving conditions or even in anoxia (Nilsson and Lutz, 2004; Storey and Storey, 1990). In these conditions, most of them switch to anaerobic metabolic pathways to supply energy (Fig. 1) (Gorr, 2004; Richards, 2011). In the shrimp *Litopenaeus vannamei*, hypoxia induces lactate concentration and lactate dehydrogenase (LDH) activity (Soñanez-Organis et al., 2012), while in goldfish an accumulation of lactate is reported (Fu et al., 2011). In the crab *Neohelice granulate*, anoxia rise pyruvate kinase (PK) activity (Marqueze et al., 2011). These findings indicate an increment in the glycolytic flux. However, ATP generation in anaerobic metabolism is much lower compared to that generated in aerobic conditions. As a result, ATP production resides on the availability of glycogen or, in some cases, on high energy phosphates, as occurs in kuruma prawn *Marsupenaeus japonicus,* where phosphoarginine functions in muscle as an ATP recharger during hypoxia (Abe et al., 2007). In many aquatic animals, anaerobic metabolism produces L-lactate as a metabolic end-product. However, accumulation of lactate (plus an associated proton) can have devastating effects on tissues. To solve this, turtles tolerate acidosis by its high buffering capacity and compensatory ion changes (Fig. 1) (Storey and Storey, 1990). In crustaceans, it has been suggested that clearance of L-lactate could be possible by: complete oxidation, conversion back into storage products such as glycogen, or excretion (Fig. 1). Oxidation may take place in the dark muscle and heart of the blue crab *Callinectes sapidus* mitochondria, due to the high citrate synthase activities presented by these tissues (Lallier and Walsh, 1991). Moreover, It has been reported that hepatopancreas of crustaceans plays a reasonable role in lactate removal (Lallier and Walsh, 1992). However, some data point that the Cori cycle seems not to be as significant as occur in mammals since only a small amount of lactate is converted to glucose through gluconeogenesis (Walsh, 1989). Despite the negative effects of L-lactate accumulation in crustacean blood, it binds to the extracellular pigment hemocyanin and enhance its affinity to oxygen (Weber et al., 2008).

Some fishes avoid lactic acidosis even in anoxia by generating ethanol as anaerobic end-product (Fig. 1). Between fishes, species of the genus *Carassius* are the best known anaerobes. These organisms, called goldfish, transform L-lactate to ethanol by

adaptations in the pyruvate dehydrogenase complex, which catalyzes the ethanol pathway by first producing acetaldehyde. The acetaldehyde can consequently be converted to ethanol by alcohol dehydrogenase, this conversion apparently takes place in skeletal muscle (Fagernes et al., 2008). Finally, ethanol and CO_2 produced can be excreted to the surrounding water through gills. Although a carbon wasteful, excretion of ethanol gives to goldfish the advantage to extend its survival beyond from which has been observed in other vertebrates (Aleleye-Wokoma, 2001).

Another biochemical adaptation that contributes to anaerobiosis includes the use of an alternative pathway of substrate level phosphorylation to produce more ATP. The mollusk channeled whelk *Busycotypus canaliculatusis* exemplifies this metabolic response by coordinating the control of glycolytic rate depression in anoxia. Anoxia induces pyruvate kinase phosphorylation causing arrest in PK activity. In these circumstances, phosphoenol pyruvate accumulates and can instead be channeled through PEP carboxykinase, and later into the succinate or propionate pathways harvesting additional ATP from substrate-level phosphorylations (Fig. 1). In addition to PK inhibition, reversible phosphorylation of glycogen phosphorylase, glycogen synthase, and pyruvate dehydrogenase convert the enzymes to the less active forms promoting glycogen degradation (Russell and Storey, 1995).

4. Regulation of responses to hypoxia: molecular mechanisms

More than two decades ago, studies performed in the human EPO gene revealed that the Hypoxia Inducible Factor (HIF-1) is involved in its regulation (Semenza and Wang, 1992). Since that discovery, It has been demonstrated that HIF-1 is involved in the control of multiple genes, and so far, more than 100 genes have been reported in mammalian systems (Semenza, 2003) and the list continues to increase. The hypoxia-inducible factors (HIFs) are transcriptional factors that work as master oxygen-dependent gene regulators in all metazoan species (Semenza, 2012). They bind a core sequence of the Hypoxia Responsive Elements (HRE) in the promoter gene regions inducing their expressions (Lee et al., 2004). Through the metazoan species, from nematodes, insects and crustaceans to mammals, the functional complex consists of a heterodimer composed of a labile α and a constitutive β subunit (also known as aryl hydrocarbon receptor nuclear translocator or ARNT). HIF proteins are members of the family of basic

helix-loop-helix/PAS (Per/ARNT/Sim) transcription factors (Gorr et al., 2004), recognizing the sequence RCGTG (Wang et al., 2015).

Multiple isoforms of HIF proteins have been reported in fish, amphibians, birds, mammals, and even in marine mammals (Soñanez-Organis et al, 2013). However, most studies about HIF have been done in higher organisms. In humans, it is known that under aerobic conditions, HIF-1α is hydroxylated by specific prolyl hydroxylases (PHDs) at two conserved proline amino-acids located in the oxygen-dependent degradation domain (ODD), the reaction requires oxygen, 2-oxoglutarate, ascorbate, and iron (Fe^{2+}). One oxygen atom is inserted into the prolyl residue, and the other atom is inserted into the α-ketoglutarate, generating CO_2 and succinate (Kaelin and Ratcliffe, 2008). In these conditions, HIF-1α is attached by the von Hippel-Lindau (VHL) protein that binds to an ubiquitin ligase, targeting HIF-1α for proteasomal degradation (Jaakkola et al., 2001).

