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

HIPOXIA, REOXIGENACIÓN Y SILENCIAMIENTO DE SUPERÓXIDO DISMUTASA CITOSÓLICA MANGANOSA (cMnSOD) Y SELENOPROTEÍNA M (SelM) EN *Litopenaeus vannamei*: IMPLICACIONES EN EL METABOLISMO REDOX

POR:

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Los miembros del comité designado para revisar la tesis de Antonio García Triana, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

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Resumen

Las proteínas Selenoproteína M (SelM) y Superóxido Dismutasa citosólica Manganosa (cMnSOD) están involucradas en el sistema REDOX de *Litopenaeus vannamei*. Para entender su función en la regulación de especies reactivas de oxígeno, ambos transcritos fueron silenciados por separado. Adicionalmente, se sometieron camarones a hipoxia y reoxigenación. En los organismos silenciados y los sometidos a estrés por hipoxia y reoxigenación, se midió la cantidad de transcritos de SelM, la actividad de peroxidasas y la concentración de peróxido de hidrógeno. Por otro lado, de manera homóloga, se midieron transcritos de cMnSOD, actividad de Superóxido Dismutasas (SODs) y capacidad de producir anión superóxido. Los resultados mostraron que la expresión y silenciamiento de SelM y cMnSOD es regulada de manera diferencial en branquias y hepatopáncreas. En branquias, el silenciamiento de SelM y cMnSOD correlaciona con la disminución de actividad de peroxidasas y SOD, respectivamente. En hepatopáncreas no hay disminución de la actividad de peroxidasas y SOD cuando son silenciados. En hipoxia y reoxigenación, el aumento de transcritos de SelM en branquias concuerda con el incremento de actividad de peroxidasas, y no se detectan cambios en la concentración de H_2O_2. En hepatopáncreas, disminuye la actividad de peroxidasas e incrementa la concentración de H_2O_2, esto podría estar relacionado a la utilización del H_2O_2 como segundo mensajero ante variaciones en la concentración de O_2. En ambos tejidos, los transcritos de cMnSOD disminuyen en hipoxia y aumentan en reoxigenación, esto concuerda con la actividad de SOD y con las variaciones en la capacidad de producir $O_2^{\cdot -}$ en hemocitos. SelM y cMnSOD son clave en la regulación del metabolismo antioxidante en branquias. En hepatopáncreas, al parecer tienen funciones diferentes, cMnSOD tiene inferencia en la regulación del estrés oxidativo y SelM podría ser reguladora de segundos mensajeros. Ambas proteínas son importantes en la regulación del metabolismo REDOX de *L. vannamei*.

Sinopsis

El cultivo del camarón blanco *L. vannamei* es la actividad más rentable dentro de la camaronicultura. La salud de los camarones es de gran interés en esta industria debido a que condiciones inadecuadas del cultivo pueden llegar a ocasionar pérdidas cuantiosas. Parte importante del mantenimiento de la salud en los camarones es la regulación adecuada del sistema de óxido-reducción (REDOX), ya que desequilibrios en este sistema pueden generar daño celular y genético (Li, Jackson, 2002). Las variaciones físicas y químicas del medio ambiente pueden generar desequilibrio en el metabolismo REDOX del camarón. La hipoxia se define como la baja concentración de oxígeno en el medio y la reoxigenación como el retorno a concentraciones normales de oxígeno después de un periodo de hipoxia. Tanto la hipoxia como la reoxigenación suceden comúnmente en las prácticas de cultivo de camarones. Dichas variaciones en la concentración de oxígeno ocasionan daños en los tejidos (Zenteno-Savin*, et al.*, 2006) y decremento en la respuesta inmune (Dantzler*, et al.*, 2001; Jiang*, et al.*, 2005), en gran medida por las especies reactivas de oxígeno (ERO) generadas (Li, Jackson, 2002). Para regular las ERO y mantener un sistema REDOX estable, los organismos contamos con proteínas antioxidantes.

La Selenoproteína M (SelM) (Clavero-Salas*, et al.*, 2007; Garcia-Triana*, et al.*) y la superóxido dismutasa citosólica manganosa (cMnSOD) (Gomez-Anduro*, et al.*, 2007; Gomez-Anduro*, et al.*, 2006) son enzimas relacionadas al sistema REDOX recientemente descubiertas en *L. vannamei*. Poco se sabe de estas proteínas, y la determinación de su papel en el sistema REDOX de camarones es importante para establecer estrategias que conlleven a mejores prácticas de cultivo y aumento de la rentabilidad del mismo.

SelM es una proteína con actividad REDOX que contiene el llamado veintiunavo aminoácido selenocisteína (Ferguson*, et al.*, 2006). Se sabe muy poco acerca de la fisiología de SelM en camarones. Ante la infección con el virus de la mancha blanca (WSSV) la expresión de SelM es inducida transitoriamente en forma tiempo-específica (Clavero-Salas*, et al.*, 2007). Las peroxidasas juegan un papel importante en la degradación de ERO producidas durante el estrés oxidativo. La actividad de peroxidasas ha sido estudiada en camarones para evaluar su papel en el metabolismo y la respuesta es variada ante diferentes estresores. En *Penaeus monodon* infectado con WSSV, la actividad de peroxidasas disminuyó en branquias y hepatopáncreas (Rameshthangam, Ramasamy, 2006). Por otro lado, en *Litopenaeus schmitti* no se identificaron cambios de la actividad de peroxidasas en la hemolinfa en respuesta a ambientes hiposalinos como 18 y 8 ups (Lamela*, et al.*, 2005).

Por otro lado, la cMnSOD es una enzima antioxidante que dismuta el anión superóxido ($O_2^{\cdot -}$) en una molécula de peróxido de hidrógeno (H_2O_2) y oxígeno molecular (O_2). Se sabe muy poco acerca

de la fisiología de las cMnSODs de camarones, aunque se sabe que la cMnSOD de *L. vannamei* es afectada por factores ambientales. Cuando los camarones se exponen a pH 5.6 y 9.3 se induce su expresión (Wang*, et al.*, 2009). También se induce cMnSOD de manera transitoria en hemocitos después de la infección con el WSSV, alcanzando el máximo nivel 1 hora después de la infección (Gomez-Anduro, *et al.*, 2006). Aunque estos factores estresantes afectan la expresión de cMnSOD, no se sabía nada del efecto de la hipoxia y reoxigenación en la expresión de cMnSOD de camarones. Las superóxido dismutasas (SODs) juegan un papel importante en la degradación de $O_2^{\cdot -}$. La actividad de SOD ha sido estudiada en *L. vannamei* para evaluar su rol en el metabolismo y ante la respuesta a estrés generado por el ambiente. A 32 °C durante 24 h disminuye la actividad (Cheng*, et al.*, 2005), a bajas salinidades (25, 20 y 15 ups) y ante *Vibrio alginolyticus* disminuye su actividad (Li*, et al.*, 2010), y cuando se exponen a pH de 6.4 y 10.1 después de 6 a 24 h la actividad también disminuye (Li, Chen, 2008). De manera interesante, en salinidades muy bajas (3 ups) la actividad de SODs aumenta (Li, Chen, 2008). Hasta este trabajo, no se tenían reportes del efecto de la hipoxia y reoxigenación en la actividad de las SODs de *L. vannamei*.

En organismos de estudio donde no se pueden aplicar técnicas de genética clásica para generar mutantes nulos como *L. vannamei*, la activación del sistema de RNA de interferencia (RNAi) por inyección de RNA de doble cadena (dsRNA) es una técnica poderosa que permite estudiar la función de genes al eliminar transcritos específicos. El sistema de RNAi es un complejo proteico de eucariotas que con base en dsRNA, identifica de manera secuencia-específica fragmentos de RNA y genera sitios de corte internos que exponen el RNA a exonucleasas para su posterior degradación. La aplicación del silenciamiento en camarones por RNAi es relativamente reciente, con el primer reporte en el 2004, en donde se silenciaron genes del WSSV (Robalino*, et al.*, 2004) y se ha demostrado que el silenciamiento es gen-específico (Robalino*, et al.*, 2005). Esta es la primera tesis en CIAD que utiliza la activación del sistema RNAi.

La hipótesis propuesta en este trabajo fue que la función de la cMnSOD y de la SelM de *L. vannamei* es esencial en condiciones de normoxia, así como para responder al estrés causado por hipoxia y reoxigenación.

Para contrastar la hipótesis, primeramente se evaluó el efecto del silenciamiento de SelM o cMnSOD en condiciones de normoxia a través de la activación del sistema de RNAi mediante la inyección de dsRNA. Aunado a la corroboración del silenciamiento de los transcritos, se midió el efecto sobre la actividad de peroxidasas y concentración de H_2O_2 en camarones silenciados con dsRNA de SelM, y en la actividad de SODs en camarones silenciados con dsRNA de cMnSOD. Posteriormente, se determinó el efecto de hipoxia y reoxigenación en la cantidad de transcritos de SelM, así como en la actividad de peroxidasas y concentración de H_2O_2. Con el fin de entender en mayor medida el selenoproteoma de *L. vannamei*, se realizó una revisión sobre selenoproteínas de

invertebrados para identificar candidatos interesantes que no hayan sido descubiertos en *L. vannamei*.

De forma independiente, se determinó el efecto de hipoxia y reoxigenación en la cantidad de transcritos de cMnSOD, así como en la actividad de SODs y capacidad de producir $O_2^{\cdot -}$. El análisis en conjunto del silenciamiento de SelM y cMnSOD, así como el efecto de de la hipoxia y reoxigenación en transcritos de SelM y cMnSOD, la actividad de las proteínas antioxidantes y ERO involucradas en el sistema REDOX descritos previamente, permitieron determinar el papel que desempeñan estas proteínas en la regulación de ERO y en el mantenimiento del metabolismo REDOX de *L. vannamei*. El desarrollo de los estudios para cumplir los objetivos propuestos, se encuentra descrito en 3 capítulos que, junto a la presente sinopsis, conforman esta tesis.