In terrestrial vertebrates and mammals, HIF-1 regulates the expression of glycolytic enzymes (Semenza, 2003), as well as the expression of glucose transporters, involved in glucose uptake (Chen et al., 2001). HIF-1 binds to the promoter HRE of the corresponding genes. On the other hand, in marine animals there are few studies focused to identify HRE in promoter regions of the hypoxia-responsive genes and to evaluate the rol of HIF in hypoxic conditions. The fact that there is hypoxia tolerance among several marine organisms inquiry whether there is a relationship between HIF function and hypoxia tolerance. HIF was implicated in the hypoxic induction of *Daphnia magna* globin genes that contributes to incresease/improve oxygen carrying capacities of this species (Gorr et al., 2006). In the killifish *Fundulus heteroclitus,* there are DNA elements in intron 2 of the LDH-B gene that is somehow similar to the HRE reported in mammalian genes, which suggest that they may be interacting with HIF (Rees et al., 2001). Furthermore, silencing of HIF-1 showed a reduction or complete depletion in lactate concentration in a tissue-specific fashion in shrimp subjected to hypoxia (Soñanez-Organis et al., 2010). Also, a reduction of PFK (Cota-Ruiz et al., 2016) and LDH transcripts and their corresponding enzymatic activities by the silencing of HIF-1, indicate that HIF-1 participates in anaerobic glycolysis regulation (Soñanez-Organis et al., 2012). These studies point that in hypoxic conditions, the regulation of target genes by HIF could be a crucial factor to support ATP production via anaerobic metabolism in crustaceans (Fig. 2). Nevertheless, more studies in more species describing the transcriptional role of HIF

need to be done to understand how marine animals survive through this energy conservation-based pathway.

In addition, some important physiological (*i.e.* metabolic depression and re-distribution of blood flow) responses implemented by hypoxic/anoxic tolerant species are molecularly regulated, at least in part, by the smallest signalling molecule known: nitric oxide (NO) (Fago and Jensen, 2015). As demonstrated by several studies in mammals, NO plays an important role in regulating vasodilatation under hypoxic conditions (Ho et al., 2012). NO is synthetized by three isoforms of NO synthase (NOS; EC 1.14.13.39) that oxidize L-arginine in the presence of the cofactors reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-) 5,6,7,8-tetrahydrobiopterin (BH4), to produce L-citrulline and NO (Förstermann and Sessa, 2012). In fish species subjected to hypoxic conditions, nitrite reacts with the ferrous heme of deoxygenated hemoglobin to form NO and oxidized (ferric) heme, then, NO acts as a messenger molecule by stimulating NO-sensitive guanylyl cyclase, that is directly involved in smooth muscle cell relaxation and vasodilatation processes (Cossins et al., 2009).

5. Activation of energy consuming process: the payment of inversion

Under normoxic conditions, the main energy consumption processes are: 1) protein synthesis, 2) protein degradation, 3) Na/K pumping, and 4) glucose biosynthesis, which almost represent the 100% of the ATP consumption (Hochachka et al., 1996). Evidently, in hypoxic conditions cells must try to save energy and "arrest" those processes that are not essentials for living. If it results necessary to consume energy for certain process, they will have to do it by orchestrating a coordinated response. In this sense, fishes and crustaceans can synthesize myoglobin and hemocyanin, respectively, under these circumstances (Fraser et al., 2006; Gorr et al., 2006). Although this is a demanding energy process, oxygen capture and delivery by these biomolecules to critical tissues could compensate this inversion. Moreover, there is some evidence that another metabolic energy consuming process such as gluconeogenesis could occur in a tissue-specific manner in some marine animals. In the killifish *Fundulus grandis* the specific activities of some gluconeogenic enzymes showed to increase in liver during long-term hypoxic exposure (Martínez et al., 2006). In line with this, a strong induction in the liver

of the genes needed for gluconeogenesis occurs in the eurioxyc fish *Gillichthys mirabilis* (Gracey et al., 2001). In shrimp hepatopancreas, it has been reported an induction on fructose bisphosphatase (FBP) transcripts (Cota-Ruiz et al., 2015). These data point that in liver/hepatopancreas from these marine animals an activation of the gluconeogenic metabolism may take place.

However, does an energy metabolic consuming process such as gluconeogenesis give to marine animals some advantages? The explanation of this question has been recently planted and discussed (Cota-Ruiz et al., 2015) and it is proposed that glucose production through gluconeogenesis could be sustained by the fact that some tissues, like brain, mandatory needs glucose as a principal sugar to maintain its ATP levels. For instance, hypoxia-tolerant animals can be able to maintain ATP levels in vital tissues when exposed to very low oxygen conditions. Additionally, when the gluconeogenic pathway is activated, glucose 6-phosphate, a key intermediary compound is produced and serves as the substrate for the pentose phosphate pathway; activation of this pathway can led to increase cellular glutathione that represent the reducing power for antioxidant defense. Also, glutathione could function as inhibitor of some forms of apoptosis as occur in mammalian cells (Fig. 3).

6. Conclusion remarks and perspectives

Hypoxia/anoxia tolerant organisms display a harmonized physiological and biochemical-molecular responses to either maximize oxygen uptake and tissue delivery, and to obtain energy in oxygen limitating conditions. Marine animals respond to oxygen scarcity by first attempting to maintain oxygen delivery, then by metabolic rate depression, and finally by producing energy from anaerobic metabolism. Available information point that HIF-1 could control the glycolitic gene expression of marine animals and thus govern the anaerobic energy production that represents a fundamental adaptation to oxygen limitation. However, further work is requiered to confirm this and to evaluate if HIF molecules control ATP costly process such as gluconeogenesis or apoptosis in hypoxia-tolerant cells. Adittionally, deeper kwnowledge on the molecular basis (*i.e.* the NO signaling pathway) that permit marine organisms to survive in hypoxia/anoxia environments will give us insights to study if these mechanisms explain the high sensitivity of mammalian cells to hypoxia/anoxia or the resilience of hypoxic tumoral cells.

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References

Abe, H., Hirai, S., Okada, S., 2007. Metabolic responses and arginine kinase expression under hypoxic stress of the kuruma prawn *Marsupenaeus japonicus*. Comp. Biochem. Physiolog. A: Mol. Integ. Physiolog. 146, 40-46.

Airriess, C., McMahon, B., 1994. Cardiovascular Adaptations Enhance Tolerance of Environmental Hypoxia in the Crab *Cancer Magister*. J. Exp. Biol. 190, 23-41.

Al Hasawi, N., Alkandari, M.F., Luqmani, Y.A., 2014. Phosphofructokinase: a mediator of glycolytic flux in cancer progression. Crit. Rev. Oncol. Hematol. 92, 312-321.

Aleleye-Wokoma, L.P., 2001. The use of the ethanol pathway in goldfish *Carassius auratus* (L.) following anoxia, in: -.J. 14th Annual Conference of the Fisheries Society of Nigeria (FISON) , Ibadan, Nigeria. (Ed.), pp. 89-95.

Aragonés, J., Fraisl, P., Baes, M., Carmeliet, P., 2009. Oxygen Sensors at the Crossroad of Metabolism. Cell metabolism 9, 11-22.

Bishop, T., Ratcliffe, P., 2014. Signaling hypoxia by hypoxia-inducible factor protein hydroxylases: a historical overview and future perspectives. Hypoxia 2, 197-213.

Brown-Peterson, N.J., Manning, C.S., Patel, V., Denslow, N.D., Brouwer, M., 2008. Effects of Cyclic Hypoxia on Gene Expression and Reproduction in a Grass Shrimp, *Palaemonetes pugio*. Biol. Bull. 214, 6-16.

Cossins, A.R., Williams, D.R., Foulkes, N.S., Berenbrink, M., Kipar, A., 2009. Diverse cell-specific expression of myoglobin isoforms in brain, kidney, gill and liver of the hypoxia-tolerant carp and zebrafish. J. Exp. Biol. 212, 627-638.

Cota-Ruiz, K., Leyva-Carrillo, L., Peregrino-Uriarte, A.B., Valenzuela-Soto, E.M., Gollas-Galván, T., Gómez-Jiménez, S., Hernández, J., Yepiz-Plascencia, G., 2016. Role of HIF-1 on phosphofructokinase and fructose 1, 6-bisphosphatase expression during hypoxia in the white shrimp Litopenaeus vannamei. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 198, 1-7.

Cota-Ruiz, K., Peregrino-Uriarte, A.B., Felix-Portillo, M., Martínez-Quintana, J.A., Yepiz-Plascencia, G., 2015a. Expression of fructose 1, 6-bisphosphatase and phosphofructokinase is induced in hepatopancreas of the white shrimp *Litopenaeus vannamei* by hypoxia. Mar. Environ. Res. 106, 1-9.

Czech-Damal, N., Geiseler, S., Hoff, M., Schliep, R., Ramirez, J.-M., Folkow, L., Burmester, T., 2014. The role of glycogen, glucose and lactate in neuronal activity during hypoxia in the hooded seal (Cystophora cristata) brain. Neuroscience 275, 374-383.

Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F., Maity, A., 2001. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J. Biol. Chem. 276, 9519-9525.

Choi, J.H., Park, M.J., Kim, K.W., Choi, Y.H., Park, S.H., An, W.G., Yang, U.S., Cheong, J., 2005. Molecular mechanism of hypoxia-mediated hepatic gluconeogenesis by transcriptional regulation. FEBS Lett. 579, 2795-2801.

Davis, R.W., 2014. A review of the multi-level adaptations for maximizing aerobic dive duration in marine mammals: from biochemistry to behavior. J Comp Physiol B 184, 23-53.

Denko, N.C., 2008. Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat. Rev. Cancer 8, 705-713.

Diaz, R.J., Rosenberg, R., 2008. Spreading Dead Zones and Consequences for Marine Ecosystems. Science 321, 926-929.

Fagernes, C., Ellefsen, S., Stenslokken, K., Berenbrink, M., Nilsson, G., 2008. Molecular background to ethanol production in crucian carp (Carassius carassius). Pergamon, S112.

Fago, A., Jensen, F.B., 2015. Hypoxia Tolerance, Nitric Oxide, and Nitrite: Lessons From Extreme Animals. Physiol. 30, 116-126.

Förstermann, U., Sessa, W.C., 2012. Nitric oxide synthases: regulation and function. Eur. Heart J. 33, 829-837.

Fraser, J., Vieira de Mello, L., Ward, D., Rees, H.H., Williams, D.R., Fang, Y., Brass, A., Gracey, A.Y., Cossins, A.R., 2006. Hypoxia-inducible myoglobin expression in nonmuscle tissues. Proc. Natl. Acad. Sci. U. S. A. 103, 2977-2981.

Fu, S.-J., Brauner, C.J., Cao, Z.-D., Richards, J.G., Peng, J.-L., Dhillon, R., Wang, Y.-X., 2011. The effect of acclimation to hypoxia and sustained exercise on subsequent hypoxia tolerance and swimming performance in goldfish (*Carassius auratus*). The J Exp Biol 214, 2080-2088.

Geihs, M.A., Maciel, F.E., Vargas, M.A., Cruz, B.P., Nery, L.E.M., 2013. Effects of hypoxia and reoxygenation on the energetic metabolism of the crab Neohelice granulata (Decapoda, Varunidae). J. Exp. Mar. Biol. Ecol. 445, 69-78.