CAPÍTULO 1: Importancia de SelM en el metabolismo redox de *L. vannamei* y el papel de selenoproteínas en crustáceos.

Este capítulo contiene dos artículos de datos originales y un artículo de revisión. Uno de estos trabajos se publicó en el 2010 y los otros dos están por ser enviados. El primero lleva por título: *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,* fue publicado en Comparative Biochemistry and Physiology 2010; A155:200-204. El segundo artículo lleva por título: *Effect of hypoxia and reoxygenation on the expression of Selenoprotein M (SelM), peroxidases activity and hydrogen peroxide concentration in gills and hepatopancreas of the white shrimp Litopenaeus vannamei*, será enviado al Journal of Experimental Marine Biology and Ecology. Finalmente, el tercer artículo de revisión tiene como título: Is the crustacean *selenoproteome similar to other arthropods homologs? A mini review*, y será enviado al Aquaculture Research.

En el primer artículo se determinó la importancia de SelM en condiciones de normoxia al detectarla en diferentes tejidos y se estudió el efecto de su silenciamiento. Asociado al silenciamiento se determinó su efecto sobre la actividad de peroxidasas y en la concentración de H_2O_2. De esta manera, se detectó la expresión de SelM en branquias, músculo, hepatopáncreas y pleópodos, encontrándose mayor abundancia en hepatopáncreas y branquias. Se llevó a cabo el silenciamiento por dsRNA y fue corroborado por qRT-PCR en branquias y hepatopáncreas. La actividad de peroxidasas disminuyó en branquias de los organismos silenciados y no se detectaron diferencias en hepatopancreas. La concentración de H_2O_2 no varió en branquias y hepatopáncreas de camarones silenciados.

En el segundo artículo de este Capítulo 1, se estudió el efecto de la hipoxia y reoxigenación en la expresión de SelM, la actividad de peroxidasas y la concentración de H_2O_2. Se encontró que la

expresión de SelM aumentó en hepatopáncreas y branquias después de 24 horas de hipoxia y 1 hora de reoxigenación. En branquias, la actividad de peroxidasas aumentó a las 24 horas de hipoxia, en 24 horas de hipoxia y una hora de reoxigenación y no se detectaron diferencias en la concentración de H_2O_2 en los diferentes tratamientos. Por otro lado, se detectó una disminución en la actividad de peroxidasas en hepatopáncreas después de los tratamientos de hipoxia y reoxigenación. De manera concordante a la actividad de peroxidasas, la concentración de H_2O_2 aumentó en hepatopáncreas.

En el artículo de revisión de este Capítulo 1 se estudian selenoproteomas de artrópodos para identificar candidatos interesantes de posibles selenoproteínas en crustáceos, entre las selenoproteínas más prometedoras se encuentran homólogos de SelD y SelB de *Drosophila melanogaster* involucrados en síntesis de selenoproteínas, así como SelBthD que se ha identificado como importante en la embriogénesis y en la viabilidad animal.

CAPÍTULO 2: Importancia de cMnSOD en el metabolismo redox de *L. vannamei.*

Este capítulo está conformado por un estudio, *Hypoxia, reoxygenation and cytosolic manganese superoxide dismutase (cMnSOD) silencing in Litopenaeus vannamei: effects on cMnSOD transcripts, superoxide dismutase activity and superoxide anion production capacity,* que fue preparado para ser enviado a la revista Developmental and Comparative Immunology.

Este artículo tiene como finalidad identificar la importancia de cMnSOD en el metabolismo REDOX de *L. vannamei*. En primera instancia, estudia el silenciamiento de cMnSOD en condiciones de normoxia. Asociado al silenciamiento, se determinó el efecto sobre la actividad de SOD en branquias y hepatopáncreas. En otro experimento, se estudió el efecto de la hipoxia y reoxigenación en la expresión de cMnSOD, la actividad de SOD y la capacidad de producir $O_2^{\cdot -}$ en hemocitos. Para llevar a cabo el silenciamiento, se inyectaron camarones con dsRNA correspondiente a la porción N-terminal de cMnSOD, ya que ésta es específica para cMnSOD y descarta otras MnSOD de ser silenciadas, haciendo este proceso totalmente específico. Los camarones inyectados con dsRNA tuvieron menor cantidad de transcritos en branquias y hepatopáncreas. La actividad de SOD también disminuyó en branquias en los camarones silenciados, pero no tuvo variación en hepatopáncreas. Por otro lado, los camarones sometidos a hipoxia tuvieron menor cantidad de transcritos y menor actividad de SOD en branquias y hepatopáncreas, e inclusive, disminuyó la capacidad de producir $O_2^{\cdot -}$ de los hemocitos. La reoxigenación revirtió los efectos de la hipoxia e incrementó los niveles de transcritos de cMnSOD, la actividad de SOD y la capacidad de producir $O_2^{\cdot -}$ de los hemocitos.

El **CAPÍTULO 3: Conclusiones**, cierra el estudio aprovechando los resultados y técnicas desarrolladas en los 2 primeros capítulos. El Capítulo 3 integra la información generada sobre SelM y cMnSOD y dilucida su papel en el metabolismo REDOX de *L. vannamei*.

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CAPÍTULO 1

Importancia de SelM en el metabolismo REDOX de *L. vannamei* y el papel de selenoproteínas en crustáceos

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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

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Silencing

a b s t r a c t

Selenoprotein M (SelM), is a selenocysteine containing protein with redox activity involved in the antioxidant response. In the white shrimp Litopenaeus vannamei, SelM expression in gills is induced transiently during viral infection by the White Spot Syndrome Virus (WSSV). We report that SelM expression was detected in healthy shrimp L. vannamei in gills, muscle, hepatopancreas and pleopods, with more abundance in the hepatopancreas and gills. SelM transcripts were silenced by intramuscular injection with double-stranded RNAs (dsRNAs). In gills and hepatopancreas, all shrimp injected with long dsRNAs had lower SelM transcripts levels compared with controls. Peroxidase activity and hydrogen peroxide concentration were measured to detect effects on antioxidants. Peroxidase activity decreased upon silencing of SelM in gills, but no significant effect was detected in hepatopancreas. In contrast, total cell hydrogen peroxide concentration did not change in gills and hepatopancreas of silenced shrimp. Non-heme peroxidases are new players in the oxidative stress system that need to be addressed in detail, as well as selenium as a critical micronutrient for the antioxidant and innate immune systems in crustaceans.

1. Introduction

Modulation of reactive oxygen species (ROS) is critical in all known organisms. ROS are part of the defense mechanisms and important regulators of metabolism, but their accumulation can be detrimental for many cellular components. Peroxidases are antioxidant enzymes that catalyze the reduction of peroxide or hydroperoxides using a donor substrate that is oxidized, regulating H_2O_2 levels. Many of them have heme as the prosthetic group but some others more recently discovered contain selenocysteine, the 21st amino acid. Selenoprotein M (SelM) is a selenocysteine containing protein with redox activity (Ferguson et al., 2006) and its expression and regulation is actively studied (Stadtman, 2005). Selenoproteins are differentially expressed. During development of zebrafish embryos, twenty-one selenoproteins mRNAs were analyzed and all of them exhibited expression patterns restricted to specific tissues and developmental stage (Thisse et al., 2003). The introduction of human SelM in a transgenic rat, resulted in higher peroxidase activity than in the non-transgenic animals, furthermore, the enzyme activity varied in the different tissues examined (Hwang et al., 2008).

Very little information is available about crustacean SelM physiology. The first marine invertebrate SelM was reported in the demosponge Suberites domuncula, (Muller et al., 2005) followed by its identification in the shrimp Litopenaeus vannamei (Clavero-Salas et al., 2007). L. vannamei SelM expression is transiently induced in gills after infection with the white spot syndrome virus (WSSV) on a time dependent manner (Clavero-Salas et al., 2007), suggesting that it plays an important role in the defense against the virus. However, much remains to be investigated about SelM in normal conditions of the shrimp and in many other processes.

In non-model organisms as shrimp, silencing by RNA interference (RNAi) is an alternative to understand gene function, opening an opportunity and a strategy when knowledge and methodology for classical genetics are lacking. Until now, there is no information about selenoprotein M expression and silencing in crustaceans.

Peroxidases play key roles in the degradation of ROS produced during the oxidative stress. Peroxidase activity has been studied in shrimps and prawns to evaluate their roles in metabolism and in the response to stress. Important changes in peroxidase activity have been detected in different tissues. During embryonic and larval developmental stages of the prawn Macrobrachium malcolmsonii, higher peroxidase activity was found in hepatopancreas than in gills (Arun and Subramanian, 1998). In the shrimp Penaeus monodon infected with WSSV, peroxidase activity in gills and hepatopancreas decreased (Rameshthangam and Ramasamy, 2006). In contrast, no

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changes were found in response to osmotic alteration, since peroxidase activity in the haemolymph of the shrimp Litopenaeus schmitti is not affected by hyposaline environments as 18 and 8 psu (Lamela et al., 2005). Selenoproteins are widely distributed among eukaryotes. In mouse, mutations that render the cell unable to incorporate selenium into proteins result in embryonic lethality (Stillwell and Berry, 2005). Phylogenetic analysis shows that aquatic organisms apparently retained and sometimes expanded their selenoproteomes, whereas the selenoproteomes of some insects and other terrestrial organisms were reduced or completely lost (Chapple et al., 2009).

We detected the expression of SelM in different shrimp tissues and evaluated the effect of silencing by RNAi in the transcripts of gills and hepatopancreas. As indicators of an effect in antioxidants, peroxidase activity and H_2O_2 concentration after silencing with SelM dsRNA were determined in gills and hepatopancreas.