Ghaffari, N., Sanchez-Flores, A., Doan, R., Garcia-Orozco, K.D., Chen, P.L., Ochoa-Leyva, A., Lopez-Zavala, A.A., Carrasco, J.S., Hong, C., Brieba, L.G., Rudiño-Piñera, E., Blood, P.D., Sawyer, J.E., Johnson, C.D., Dindot, S.V., Sotelo-Mundo, R.R., Criscitiello, M.F., 2014. Novel transcriptome assembly and improved annotation of the whiteleg shrimp (Litopenaeus vannamei), a dominant crustacean in global seafood mariculture. Sci. Rep. 4, 1-10.

Gorr, T.A., 2004. Daphnia and Drosophila: two invertebrate models for O2 responsive and HIF-mediated regulation of genes and genomes. International Congress Series 1275, 55-62.

Gorr, T.A., Cahn, J.D., Yamagata, H., Bunn, H.F., 2004. Hypoxia-induced Synthesis of Hemoglobin in the Crustacean *Daphnia magna* Is Hypoxia-inducible Factor-dependent. J. Biol. Chem. 279, 36038-36047.

Gorr, T.A., Gassmann, M., Wappnerb, P., 2006. Sensing and responding to hypoxia via HIF in model invertebrates. J. Insect Physiol. 52, 349-364.

Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N.B., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83(5), 733-752.

Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. Proc. Natl. Acad. Sci. U. S. A. 98, 1993-1998.

Guppy, M., Withers, P., 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biol. Rev. Camb. Philos. Soc. 74, 1-40.

Hardy, K.M., Burnett, K.G., Burnett, L.E., 2013. Effect of hypercapnic hypoxia and bacterial infection (*Vibrio campbellii*) on protein synthesis rates in the Pacific whiteleg shrimp, *Litopenaeus vannamei*. Am J Physiol-Reg I 305, R1356-R1366.

Havird, J.C., Vaught, R.C., Weeks, J.R., Fujita, Y., Hidaka, M., Santos, S.R., Henry, R.P., 2014. Taking their breath away: Metabolic responses to low-oxygen levels in anchialine shrimps (Crustacea: Atyidae and Alpheidae). Comp Biochem Phys A 178, 109-120.

Havula, E., Teesalu, M., Hyötyläinen, T., Seppälä, H., Hasygar, K., Auvinen, P., Orešič, M., Sandmann, T., Hietakangas, V., 2013. Mondo/ChREBP-Mlx-regulated transcriptional network is essential for dietary sugar tolerance in *Drosophila*. PLoS Genet 9, 1-13.
Hermes-Lima, M., Moreira, D.C., Rivera-Ingraham, G.A., Giraud-Billoud, M., Genaro-Mattos, T.C., Campos, É.G., 2015. Preparation for oxidative stress under hypoxia and metabolic depression: Revisiting the proposal two decades later. Free Radical Biol. Med. 89, 1122-1143.
Hermes-Lima, M., Zenteno-Savín, T., 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp. Biochem. Phys. C. 133, 537-556.
Ho, J.D., Man, H.J., Marsden, P.A., 2012. Nitric oxide signaling in hypoxia. J. Mol. Med. 90, 217-231.
Hochachka, P.W., Buck, L.T., Doll, C.J., Land, S.C., 1996. Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. P Natl Acad Sci USA 93, 9493-9498.
Hochachka, P.W., Lutz, P.L., 2001. Mechanism, origin, and evolution of anoxia tolerance in animals☆. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 130, 435-459.
Hochachka, P.W., Somero, G.N., 2002. Biochemical adaptation: Mechanism and process in physiological evolution. Biochem. Mol. Biol. Edu. 30, 215-216.
Hochachka, P.W., Somero, G.N., 2014. Biochemical adaptation. Princeton University Press.
Hopkins, S.R., Powell, F.L., 2001. Common themes of adaptation to hypoxia. Insights from comparative physiology. Adv. Exp. Med. Biol. 502, 153-167.
Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., von Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J., 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292, 468-472.
Jenkins, C.M., Yang, J., Sims, H.F., Gross, R.W., 2011. Reversible High Affinity Inhibition of Phosphofructokinase-1 by Acyl-CoA: A Mechanism Integrating Glycolytic Flux With Lipid Metabolism. J. Biol. Chem. 286, 11937-11950.
Johnston, M.A., Elder, H.Y., Davies, P.S., 1973. Cytology of carcinus haemocytes and their function in carbohydrate metabolism. Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol. 46, 569-581.
Kaelin, W.G., Ratcliffe, P.J., 2008. Oxygen Sensing by Metazoans: The Central Role of the HIF Hydroxylase Pathway. Mol. Cell. 30, 393-402.
Lai, J.C., Kakuta, I., Mok, H.O., Rummer, J.L., Randall, D., 2006. Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. J Exp Biol 209, 2734-2738.
Lallier, F.H., Walsh, P.J., 1992. Metabolism of Isolated Hepatopancreas Cells from the Blue Crab (Callinectes sapidus) under Simulated Postexercise and Hypoxic Conditions. Physiol. Zool. 65, 712-723.
Lallier, F.o.H., Walsh, P.J., 1991. Metabolic Potential in Tissues of the Blue Crab, <i>Callinectes Sapidus</i>. Bull. Mar. Sci. 48, 665-669.
Le Moullac, G., Bacca, H., Huvet, A., Moal, J., Pouvreau, S., Van Wormhoudt, A., 2007. Transcriptional regulation of pyruvate kinase and phosphoenolpyruvate carboxykinase in the adductor muscle of the oyster *Crassostrea gigas* during prolonged hypoxia. J. Exp. Zool. A. Ecol. Genet. Physiol. 307A, 371-382.
Lee, J.W., Bae, S.H., Jeong, J.W., Kim, S.H., Kim, K.W., 2004. Hypoxia-inducible factor (HIF-1)[alpha]: its protein stability and biological functions. Exp. Mol. Med. 36, 1-12.

Levin, L.A., Ekau, W., Gooday, A.J., Jorissen, F., Middelburg, J.J., Naqvi, W., Neira, C., Rabalais, N.N., Zhang, J., 2009. Effects of natural and human-induced hypoxia on coastal benthos. Biogeosciences Discuss. 6, 3563-3654.

Li, X.-b., Gu, J.-d., Zhou, Q.-h., 2015. Review of aerobic glycolysis and its key enzymes – new targets for lung cancer therapy. Thoracic Cancer 6, 17-24.

Li, Y., Wei, L., Cao, J., Qiu, L., Jiang, X., Li, P., Song, Q., Zhou, H., Han, Q., Diao, X., 2016. Oxidative stress, DNA damage and antioxidant enzyme activities in the pacific white shrimp (Litopenaeus vannamei) when exposed to hypoxia and reoxygenation. Chemosphere 144, 234-240.

López-Cruz, R.I., Pérez-Milicua, M.B., Crocker, D.E., Gaxiola-Robles, R., Bernal-Vertiz, J.A., de la Rosa, A., Vázquez-Medina, J.P., Zenteno-Savín, T., 2014. Purine nucleoside phosphorylase and xanthine oxidase activities in erythrocytes and plasma from marine, semiaquatic and terrestrial mammals. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 171, 31-35.

Lugo, J.M., Morera, Y., Rodriguez, T., Huberman, A., Ramos, L., Estrada, M.P., 2006. Molecular cloning and characterization of the crustacean hyperglycemic hormone cDNA from *Litopenaeus schmitti*. Functional analysis by double-stranded RNA interference technique. FEBS J 273, 5669-5677.

Lutz, P.L., Storey, K.B., 2010. Adaptations to Variations in Oxygen Tension by Vertebrates and Invertebrates, Comprehensive Physiology. John Wiley & Sons, Inc.

Marqueze, A., Ribarcki, F., Kirst, I., Kucharski, L.C., Da Silva, R.S.M., 2011. Glucose metabolism in the hepatopancreas of the crab Neohelice granulata maintained on carbohydrate-rich or high-protein diets: Anoxia and recovery. J. Exp. Mar. Biol. Ecol. 404, 40-46.

Martínez-Quintana, J.A., Kikuta, S., Felix-Portillo, M., Peregrino-Uriarte, A.B., Yepiz-Plascencia, G., 2015. A novel functional glucose transporter in the white shrimp Litopenaeus vannamei-LvGLUT2-is up-regulated during hypoxia in hepatopancreas. Mar. Environ. Res. 112, 61-67.

Martínez-Quintana, J.A., Valenzuela-Soto, E.M., Martínez-Téllez, M.A., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Kikuta, S., Yepiz-Plascencia, G., 2016. Functionality of the white shrimp glucose transporter 1: Expression in *Xenopus oocytes* and gene silencing during hypoxia. J. Exp. Mar. Biol. Ecol. 474, 109-115.

Martínez, M.L., Landry, C., Boehm, R., Manning, S., Cheek, A.O., Rees, B.B., 2006. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*. J. Exp. Biol. 209, 3851-3861.

Meister, G., Tuschl, T., 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343-349.

Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M., Bange, H., Hansen, H., Körtzinger, A., 2013. Future ocean acidification will be amplified by hypoxia in coastal habitats. Mar. Biol. 160, 1875-1888.

Mitrovic, D., Dymowska, A., Nilsson, G.E., Perry, S.F., 2009. Physiological consequences of gill remodeling in goldfish (Carassius auratus) during exposure to long-term hypoxia. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 297, R224-R234.

Nilsson, G.E., 2007. Gill remodeling in fish--a new fashion or an ancient secret? J Exp Biol 210, 2403-2409.

Nilsson, G.E., Lutz, P.L., 2004. Anoxia tolerant brains. J. Cereb. Blood Flow Metab. 24, 475-486.

Obenour, D.R., Michalak, A.M., Zhou, Y., Scavia, D., 2012. Quantifying the Impacts of Stratification and Nutrient Loading on Hypoxia in the Northern Gulf of Mexico. Environ. Sci. Technol. 46, 5489-5496.

Panneton, W.M., 2013. The mammalian diving response: an enigmatic reflex to preserve life? Physiology 28, 284-297.

Pegoraro, C., Maczkowiak, F., Monsoro-Burq, A.H., 2013. Pfkfb (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase) isoforms display a tissue-specific and dynamic expression during Xenopus laevis development. Gene Expression Patterns 13, 203-211.

Pérez-Rostro, C.I., Racotta, I.S., Ibarra, A.M., 2004. Decreased genetic variation in metabolic variables of Litopenaeus vannamei shrimp after exposure to acute hypoxia. J. Exp. Mar. Biol. Ecol. 302, 189-200.

Perry, S.F., Jonz, M.G., Gilmour, K.M., 2009. Chapter 5 Oxygen Sensing And The Hypoxic Ventilatory Response, in: A.P.F. Jeffrey G. Richards, J.B. Colin (Eds.), Fish Physiology. Academic Press, 193-253.

Podrabsky, J.E., Hand, S.C., 2015. Physiological strategies during animal diapause: lessons from brine shrimp and annual killifish. J. Exp. Biol. 218, 1897-1906.

Ponganis, P.J., Meir, J.U., Williams, C.L., 2011. In pursuit of Irving and Scholander: a review of oxygen store management in seals and penguins. J. Exp. Biol. 214, 3325-3339.