2. Materials and methods

2.1. Animals

White shrimp L. vannamei (average weight 15 g) were donated by the University of Sonora, DICTUS, Kino Bay Unit. Shrimp were acclimated for 14 days at 28 °C, 37 psu (Practical Salinity Units), under constant aeration (6 mg/L dissolved oxygen) and fed ad libitum twice daily with commercial feed (Camaronina 35®, Agribrands Purina, Mexico). One-third of the water volume was changed daily, and uneaten food particles and feces were removed daily. Before injecting the shrimp, randomly selected healthy intermolt shrimp were placed in separate aquaria.

2.2. Total RNA preparation, first strand cDNA synthesis and SelM mRNA detection

Gills, muscle, hepatopancreas and pleopods were dissected from shrimp, immediately frozen in liquid nitrogen and kept at −80 °C until used. Total RNA was extracted using Trizol® (Invitrogen) according to instructions of the manufacturer. Reverse transcription (RT) was performed using Quantitect Reverse transcription (Qiagen)®. For this, 1 μg of total RNA was reverse transcribed using oligo dT (12–18). For a 20 μL final volume PCR reaction, the following were added: 18 μL of Platinum® PCR Supermix (Invitrogen), 0.5 μL of each primer (20 μM) and 1 μL of cDNA (equivalent to 50 ng of total RNA). Detection of SelM transcripts was done using the primers BSelMF (5′-GATTTGACCAGGTTGTGGAG-3′) and BSelMR (5′-AAGCTGCATTTTGGAGTCTG-3′) designed based on our previously reported sequence (positions 255–648 of the sequence, GenBank accession no. DQ907947). These primers amplify a 393 bp fragment and were used under the following conditions: 95 °C, 1 min, 95 °C, 30 s, 60 °C, 1 min, 68 °C, 1 min (31 cycles); 72 °C, 10 min and kept at 4 °C until used. A ribosomal protein L8 producing a 428 bp fragment, (positions 72–500 of the sequence, DQ316258) was amplified side by side for comparisons using the forward and reverse primers (5′-GAAGAGGATCGGTGTTCAAGT-3′) and (5′-CTCCTGAAGGAAGCTTTACAC-3′), respectively and using the conditions mentioned before. Positive and negative controls were included. The PCR products were analyzed by agarose gel electrophoresis.

2.3. Synthesis of dsRNA

Two DNA templates were used to produce separately each ssRNA by in vitro transcription for each strand of SelM. Also, ssRNA was synthesized from a clone for a mango ethylene receptor (ER) that was used as an unrelated negative dsRNA control. The DNA templates were first cloned in the pGEM T Easy Vector (Promega®) in both orientations. A 454 pb fragment of the coding sequence of SelM (positions 194–648 of the sequence, DQ907947) was obtained with the primers SelMFwa1 (TTCCACAATGCCAAGTTCAA) and BSelMR (5′-AAGCTGCATTTTGGAGTCTG-3′), while an 850 pb of the ER clone was used (clone graciously donated by Dr. Islas-Osuna, unpublished data). The clones obtained in pGEM T Easy contain a T7 promoter adjacent to the inserted DNA fragments. The T7 primer was combined with specific primers to obtain from each clone a PCR product containing the T7 promoter. Single-stranded RNA (ssRNA) was transcribed in vitro from the PCR products templates using T7 phage RNA polymerases RiboMAX (Promega®). Then the DNA template was degraded with DNase I using 1 U/μg of template DNA. The in vitro synthesized RNAs were purified according to the manufacturer's protocol. Equal amounts of the two cRNA strands were mixed and annealed by incubation at 80 °C for 10 min and slowly cooled to room temperature for 50 min. The formation of dsRNA was detected by the changes in migration of the dsRNA vs the ssRNA by agarose gel electrophoresis.

2.4. Knockdown assay for Sel M

L. vannamei adults at intermolt stage were placed in a 50 L glass aquarium with seawater (37 psu), temperature controlled (28 °C) and under constant aeration. Shrimp were randomly divided into seven groups. The following treatments were included: non-injected (NI), injected with shrimp saline solution (ISS, 400 mM NaCl, 20 mM Tris, pH, 7.5), injected with 20 μg of dsRNA mango ethylene receptor (ER) as controls, and with varying quantities of SelM dsRNA including 5, 10, 20 and 40 μg; all the dsRNA were resuspended in shrimp saline solution. The shrimps were injected intramuscularly through the dorsal area of the second abdominal segment. The samples were collected 24 h after injection of the dsRNA, frozen on liquid nitrogen and stored at −80 °C. SelM and ribosomal protein L8 transcripts were detected by real time quantitative RT-PCR (qRT-PCR) in gills and hepatopancreas. Two separate cDNA reactions and two PCR reactions for each individual shrimp and tissue were done (n = 8) for qRT-PCR on an iQ5 Real-Time PCR Detection System (Bio-Rad) in 20 μL final volume containing 10 μL of iQ SYBR Green Supermix (Bio-Rad), 8 μL of H_2O, 0.5 μL of each primer (20 μM) and 1 μL of cDNA (equivalent to 50 ng of total RNA). A fragment of 393 bp for SelM was obtained using the primers BSelMF and BSelMR under the following conditions: 95 °C, 5 min, 95 °C, 30 s, 65 °C, 35 s, 75 °C, 55 s (40 cycles). A single fluorescence measurement and a final melting curve program increasing 0.3 °C each 20 s from 60 °C to 95 °C were run to discard unspecific amplifications. The L8 cDNA (positions 333–500 of the sequence, GenBank accession no. DQ316258) was amplified side by side for comparisons using the L8F2 (5′-TAGGCAATGTCATCCCCATT-3′) and L8R2 (5′-TCCTGAAGGGAGCTTTACACG-3′) primers, producing a fragment of 167 bp and under the same conditions. Positive and negative controls were included. Standard curves of SelM and L8 were run to determine the efficiency of amplification using dilutions from 5×10^{-3} to 5×10^{-8} ng/μL of PCR fragments. For each measurement, expression levels (ng/μL) were normalized to L8 and expressed as relatives values (SelM/L8).

2.5. Peroxidase activity

This activity was measured using guaiacol as the substrate (Pérez-Tello et al., 2009) with the following modifications. Hepatopancreas and gills (60 mg, n = 8) were homogenized in 120 μl of 0.1 M Tris–HCl, 5 mM β-mercaptoethanol, pH 8, centrifuged at 12,000 g/20 min/4 °C and the aqueous extract separated. In a 96-well microplate, 25 μL of the extract from gills or hepatopancreas was mixed with 160 μL of 0.01 M sodium acetate, 0.5% guaiacol, pH 5.3, and then 25 μL of 0.1% H_2O_2 were added. This reaction was immediately mixed and the absorbance was recorded at 490 nm after 60 s in a microplate reader. Activity was expressed as the difference in absorbance after 60 s of reaction per mg of protein. Protein concentration was determined

using the BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Specific activity unit was defined as the amount of enzyme that causes an absorbance change of 0.001/min/mg protein.

2.6. Hydrogen peroxide determination

The H_2O_2 concentration was determined using phenol red as substrate (Messner and Boll, 1994) with the following modifications. Hepatopancreas and gills (25 mg, n = 8) were sonicated in 200 µl of 0.1 M Tris–HCl, 5 mM β-mercaptoethanol, pH 8, then 320 µl of 10% SDS were added, centrifuged at 12,000 g/20 min/ 4 °C and the aqueous extract was separated. In a 96-well microplate, to 6 µL of the extract from gills or hepatopancreas, 194 µL of phenol red mix (10 mM Mes, 40 µM phenol red and 0.01 mg/mL horseradish peroxidase, pH 6.5) was added and mixed, incubated 3 min at room temperature and the reaction stopped by adding 4 µL of 0.5 N NaOH. The absorbance was measured at 550 nm in a microplate reader. A standard curve from 0 to 40 nM of H_2O_2 was used.

2.7. Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple comparison test (α = 0.01) were applied to the data. The NCSS and PASS Statistical Systems software was used.

3. Results

3.1. Silencing of SelM reduced transcript levels differentially in gills and hepatopancreas

We have previously reported transient induction of SelM mRNA after WSSV infection in gills, although the expression in other tissues was not analized. In this study we investigated the expression of SelM during normal conditions and also, the effect of silencing using SelM dsRNA. In normal conditions, SelM expression was detected in all tissues tested (gills, muscle, hepatopancreas and pleopods) with different relative expressions (Fig. 1). Judging from the intensity of the bands, SelM higher expression was detected in hepatopancreas, followed by gills, muscle and pleopods.

Intramuscular injection of SelM dsRNA appeared to knock down most of the transcripts in gills after 24 h of treatment (Fig. 2). No differences were detected between NI, ISS and ER intramuscularly injected shrimp (α = 0.01). In the shrimp injected with 5, 10, 20 and 40 µg of Sel M dsRNA, gills SelM transcript levels were lower than in NI, ISS and ER (α = 0.01). As dsRNA doses increased, a decrease in SelM transcripts was detected (Fig. 2). Injection of 5 µg dsRNA reduced SelM transcripts to 76% of the ISS expression, while for 10 µg dsRNA, the reduction was 79% and at 20 µg dsRNA, the value was even lower, corresponding to 92% less of the ISS expression. Finally, for 40 µg, the relative expression was 90% less of the ISS. Interestingly, handling of the shrimp caused 12 and 13% less transcript levels in the ISS and ER treatments, although these differences were not significant (α = 0.01) to NI (Fig. 2), probably due to the high individual variability of the experimental animals. SelM transcripts in hepatopancreas in non-injected shrimp were almost 4-fold higher than in gills. In

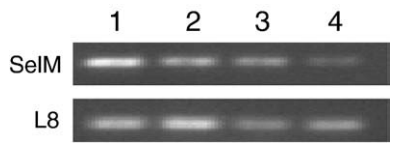

Fig. 1. Detection of SelM transcripts by RT-PCR. RT-PCR products of SelM were analyzed in a 1.5% agarose gel and compared to ribosomal protein L8. Lanes 1, 2, 3 and 4 are for gills, muscle, hepatopancreas and pleopods, respectively. Ribosomal protein L8 mRNA levels were also determined by RT-PCR and used for comparisons.