Rabalais, N.N., Turner, R.E., Wiseman, W.J., 2001. Hypoxia in the Gulf of Mexico. Journal of Environmental Quality 30, 320-329.

Racotta, I.S., Palacios, E., Méndez, L., 2002. Metabolic responses to short and long-termexposure to hypoxia in white shrimp (*Penaeus vannamei*),. Mar. Freshwater Behav. Physiol. 35, 269-275.

Rathburn, C.K., Sharp, N.J., Ryan, J.C., Neely, M.G., Cook, M., Chapman, R.W., Burnett, L.E., Burnett, K.G., 2013. Transcriptomic responses of juvenile Pacific whiteleg shrimp, Litopenaeus vannamei, to hypoxia and hypercapnic hypoxia.

Rees, B.B., Bowman, J.A.L., Schulte, P.M., 2001. Structure and Sequence Conservation of a Putative Hypoxia Response Element in the Lactate Dehydrogenase-B Gene of Fundulus, 247-251.

Richards, J.G., 2011. Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. The Journal of Experimental Biology 214, 191-199.

Rosas, C., Cuzon, G., Gaxiola, G., Le Priol, Y., Pascual, C., Rossignyol, J., Contreras, F., Sanchez, A., Van Wormhoudt, A., 2001. Metabolism and growth of juveniles of *Litopenaeus vannamei*: effect of salinity and dietary carbohydrate levels. J. Exp. Mar. Biol. Ecol. 259, 1-22.

Rose, I.A., Rose, Z.B., 2014. Glycolysis: regulation and mechanisms of the enzymes. Comprehensive biochemistry 17, 93-161.

Russell, E.L., Storey, K.B., 1995. Anoxia and freezing exposures stimulate covalent modification of enzymes of carbohydrate metabolism in Littorina littorea. J Comp Physiol B 165, 132-142.

Sánchez-Paz, A., Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Muhlia-Almazan, A., Yepiz-Plascencia, G., 2008. Response of the phosphofructokinase and pyruvate kinase genes expressed in the midgut gland of the Pacific white shrimp *Litopenaeus vannamei* during short-term starvation. J. Exp. Mar. Biol. Ecol. 362, 79–89.

Schein, V., Wache, Y., Etges, R., Kucharski, L.C., van Wormhoudt, A., Da Silva, R.S., 2004. Effect of hyperosmotic shock on phosphoenolpyruvate carboxykinase gene expression and gluconeogenic activity in the crab muscle. FEBS Lett. 561, 202-206.

Selman, M., Greenhalgh, S., Díaz, R., Sugg, Z., 2008. Eutrophication and hypoxia in coastal areas: a global assessment of the state of knowledge. World Resources Institute 284.

Semenza, G.L., 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J. Appl. Physiol. 88, 1474–1480.

Semenza, G.L., 2003. Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer. 3, 721-732.

Semenza, G.L., 2012. Hypoxia-Inducible Factors in Physiology and Medicine. Cell. 148, 399-408.

Semenza, G.L., Wang, G.L., 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. 12, 5447-5454.

Silva-Castiglioni, D.d., Oliveira, G.T., Buckup, L., 2011. Metabolic responses in two species of crayfish (Parastacus defossus and Parastacus brasiliensis) to post-hypoxia recovery. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 159, 332-338.

Soñanez-Organis, J.G., Peregrino-Uriarte, A.B., Sotelo-Mundo, R.R., Forman, H.J., Yepiz-Plascencia, G., 2011. Hexokinase from the white shrimp *Litopenaeus vannamei*: cDNA sequence, structural protein model and regulation via HIF-1 in response to hypoxia. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 158, 242-249.

Soñanez-Organis, J.G., Racotta, I.S., Yepiz-Plascencia, G., 2010. Silencing of the Hypoxia Inducible Factor 1 -HIF-1- obliterates the effects of hipoxia on glucose and lactate concentrations in a tissue-specific manner in the white shrimp *Litopenaeus vannamei*. J. Exp. Mar. Biol. Ecol. 393, 51-58.

Soñanez-Organis, J.G., Rodriguez-Armenta, M., Leal-Rubio, B., Peregrino-Uriarte, A.B., Gómez-Jiménez, S., Yepiz-Plascencia, G., 2012. Alternative splicing generates two lactate dehydrogenase subunits differentially expressed during hypoxia via HIF-1 in the shrimp *Litopenaeus vannamei*. Biochimie, 1-11.

Storey, K.B., Storey, J.M., 1990. Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. Q. Rev. Biol. 65, 145-174.

Sun, S., Xuan, F., Ge, X., Fu, H., Zhu, J., Zhang, S., 2014. Identification of differentially expressed genes in hepatopancreas of oriental river prawn, *Macrobrachium nipponense* exposed to environmental hypoxia. Gene 534, 298-306.

Thabrew, M.I., Poat, P.C., Munday, K.A., 1971. Carbohydrate metabolism in *Carcinus maenas* gill tissue. Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 40, 531-541.

Tillmann, H., Bernhard, D., Eschrich, K., 2002. Fructose-1,6-bisphosphatase genes in animals. Gene 291, 57-66.

Uyeda, K., 2006. Phosphofructokinase. Advances in Enzymology and Related Areas of Molecular Biology, Volume 48, 193-244.

Vaquer-Sunyer, R., Duarte, C.M., 2008. Thresholds of hypoxia for marine biodiversity. Proc. Natl. Acad. Sci. U. S. A. 105, 15452-15457.

Vaquer-Sunyer, R., Duarte, C.M., 2011. Temperature effects on oxygen thresholds for hypoxia in marine benthic organisms. Global Change Biol. 17, 1788-1797.

Vinatea, L., Gálvez, A.O., Venero, J., Leffler, J., Browdy, C., 2009. Oxygen consumption of Litopenaeus vannamei juveniles in heterotrophic medium with zero water exchange. Pesquisa Agropecuária Brasileira 44, 534-538.

Vornanen, M., Stecyk, J.A.W., Nilsson, G.r.E., Jeffrey G. Richards, A.P.F.a.C.J.B., 2009. Chapter 9 The Anoxia-Tolerant Crucian Carp (Carassius Carassius L.), Fish Physiology. Academic Press, 397-441.

Walsh, P.J., 1989. An in Vitro Model of Post-Exercise Hepatic Gluconeogenesis in the Gulf Toadfish Opsanus Beta, 393-406.

Wang, J., Fu, X., Yang, K., Jiang, Q., Chen, Y., Jia, J., Duan, X., Wang, E.W., He, J., Ran, P., Zhong, N., Semenza, G.L., Lu, W., 2015. Hypoxia inducible factor 1 dependent upregulation of BMP4 mediates hypoxia-induced increase of TRPC expression in PASMCs. Cardiov. res.

Weber, R.E., Behrens, J.W., Malte, H., Fago, A., 2008. Thermodynamics of oxygenation-linked proton and lactate binding govern the temperature sensitivity of O2 binding in crustacean (Carcinus maenas) hemocyanin, 1057-1062.

Wells, R.M.G., 2009. Chapter 6 Blood-Gas Transport and Hemoglobin Function: Adaptations for Functional and Environmental Hypoxia, in: A.P.F. Jeffrey G. Richards, J.B. Colin (Eds.), Fish Physiology. Academic Press, 255-299.

Wu, R.S.S., 2002. Hypoxia: from molecular responses to ecosystem responses. Mar. Pollut. Bull. 45, 35-45.

Yoon, J.C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., Adelmant, G., Stafford, J., Kahn, C.R., Granner, D.K., Newgard, C.B., Spiegelman, B.M., 2001. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 413, 131-138.

Zhang, J., Cowie, G., Naqvi, S.W.A., 2013. Hypoxia in the changing marine environment. Environ. Res. Lett. 8, 015025.

Table 1. Summary of main physiological and biochemical strategies employed by marine animals to cope with hypoxia

Strategy	Mechanism	Reference
Increment of oxygen extraction	Gill remodeling	(Mitrovic et al., 2009; Nilsson, 2007)
Modification of respiratory and cardiovascular rate	Ventilation and perfusion	(Perry et al., 2009)
Blood flow delivery	Vasodilatation and redistribution of blood flow	(Cossins et al., 2009; Davis, 2014; Hopkins and Powell, 2001)
Energy Conservation	Metabolic depression	(Hermes-Lima et al., 2015)
Anaerobic Metabolism	Anaerobic glycolysis activation	(Cota-Ruiz et al., 2016; Martínez-Quintana et al., 2016)
	High glycogen reserves	(Czech-Damal et al., 2014; Geihs et al., 2013)
Respiratory cascade modification	Erythrocytes release or erythropoietin/mediated synthesis of new erythrocytes	(Lai et al., 2006)
	Hemoglobin/hemocyanin conformational changes leading to more oxygen tissues delivery	(Wells, 2009)

Figure legends

Figure 1. Anaerobic metabolism and its different end-products generated by aquatic animals. The diagram shows how glucose is converted to lactate or other end-products and the strategies displayed by different species to avoid or handle acidosis. Abbreviations: PEP, Phosphoenol pyruvate; OXA, Oxaloacetate; SUCC, Succinate; and, PRO, Propionate. Adapted and modified from Storey and Storey, 1990.

Figure 2. Overview of the glycolytic gene activation in shrimp cells during hypoxia via HIF-1. Next scenario is proposed: (1) in normoxic conditions HIF-1α is hydroxylated, ubiquitinated and subsequently degraded. (2) Opposite, HIF-1α stabilizes in hypoxic conditions, then migrates to the nucleus and dimerizes with HIF-1 β. Afterward, the complex becomes active and (3) induces the expression of glycolytic genes. Transcript increment is in favor of generating more protein/enzyme to (5) intensify ATP production in an oxygen independent manner through anaerobic glycolysis. Red dots represent low oxygen availability.

Fig. 3 Possible implications of gluconeogenic genes and enzymes induction in crustaceans and fish during hypoxia. Accumulation of FBP and PEPCK transcripts and increment of enzyme activities supports glucose 6-phosphate accumulation. This latter compound works as substrate for glucose production, as well as substrate for pentose phosphate pathway (see section 5 for major details and discussion). Abbreviations: PEPCK, Phosphoenol pyruvate carboxykinase; FBP, Fructose bisphosphatase; Glucose-6P, Glucose 6-phosphate.