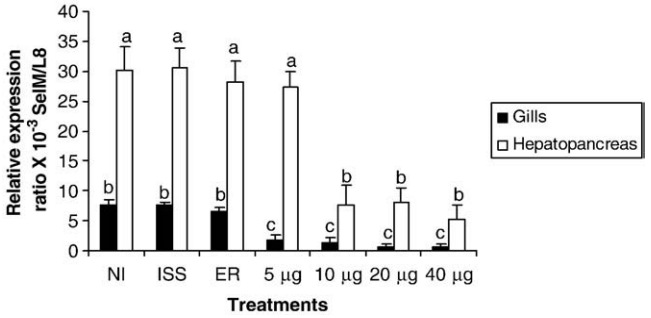

Fig. 2. Expression of SelM relative to L8 in gills and hepatopancreas of dsRNA-injected and control shrimp by real time qRT-PCR. Non-injected (NI), injected with saline solution (ISS), injected with 20 µg of dsRNA mango ethylene receptor (ER), injected with 5 µg of dsRNA (5 µg), injected with 10 µg of dsRNA (10 µg), injected with 20 µg of dsRNA (20 µg) and injected with 40 µg of dsRNA (40 µg). Levels of transcripts were measured in duplicates for every individual shrimp. Bars represent mean ± standard errors (n = 8). SelM levels significantly different are labeled with different literals (ANOVA $p < 0.01$).

contrast to the case in gills, intramuscular injection of 5 µg of dsRNA did not decrease SelM levels and the effect was only noted when the amounts injected were 10, 20 and 40 µg (Fig. 2). NI shrimp did not have differences in expression compared to ISS, ER and 5 µg (α = 0.01). In 10, 20 and 40 µg injected shrimps, SelM transcripts were lower than in NI, ISS, ER and 5 µg injected shrimps (α = 0.01). At 10 µg dsRNA, relative expression decreased 4-fold of the ISS expression, at 20 µg dsRNA, relative expression was 3.8-fold less of the ISS expression and at 40 µg relative expression was reduced 5.7-fold of the ISS expression. In this case, the effect of handling of the shrimp resulted in ~7- less detected in the ER treatment, but as mentioned before and due to individual variation, the numbers are not statistically different (α = 0.01) from NI (Fig. 2).

3.2. Silencing of SelM and effects in peroxidase activity and hydrogen peroxide

Peroxidase activity in gills was significantly different between NI and ER (α = 0.01) (Fig. 3). Also, peroxidase activities detected in ISS and ER are statistically different (α = 0.01) in the shrimp injected with 5, 10, 20 and 40 µg dsRNA and there are no differences between ISS and ER. After injection of 5, 10, 20 and 40 µg dsRNA treatments (α = 0.01), no differences in the response to the injected dsRNA were detected. SelM dsRNA treatments decreased peroxidase activity to 77% with respect to RE in gills. In contrast, in hepatopancreas, significant differences in peroxidase activity were detected among NI and the ISS, ER, 5, 10, 20 and

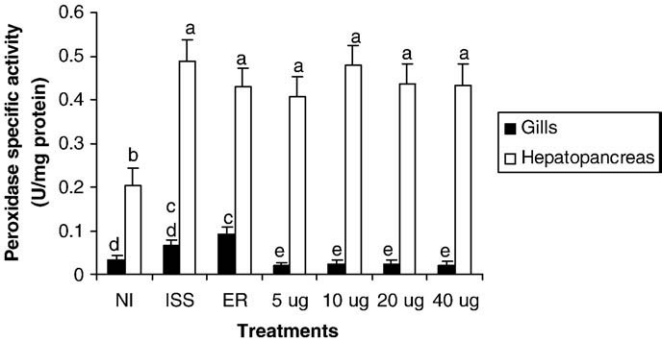

Fig. 3. Peroxidase activity in gills and hepatopancreas of dsRNA-injected and control shrimp. Non-injected (NI), injected with saline solution (ISS), injected with 20 µg of dsRNA mango ethylene receptor (ER), injected with 5 µg of dsRNA (5 µg), injected with 10 µg of dsRNA (10 µg), injected with 20 µg of dsRNA (20 µg) and injected with 40 µg of dsRNA (40 µg). Levels of peroxidase activity were measured in triplicate for every shrimp. Bars represent mean ± standard errors (n = 8). SelM levels significantly different are labeled with different literals (ANOVA $p < 0.01$).

40 µg dsRNA treatments ($\alpha = 0.01$) (Fig. 3). There is no statistically significant difference ($\alpha = 0.01$) in the mean values among ISS, RE, 5, 10, 20 and 40 µg dsRNA. It is important to mention that the ISS injection by itself increased 2.5-fold the peroxidase activity compared with the non-injected shrimp and these values were basically kept in the dsRNA-injected animals, suggesting that perhaps, other peroxidases are induced by stress.

Differences in H_2O_2 between gills and hepatopancreas were detected. In gills there are no differences ($\alpha = 0.01$) in H_2O_2 content among all the controls and treatments ($\alpha = 0.01$) (Fig. 4), and this group is statistically different ($\alpha = 0.01$) to hepatopancreas. Similarly, in hepatopancreas, no significant differences in H_2O_2 concentrations among all the treatments were detected ($\alpha = 0.01$) (Fig. 4). It is worthwhile to mention that the H_2O_2 content in hepatopancreas is 3-fold compared to gills, perhaps related to the functions of these tissues.

4. Discussion

SelM expression analyzed by RT-PCR indicated that this transcript is more abundant in gills and hepatopancreas, while the expression in muscle and pleopods are lower. The higher abundance of SelM in gills and hepatopancreas might be related to the higher enzymatic activity in hepatopancreas and to the vastly respiratory and mitochondrial activity in the gills. These kinds of results were only found in a gasteropod previously (De Zoysa et al., 2008). This is the first report of SelM tissue-specific differential expression in crustaceans.

Silencing of SelM was studied in gills and hepatopancreas. Injection of the SelM dsRNA significantly reduced the steady-state levels of mRNA ($\alpha = 0.01$) compared with the negative controls (ISS or ER). The RNAi gene-silencing effect is mediated by dsRNA. In this study, long dsRNA was produced and used for the RNAi experiments. The amount of dsRNA needed for silencing is directly proportional to the abundance of the target mRNA and it is strongly influenced by the tissue (Maningas et al., 2008). In gills, at least 5 µg were sufficient to activate the process of SelM transcripts degradation, although a small amount of the transcripts did remain. Conversely, in hepatopancreas, the amount of dsRNA necessary was much higher, where at least 10 µg of SelM dsRNA was needed to lower SelM transcript levels. From the data of SelM transcript abundance in the non-injected shrimp, it is clear that hepatopancreas has higher amounts than gills, and this may explain the need for more dsRNA in the former tissue. In L. schmitti

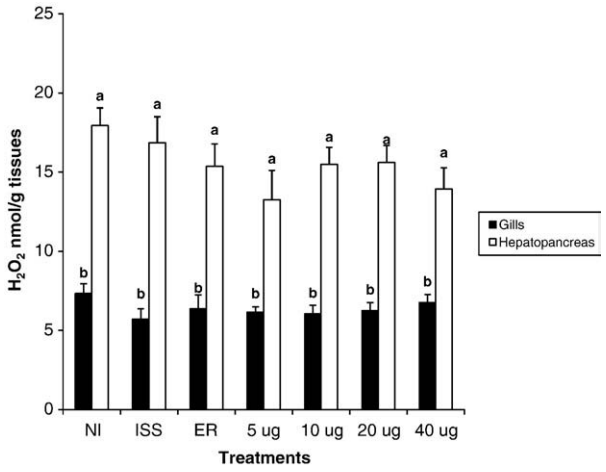

Fig. 4. H_2O_2 concentration in gills and hepatopancreas of dsRNA-injected and control shrimp. Non-injected (NI), injected with saline solution (ISS), injected with 20 µg of dsRNA mango ethylene receptor (ER), injected with 5 µg of dsRNA (5 µg), injected with 10 µg of dsRNA (10 µg), injected with 20 µg of dsRNA (20 µg) and injected with 40 µg of dsRNA (40 µg). Levels of H_2O_2 were measured in triplicate for every individual shrimp. Bars represent mean ± standard errors ($n = 8$). SelM levels significantly different are labeled with different literals (ANOVA $p < 0.01$).

injection of 20 µg of dsRNA per shrimp (10 g animals) was sufficient to silence the crustacean hyperglycemic hormone (CHH) expression (Lugo et al., 2006), while 3–5 µg dsRNA was used in 23–28 g Metapenaeus ensis shrimp to silence the gonad stimulating hormone (Tiu et al., 2007) and 1 and 10 µg dsRNA for transglutaminase and clotting protein, respectively, in P. monodon, to result in silencing (Maningas et al., 2008). In our study, the amount of dsRNA injected into each animal was between 5 and 40 µg for each shrimp (15 g). From 10 to 40 µg of SelM dsRNA successfully reduced the SelM transcripts in gills and hepatopancreas, indicating an appropriate SelM RNAi silencing activation.