Figure 1

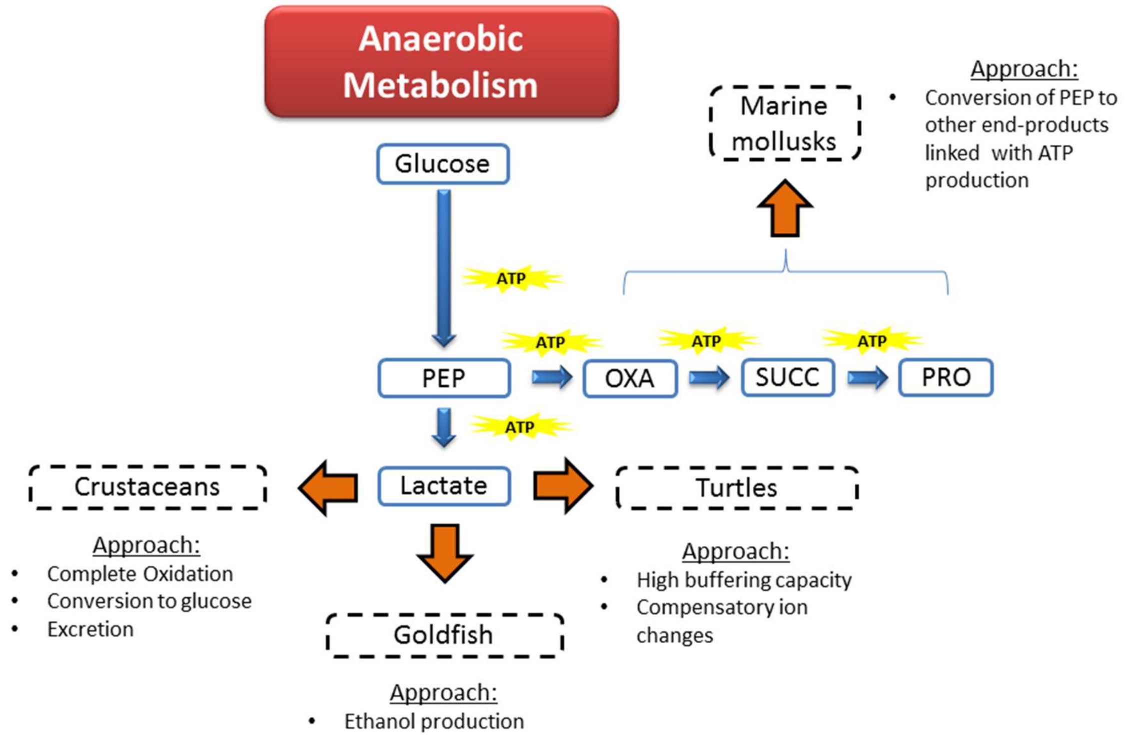

Figure 2

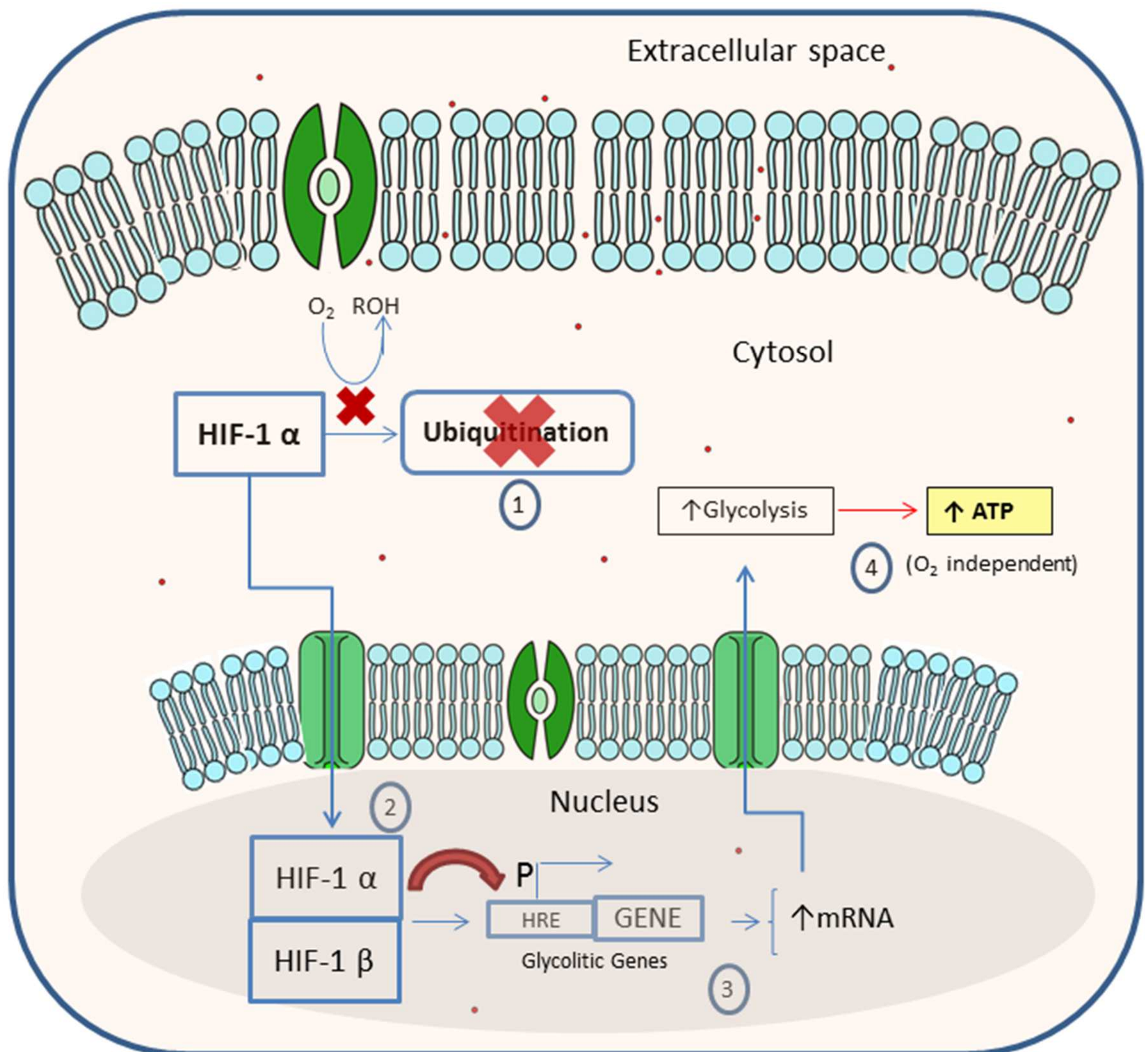

Fig. 3