It was also shown that the dsRNAs effect is detected 24 h after injection, although a time-course experiment was not done. Shrimp RNAi activation by dsRNA injection appears to be variable and the identification of adequate injection quantities is very important in this process. It has been suggested that more abundant mRNAs may need larger quantities of dsRNA for appropriate silencing (Tirasophon et al., 2005). When 20 µg of dsRNA was injected, the transcript decrease was 85% in hepatopancreas and 92% in gills 24 h after injection, therefore, it appears that 20 µg is an adequate quantity to silence L. vannamei (15 g) SelM transcripts. No visual harmful effects were detected in the shrimp injected with the SelM dsRNA during the experiments; the animals apparently kept behaving as before the injection.

Interestingly, the silencing effect is different among tissues in shrimps. As far as we know; there is only one report of differential tissue-specific silencing by dsRNA. In the shrimp P. monodon, the clotting protein transcript was silenced only in gills and heart tissues one day post-injection, and no silencing was detected in hemocytes, hepatopancreas, intestine and lymphoid organ during that period of time, however at day 7 post-injection, systemic gene silencing was detected (Maningas et al., 2008). These results open several questions. Is the differential tissue-specific silencing primarily related to receptors or membranes of the tissues or to the amount and availability of the transcript? In mammalians, several ligands are reported to deliver siRNA into the cell cytoplasm (Yu et al., 2009). In the black tiger shrimp P. monodon, quantitative RT-PCR analysis showed that dicer mRNA – a component of the silencing complex – is highly expressed in haemolymph cells and lymphoid organ tissues (Su et al., 2008), if siRNAs are produced in the haemolymph or lymphoid organ, which kind of natural receptors exists to internalize the silencing effect to the shrimp tissues as gills and hepatopancreas? And what would be the main differences in the gills and hepatopancreas siRNA receptors?

In our study, peroxidase activity was higher in hepatopancreas than in gills and is consistent with the results obtained in the prawn Macrobrachium malcolmsonii (Arun and Subramanian, 1998). However a higher silencing effect was seen in gills, where a decrease of 73% was detected in all the SelM silenced treatments; indicating that SelM is a very important component of the antioxidant response in gills. Reports of peroxidase changes in other invertebrates are known. For example, the selenoprotein glutathione peroxidase mRNA expression was significantly up-regulated in gills of the disk abalone Haliotis discus after H_2O_2 injection and Vibrio alginolyticus infection (De Zoysa et al., 2008). Although the abundance of SelM transcripts is higher in hepatopancreas compared with all the tissues studied, hepatopancreas peroxidase activity was not affected significantly by SelM silencing by dsRNA. Interestingly, the handling and injection of control and treated shrimp seem to have a more important effect in peroxidase activity compared to the NI shrimp.

In agreement to SelM transcripts and peroxidase activity, H_2O_2 concentration was higher in hepatopancreas than in gills, presuming a more profuse role of this ROS in hepatopancreas. The concentration of H_2O_2 in the hepatopancreas is within the order of magnitude reported in mammalian testicles, with H_2O_2 values of 24 nmol/g of tissue in rats and 16 nmol/g of tissue in mice (Samanta and Chainy, 2002). In gills and hepatopancreas, the silencing of SelM did not induce changes in H_2O_2 concentration. H_2O_2 plays key roles in metabolism as a second

messenger (Rojkind et al., 2002). Our results suggest that to maintain the steady state level of H_2O_2 after the depletion of SelM, the shrimp should decrease H_2O_2 production to compensate the elimination of this enzyme. H_2O_2 producing oxidases like NADPH oxidase have been reported in Fenneropenaeus indicus (Sarathi et al., 2007) and Homarus americanus (Anderson and Beaven, 2005). Also, superoxide dismu- tases as the cytosolic manganese superoxide dismutase (cMnSOD) in

L. vannamei (Gomez-Anduro et al., 2006), Cu–Zn superoxide dis- mutase in Callinectes sapidus (Brouwer et al., 2003), Cu–Zn superoxide dismutase in Macrobrachium rosenberguii (Cheng et al., 2006) and a superoxide dismutase in Palaemonetes pugio (Brouwer et al., 2007) have been identified. To understand the mechanisms of H_2O_2 regulation in shrimp, it would be very interesting to study proteins as superoxide dismutases, NADPH oxidases and other oxidases involved in H_2O_2 production when SelM and/or other antioxidant enzymes are silenced.

SelM is differentially expressed in shrimp tissues and its silencing

is differentially regulated in gills and hepatopancreas. Silencing affects peroxidase activity also in a tissue-specific manner and the concen- tration of H_2O_2 is not affected in the silenced tissues. All these changes and the H_2O_2 concentration maintenance seem to be related to the regulation of the stationary state in shrimp physiology. Future work will uncover the complete role of selenoproteins in marine crustaceans redox system.

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Effect of hypoxia and reoxygenation on Selenoprotein M (SelM) expression, peroxidases activity and hydrogen peroxide concentration in gills and hepatopancreas of the white shrimp *Litopenaeus vannamei*

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Glossary

Hypoxia. Low environmental O_2 concentration.

Reoxygenation. increase of O_2 concentration after hypoxia.

Reactive Oxygen Species (ROS). Molecules that contain oxygen with unpaired valence shell electrons.

Hydrogen peroxide (H_2O_2). ROS constituted by two molecules of oxygen and two molecules of hydrogen.

Reduction-oxidation (redox). Chemical reaction in which atoms have their oxidation number changed.

Selenoprotein M (SelM). Protein with redox activity.

Peroxidases. Antioxidant enzymes that catalyze the reduction of peroxide or hydroperoxides using a donor substrate (typically a thiol) which is oxidized, regulating H_2O_2 levels.

Gills. Respiratory organ of aquatic organisms.

Hepatopancreas. Nutrients absorption and accumulation organ of aquatic organisms.

Abstract

Low O_2 concentration (hypoxia) and the subsequent increase of O_2 concentration (reoxygenation) in the environment are a cause of oxidative stress. Selenoprotein M (SelM), is a selenocysteine containing protein with redox activity involved in the antioxidant response. In the white shrimp *Litopenaeus vannamei*, SelM silencing by RNAi decreases peroxidase activity in gills. We report that SelM expression increased in hepatopancreas and gills after 24 h of hypoxia followed by 1 h of reoxygenation. In gills, peroxidase activity increased after 24 h hypoxia and also, after 24 h hypoxia and 1 h reoxygenation. A significant decrement was detected in peroxidase activity in hepatopancreas after hypoxia and reoxygenation treatments. A similar trend in peroxidase activity in response to hypoxia and reoxygenation was detected in hydrogen peroxide (H_2O_2) concentration that increased in hepatopancreas, but was not different in gills. SelM appears to be a key enzyme in gill oxidative stress regulation, since the increased transcripts expression is associated with an increase in peroxidases activity and unchanged H_2O_2 concentration during the hypoxia and reoxygenation stress conditions.

Keywords: Hypoxia, reoxygenation, *Litopenaeus vannamei*, peroxidase, selenoprotein M (SelM), hydrogen peroxide.

1. Introduction

Low environmental O_2 concentration (hypoxia) and the subsequent increases of O_2 concentration (reoxygenation) are physiological stressors to most multicellular obligatory aerobic organisms. Reoxygenation of hypoxic tissues restores the energy potential and results in generation of reactive oxygen species (ROS) (Li, Jackson, 2002). Modulation of ROS is vital in all known organisms. ROS are important regulators of metabolism, but their accumulation can be harmful for many cellular components. In *Litopenaeus vannamei*, superoxide anion ($O_2^{\cdot -}$) production increased while antioxidant capacity decreased in hepatopancreas at the first hours of reoxygenation (Zenteno-Savin, *et al.*, 2006). This could translate into tissue damage, which may significantly jeopardize the commercial aquaculture product (Zenteno-Savin, *et al.*, 2006). Besides, many crustaceans are known to be tolerant to hypoxia (McMahon, 2001). Little is known about the antioxidant proteins involved in the regulation of ROS generation in response to hypoxia and reoxygenation.

Peroxidases are antioxidant enzymes that catalyze the reduction of peroxide (H_2O_2) or hydroperoxides using a donor substrate (typically a thiol) which is oxidized, regulating H_2O_2 levels. Many of them have heme as the prosthetic group, but other proteins discovered recently contain selenocysteine, the 21st amino acid. Selenoprotein M (SelM) belongs to that category, as an important molecule for maintaining the cellular redox balance (Ferguson, *et al.*, 2006) and its expression and regulation is actively studied (Stadtman, 2005). Selenoproteins are differentially expressed. During development of zebrafish embryos, 21 selenoprotein mRNAs were analyzed and all of them exhibited expression patterns restricted to specific tissues and developmental stage (Thisse, *et al.*, 2003). We recently identified that SelM is expressed differentially in gills and hepatopancreas, with higher levels in hepatopancreas (Garcia-Triana, *et al.*, 2010). The

understanding of SelM, its differential expression and the importance for shrimp physiology is just beginning to be revealed.

Very little information is available about shrimp SelM physiology. *L. vannamei* SelM expression is transiently induced in gills after infection with the white spot syndrome virus (WSSV) on a time-dependent manner (Clavero-Salas, *et al.*, 2007), suggesting that it plays an important role in the defense against the virus. In our recent work, SelM was silenced by dsRNA and decreased peroxidase activity upon silencing was detected in gills, but not in hepatopancreas. In contrast, no change in H_2O_2 concentration in gills and hepatopancreas occurred in the silenced shrimp (Garcia-Triana, *et al.*, 2010). However, much remains to be investigated about SelM in normal conditions of the shrimp and in many other processes, such as hypoxia and reoxygenation, which might shed light about its function in specific conditions.

Peroxidases play key roles in the degradation of ROS produced during the oxidative stress. Peroxidase activity has been studied in shrimp and prawns to evaluate their roles in metabolism and in the response to stress (García-Triana, *et al.*, 2010). Important changes in peroxidase activity have been detected in different tissues. During embryonic and larval developmental stages of the prawn *Macrobrachium malcolmsonii*, higher peroxidase activity was found in hepatopancreas than in gills (Arun, Subramanian, 1998). In the shrimp *Penaeus monodon* infected with WSSV, the peroxidase activity in gills and hepatopancreas decreased (Rameshthangam, Ramasamy, 2006). In contrast, no changes were found in response to osmotic alteration, since peroxidase activity in the haemolymph of the shrimp *Litopenaeus schmitti* is not affected by hyposaline environments as 18 and 8 psu (Lamela, *et al.*, 2005). Differences are also due to the tissues studied, as we recently reported that peroxidase activity is 7-fold higher in hepatopancreas compared to gills (Garcia-Triana, *et al.*, 2010). In the Chinese shrimp, *Fenneropenaeus chinensis*, during hypoxia a reduced peroxidase activity was detected

(Li, *et al.*, 2006). Besides, there is no information about the effects of hypoxia and reoxygenation in shrimp peroxidase activity.

A single report determined the H_2O_2 concentration in shrimp tissues. In *L. vannamei*, H_2O_2 is 3-fold higher in hepatopancreas compared to gills (Garcia-Triana, *et al.*, 2010). Comparison of H_2O_2 production in lobsters collected from field sites and then submitted to different levels of dissolved oxygen suggests an influence of dissolved oxygen in H_2O_2 production (Moss, Allam, 2006). But, there are also no reports relating the effect of hypoxia and reoxygenation to H_2O_2 production in shrimp tissues.

We evaluated the effect of hypoxia and reoxygenation on SelM expression in gills and hepatopancreas. In addition, peroxidase activity and H_2O_2 concentration was determined in the same tissues.

2. Materials and Methods

2.1 Animals

White shrimp *L. vannamei* (average weight 15 g) were donated by the Selecta farm at Kino Bay, Sonora. Shrimps were acclimated for 14 days at 28 °C, 37 psu, constant aeration (6 mg/L dissolved oxygen) and fed *ad libitum* twice daily with commercial feed (Camaronina 35®, Agribrands Purina, Mexico). One-third of the water volume was changed daily, and uneaten food particles and feces were removed daily. Randomly selected healthy intermolt shrimp were placed in separate aquaria.

2.2 Total RNA preparation, first strand cDNA synthesis and Sel M mRNA detection

Gills and hepatopancreas were dissected from shrimp, immediately frozen in liquid nitrogen and kept at -80 °C until used. Total RNA was extracted using Trizol® (Invitrogen) according to instructions of the manufacturer. Reverse transcription (RT)

was performed using Quantitect Reverse transcription (Qiagen)®. For this, 1 µg of total RNA was reverse transcribed using oligo dT (12–18).

2.3 Hypoxia and reoxygenation assay for Sel M

L. vannamei adults at intermolt stage were placed in 150 L glass fiber aquarium with seawater (37 psu), controlled temperature (28 °C) and constant O_2 concentration (6 mg/L). Shrimp were randomly divided into five groups. The following treatments were included: Normoxia (Nor, 6 mg/L) as control, hypoxia (Hyp, 1.5 mg/L) for 6 h (Hyp6), 6 h hypoxia and 1 h reoxygenation (Reo6, 6 mg/L), 24 h hypoxia (Hyp24), 24 h hypoxia and 1 h reoxygenation (Reo24). Samples were collected at the end of the hypoxia or reoxygenation treatment, frozen on liquid nitrogen and stored at -80 °C. SelM and ribosomal protein L8 transcripts were detected by real time quantitative RT-PCR (qRT-PCR) in gills and hepatopancreas. Two separate cDNA reactions and two PCR reactions for each individual shrimp and tissue were done (n = 8) for qRT-PCR on an iQ5 Real-Time PCR Detection System (Bio-Rad) in 20 µL final volume containing 10 µL of iQ SYBR Green Supermix (Bio-Rad), 8 µL of H_2O, 0.5 µL of each primer (20 µM) and 1 µL of cDNA (equivalent to 50 ng of total RNA). A fragment of 393 bp for Sel M was obtained using the primers BSelMF and BSelMR under the following conditions: 95 °C, 5 min, 95 °C, 30 s, 65 °C, 35 s, 75 °C, 55 s (40 cycles). A single fluorescence measurement and a final melting curve program increasing 0.3 °C each 20 s from 60 °C to 95 °C was run to discard unspecific amplifications. The L8 cDNA, GenBank accession number **DQ316258** was amplified side by side for comparisons using the L8F2 (5'-TAGGCAATGTCATCCCCATT-3') and L8R2 (5'-TCCTGAAGGGAGCTTTACACG-3') primers, producing a fragment of 167 bp and under the same conditions. Positive and negative controls were included. Standard curves for SelM and L8 were run to determine the efficiency of amplification using dilutions from $5x10^{-3}$ to $5x10^{-8}$ ng/µl of PCR fragments for each gene. For each measurement, expression levels (ng/µl) were normalized with L8 and expressed as relative values (SelM/L8).

2.5 Peroxidase activity

This activity was measured using guaiacol as substrate (Pérez-Tello, *et al.*, 2009) with the following modifications. Hepatopancreas and gills (60 mg, n = 8) were sonicated in 120 µl of 0.1 M Tris-HCl, 5 mM β-mercaptoethanol, pH 8, centrifuged at 12,000 g for 20 min at 4 °C and the aqueous extract separated. In a 96 well microplate, 25 µL of the extract from gills or hepatopancreas were mixed with 160 µL of 0.01 M sodium acetate, 0.5% guaiacol, pH 5.3, and then 25 µL of 0.1% H_2O_2 were added. This reaction was immediately mixed and the absorbance was recorded at 490 nm after 60 s in a microplate reader. Activity was expressed as the difference in absorbance after 60 s of reaction per mg of protein. Protein concentration was determined using the BCA Protein Assay Kit (Thermo scientific) according to the manufacturer instructions. Specific activity unit was defined as the amount of enzyme that causes an absorbance change of 0.001/min*mg protein.

2.6 Hydrogen peroxide determination

The H_2O_2 concentration was determined using phenol red as substrate (Messner, Boll, 1994) with the following modifications. Hepatopancreas and gills (25 mg, n = 8) were sonicated in 200 µl of 0.1 M Tris-HCl, 5 mM β-mercaptoethanol, pH 8, then 320 µl of 10% SDS were added, centrifuged at 12,000 g for 20 min at 4 °C and the aqueous extract was separated. In a 96 well microplate, to 6 µL of the extract from gills or hepatopancreas, 194 µL of phenol red mix (10 mM Mes, 40 µM phenol red and 0.01 mg/ml horseradish peroxidase, pH 6.5) were added and mixed, incubated 3 min at room temperature and the reaction was stopped by adding 4 µL of 0.5 N NaOH. The absorbance was measured at 550 nm in a microplate reader. A standard curve from 0 to 40 nM of H_2O_2 was used.

2.7 Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple comparison test (α= 0.01) was applied to data. The NCSS and PASS (2001) Statistical Systems software was used.

3. Results

3.1 Hypoxia and reoxygenation increase SelM transcripts differentially in gills and hepatopancreas

We have previously reported transient induction of SelM mRNA after WSSV infection in gills (Clavero-Salas, *et al.*, 2007). Also, we reported that peroxidase activity decreased upon silencing of SelM in gills, but no significant effect was detected in hepatopancreas. In contrast, H_2O_2 concentration did not change in gills and hepatopancreas of silenced shrimp (Garcia-Triana, *et al.*, 2010). In this study we investigated the expression of SelM during normal conditions and also, the effect of hypoxia and reoxygenation. In all conditions, SelM expression was detected in gills and hepatopancreas (Fig. 1).

Hyp6, Reo6 and Hyp24 did not affect SelM expression in gills, but Reo24 increased it significantly (Fig. 1). No differences were detected between Nor, Hyp6, Reo6 and Hyp24 in hepatopancreas (α =0.01). Only Reo24 SelM transcript levels were higher than the rest of the treatments (α =0.01). In both tissues, only the longer hypoxia and reoxygenation treatments increased SelM transcript levels (Fig. 1). Reo24 increased Sel M transcripts 8-fold of the Nor expression. SelM transcripts in hepatopancreas in Nor were not different than gills (α =0.01). Similar to gills, hepatopancreas Reo24 treatment increased SelM levels (Fig. 1). SelM transcripts were lower in Nor, Hyp6, Reo6 and Hyp24 than in Reo24 (α =0.01). At Reo24, relative expression increased 3-fold above the Nor expression.

3.2 Hypoxia and reoxygenation effects in peroxidase activity and hydrogen peroxide

Peroxidase activity in gills was significantly different between Nor, Hyp6, Reo6, Hyp24 and Reo24 (α =0.01) (Fig 2). No differences were found between Nor, Hyp6, Reo6 and Hyp24. Reo24 treatment increased peroxidase activity almost 4-fold with respect to Nor

in gills. In contrast, in hepatopancreas, significant differences in peroxidase activity was observed among Nor and the Hyp6, Reo6, Hyp24 and Reo24 treatments (α =0.01) (Fig. 2). There is no statistically significant difference (α =0.01) in the mean values among Hyp6, Reo6, Hyp24 and Reo24. It is interesting to mention that the gills and hepatopancreas peroxidase activity was inverse in Reo24 with respect to Nor controls.

Differences in H_2O_2 between gills and hepatopancreas were detected. In gills there were no differences (α =0.01) in H_2O_2 content among all the controls and treatments (α =0.01) (Fig 3). In hepatopancreas, significant differences in H_2O_2 concentrations among Nor and Hyp6, Reo6, Hyp24 and Reo24 were detected (α =0.01) (Fig. 3). It is interesting to mention that there were no differences in the H_2O_2 content in hepatopancreas compared to the gills in the Nor treatment (α =0.01). In the hypoxia and reoxygenation treatments, the hepatopancreas content of H_2O_2 was almost 3-fold higher than in gills.

4. Discussion

In Nor conditions, SelM expression analyzed by qRT-PCR (Fig. 1) indicated that this transcript is more abundant in hepatopancreas than gills, but no significant differences were found. In our previous results (Garcia-Triana, *et al.*, 2010) statistical differences were detected between these organs in a similar normoxia treatment. The differences could be related to the bioassay conditions or to the individual intrinsic variability.

The effect of hypoxia and reoxygenation on SelM expression was studied in gills and hepatopancreas. In gills, Hyp6, Reo6 and Hyp24 did not affect the levels of mRNA (α =0.01) compared to the Nor treatment. It seems that relatively short stress times such as 6 h of hypoxia and the stress generated by hypoxia 6 h and reoxygenation 1 h is not enough to induce the expression of SelM. Only Reo24 treatment increased significantly (α =0.01) SelM expression levels compared to the control and all other treatments. An 8-fold increase was detected compared to Nor treatment. Since there are no reports related

to the effect of hypoxia and reoxygenation in SelM expression, it is remarkable that a relatively long period of hypoxia and the subsequent reoxygenation increased SelM expression, indicating a regulatory response during prolonged stress conditions. In hepatopancreas, a similar pattern was measured, only Reo24 treatment has significant differences (α =0.01) to the other treatments and control. SelM differential expression has been previously documented by us, with variable patterns during WSSV infection in gills (Clavero-Salas, *et al.*, 2007) and higher expression levels in hepatopancreas than in gills in normoxic conditions (Garcia-Triana, *et al.*, 2010). The SelM differential expression should be highly regulated, since in the SelM over-expressing transgenic mice, native SelM expression was significantly suppressed (Hwang, *et al.*, 2005). These results open several questions. Which transcription factors regulate SelM differential expression in *L. vannamei*? Are the pathogen activated SelM transcription factors the same as environmental stress activated factors? In mammalian osteoblast cell lines the Cbfa1 transcription factor is known as a SelM expression regulator. The study of a Cbfa1 dominant negative mutant indicated that it is involved in global changes in cellular metabolism and cell growth (Bertaux, *et al.*, 2005). The identification and characterization of shrimp SelM transcription factors is a challenging research field.

In normoxic conditions, peroxidase activity was higher in hepatopancreas than in gills. The results are concordant with previously reported findings (Garcia-Triana, *et al.*, 2010) and with the data reported in the prawn *Macrobrachium malcolmsonii* (Arun, Subramanian, 1998). Moreover, in agreement with SelM expression, gill peroxidase activity was higher (α =0.01) in Reo24 treatment. When *L. vannamei* SelM transcripts were silenced in normoxic conditions with different amounts of dsRNA, a decrease in peroxidase activity was found (Garcia-Triana, *et al.*, 2010). The analysis of SelM silencing and the effect of hypoxia for 24 h and reoxygenation for 1 h suggests SelM as a key protein in gill peroxidase activity. Activity of peroxidases in shrimp is influenced by environmental stress factors and pathogen-generated stress. In *L. vannamei*, H_2O_2-induced glutathione peroxidase (GPx) activity increased as a result of up-regulated

expression of GPx mRNA in haemocytes (Liu, *et al.*, 2007). Acidic (5.6) or alkaline (9.3) pH induced oxidative stress and activated the expression of *L. vannamei* GPx in hepatopancreas (Wang, *et al.*, 2009). The transcript of *F. chinensis* GPx increased in response to *Vibrio anguillarum* infection. GPx activity in gill tissues quickly increased 6 h after *V. anguillarum* challenge and was maintained at a relatively high level from 6 to 24 h (Ren, *et al.*, 2009). Interestingly, even hepatopancreas SelM transcripts increased in Reo24h treatment, hepatopancreas peroxidases activity diminishes (α =0.01) in all the hypoxia and reoxygenation treatments compared to normoxia control. Thus, other enzymes with redox activity besides SelM should be involved in the hypoxia and reoxygenation antioxidant response in hepatopancreas. A candidate is GPx, since it is differentially expressed between the control and hypoxia-stressed groups in adult *F. chinensis* (Jiang, *et al.*, 2009). If hepatopancreas SelM transcripts increase in acute stress, such as hypoxia 24 h and reoxygenation 1 h, and do not have a direct effect on peroxidase activity, which is the role of SelM in those conditions? Why does SelM increase only its expression in reoxygenation acute stress? An hypothesis is that SelM is involved in H_2O_2 regulation as a second messenger in hepatopancreas; the facts that support this idea are that SelM is expressed in low quantities, a reoxygenation related increase in transcripts does not correlate with peroxidase activity reduction, and SelM silencing does not affect peroxidase activity (Garcia-Triana, *et al.*, 2010).

Only in normoxia, no differences were detected in H_2O_2 concentration between gills and hepatopancreas (α =0.01). The concentration of H_2O_2 in both tissues is within the order of magnitude reported previously (Garcia-Triana, *et al.*, 2010). No differences between the normoxic control and all the treatments were detected in gills (α =0.01). Thus, hypoxia and reoxygenation treatments in this study did not affect the gills H_2O_2 stationary state.

In hepatopancreas, hypoxia and reoxygenation increased H_2O_2 concentration (α =0.01). H_2O_2 plays key roles in metabolism as second messenger (Rojkind, *et al.*, 2002). Our

results suggest that, in order to respond to environmental oxygen variations, shrimp should increase H_2O_2 production. cMnSOD is expressed in hepatopancreas and gills of *L. vannamei* (Gomez-Anduro, *et al.*, 2006). The H_2O_2 production could be mediated by cMnSOD since this protein produces H_2O_2. The study of cMnSOD in hypoxia, reoxygenation and its silencing would give information about the H_2O_2 regulatory mechanisms in *L. vannamei*, tissues.

SelM expression is increased in hypoxia 24 h and reoxygenation 1 h in gills and hepatopancreas. Under that treatment, peroxidase activity increases in gills and decreases in hepatopancreas with respect to controls. H_2O_2 concentration increases in hypoxia and reoxygenation treatments in hepatopancreas. SelM transcripts, peroxidase activity and H_2O_2 concentration in gills indicate that SelM is important in the antioxidant response in hepatopancreas. Peroxidase activity changes and the H_2O_2 concentration increases in hepatopancreas seem to be related to the regulation of the antioxidant response to environmental oxygen variations. SelM could act as a second messenger regulator in hepatopancreas. Full understanding of SelM role in *L. vannamei* is in process, its multiple functions in redox system need to be discovered.

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Captions to figures

Fig. 1. Expression of SelM relative to L8 in gills and hepatopancreas of hypoxia, reoxygenation and control shrimp by real time qRT-PCR. Normoxia (Nor), Hypoxia by 6 h (Hyp6), hypoxia by 6 h and reoxygenation 1 h (Reo6), hypoxia by 24 h (Hyp24), hypoxia by 24 h and reoxygenation 1 h (Reo24). Levels of transcripts were measured in duplicates. Bars represent mean ± standard deviation (n=8). SelM levels significantly different are labeled with different literals (ANOVA $p<0.01$).

Fig. 2. Peroxidase activity in gills and hepatopancreas of hypoxia, reoxygenation and control shrimps. Normoxia (Nor), Hypoxia by 6 h (Hyp6), hypoxia by 6 h and reoxygenation 1 h (Reo6), hypoxia by 24 h (Hyp24), hypoxia by 24 h and reoxygenation 1 h (Reo24). Levels of peroxidase activity were measured by triplicate. Bars represent mean ± standard errors (n=8). Peroxidase activity levels significantly different are labeled with different literals (ANOVA $p<0.01$).

Fig. 3. H_2O_2 concentration in gills and hepatopancreas of hypoxia, reoxygenation and control shrimps. Normoxia (Nor), Hypoxia by 6 h (Hyp6), hypoxia by 6 h and reoxygenation 1 h (Reo6), hypoxia by 24 h (Hyp24), hypoxia by 24 h and reoxygenation 1 h (Reo24). Levels of H_2O_2 were measured by triplicate. Bars represent mean ± standard errors (n=8). H_2O_2 levels significantly different are labeled with different literals (ANOVA $p<0.01$).

Figures

Fig 1

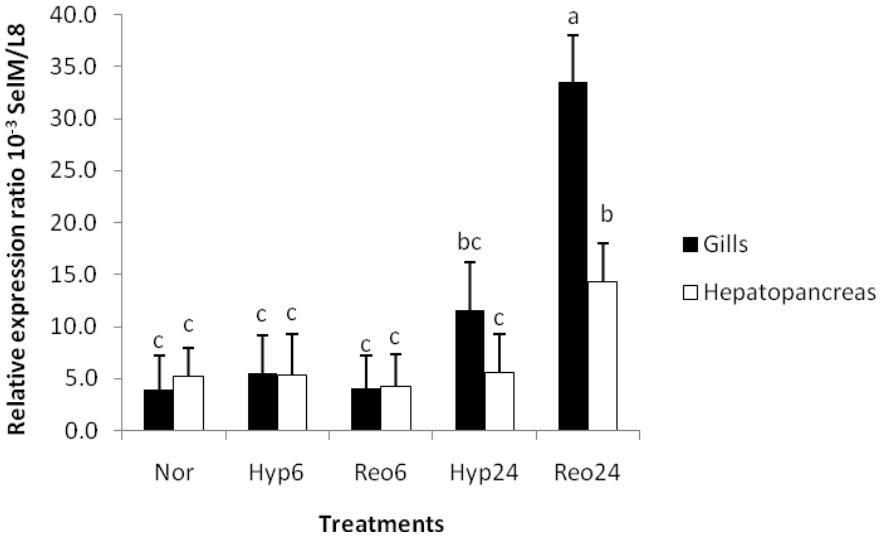


Fig 2

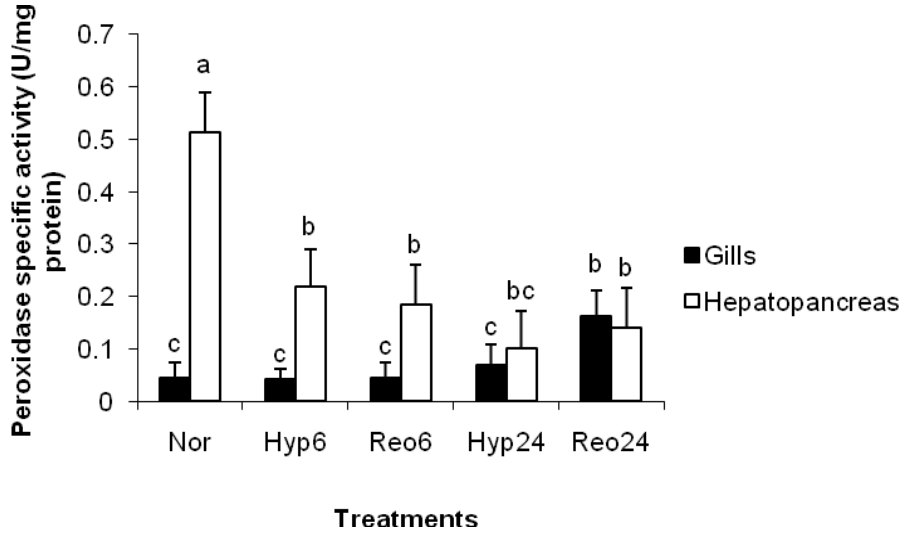
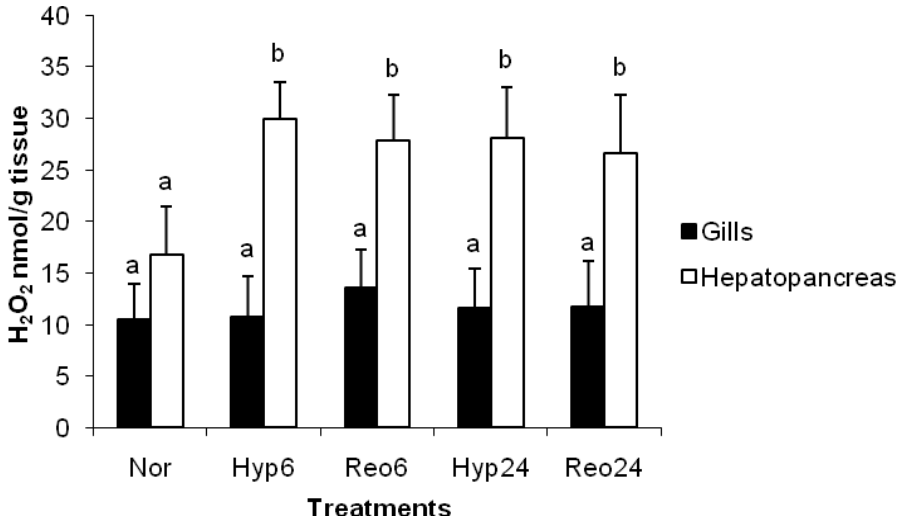


Fig 3


40
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H_2O_2 nmol/g tissue
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Nor
Hyp6
Reo6
Hyp24
Reo24
Treatments
Gills
Hepatopancreas

Is the crustacean selenoproteome similar to other arthropods homologs? A mini review

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Abstract

Selenoproteins (Sels) are involved in oxidative stress regulation. Glutathione peroxidase (GPx) and thioredoxin reductase are among the most studied Sels in crustaceans. Since their expressions and activities are affected by pathogens, environmental and metabolic factors, their functions might be key factors to orchestrate the redox cellular balance. The most studied invertebrate selenoproteome is from *Drosophila*. In this fly SelD and SelB are involved in Sel synthesis whereas SelBthD, SelH and SelK are associated with embryogenesis and animal viability. None of those Sels found in *Drosophila* have been identified in marine crustaceans yet, and their discovery and function identification is an interesting research challenge. SelM has been identified in the white shrimp *Litopenaeus vannamei* and is differentially expressed in tissues, while its function remains to be clarified. SelW and G-rich Sel were recently discovered in marine crustaceans and its function is also unknown. To fully understand the marine crustacean selenoproteome, it is still necessary to identify important Sels such as the SelD, SelBthD and SelB homologs. This knowledge will lead marine crustacean industry to better culture strategies, enhanced health and improved profits.

Key words: selenoprotein, invertebrates, crustaceans, functional

Introduction

Modulation of reactive oxygen species (ROS) plays a key role in metabolism (Alsina, 1996). All known organisms have specialized antioxidant enzymes to manage oxidative stress. Selenoproteins (Sels), proteins that contain selenocysteine (Sec), a modified cysteine residue in which S is replaced by Se, are involved in the regulation of cellular redox balance. The selective advantage of selenoenzymes compared to cysteine or serine containing enzymes is their broad range of substrates and their activity under a wide range of microenvironmental conditions (Gromer, *et al.*, 2003). Peroxidases are antioxidant enzymes that catalyze the reduction of peroxide (H_2O_2) or hydroperoxides using a donor substrate that is oxidized, regulating H_2O_2 levels. The selenocysteine tRNA ($tRNA^{Sec}$) synthetase and selenophosphate synthetase (*sps1* and *sps2*) genes have been identified in *Drosophila melanogaster* (Alsina, *et al.*, 1998; Alsina, 1996). Selenophosphate synthetase or SelD, an enzyme required for synthesis of Sels is the most studied Sel. Although the *Drosophila* Sels have been isolated and characterized, and it is thought that they are present in the cytoplasm, it is not clear whether this is their final cellular localization. The so far known invertebrate Sels have been found in insects (Martin-Romero, *et al.*, 2001), crustaceans (Clavero-Salas, *et al.*, 2007), porifera (Muller, *et al.*, 2005) and cnidaria (Bode H, 2002). Insect Sels have been a useful tool to study H_2O_2 function and degradation because of their high affinity for this ROS and *Drosophila* availability as a model organism. SelD and SelBthD from *Drosophila* have been well characterized; but their homologs have not been identified in other arthropods,

yet. Recently Selenoprotein M (SelM) was identified in a crab (Muller, Borejko, Brandt, Osinga, Ushijima, Hamer, Krasko, Cao, Muller, Schroder, 2005) and a marine shrimp (Clavero-Salas, Sotelo-Mundo, Gollas-Galvan, Hernandez-Lopez, Peregrino-Uriarte, Muhlia-Almazan, Yepiz-Plascencia, 2007), indicating that perhaps the presence of different Sels extends also to marine invertebrates, but their physiological roles are only beginning to be studied. In this article, we review the current knowledge about invertebrate Sels, with special emphasis in the search for these proteins in marine invertebrates.

Stress related Sels with peroxidase activity: glutathione peroxidase and thioredoxin reductase

One of the most studied Sels in marine invertebrates is glutathione peroxidase (GPx). GPx is an enzyme family with peroxidase activity, with approximately 84 kDa, homotetrameric (GPx1, GPx2 and GPx3) or monomeric (GPx4) contain one selenocysteine per monomer unit. Selenocysteine is key for the enzyme function. In the cladoceran *Daphnia magna* GPx has been studied as a biomarker and contaminant response enzyme. GPx appears to be among the most responsive of the induced biomarkers of oxidative stress in *D. magna* exposed to sublethal levels of menadione, paraquat, endosulfan, cadmium and copper for 48 h. This suggests that GPx is important to detoxify the H_2O_2 produced in response to the xenobiotics studied (Barata, *et al.*, 2005a). In contrast, significant decrease of *D. magna* GPx activity was found in organisms exposed to N-heterocyclic polycyclic aromatic hydrocarbons (N-PAHs), such

as phenanthrene and 1,10-phenathroline (Feldmannova, *et al.*, 2006), indicating that GPx antioxidant response could be inhibited by these aromatic hydrocarbons. Therefore *D. magna* GPx activity responds to the different stress depending on the type of molecules involved. It appears that when the antioxidant response is overwhelmed, GPx activity decreases or ceases. The same seems to occur in the crustacean, *Artemia parthenogenetica*. The therapeutic agents clofibrate and clofibric acid in *A. parthenogenetica* were responsible for significant decreases in GPx activity (Nunes, *et al.*, 2006). GPx is also involved in aging in *D. magna*, where aging was accompanied by selective loss of key antioxidant enzymes, including GPx, and age-related increases in lipid peroxides were at least partially due to the functional imbalance of enzymatic antioxidant defenses as GPx (Barata, *et al.*, 2005b).

In crabs, GPx activity has been studied in response to different contaminants. In the blue crab (*Callinectes sapidus*) copper exposure leads to increased activity of GPx (Brouwer, Brouwer, 1998). In *Parasesarma erythodactyla* GPx was higher in individuals where Pb, Cu, Cr, Zn, Cd, As and Se were elevated. Therefore, GPx may be a sensitive biomarker of metal exposure and a secondary marker when accumulated metals are high (MacFarlane, *et al.*, 2006). In gills and hepatopancreas of the portunid crab *Charybdis japonica* exposed to Cd, higher GPx was detected as soon as 0.5 day, increased continuously and was later reduced, but still, it was maintained at higher concentration than in unexposed animals, even after 15 days of exposure. Gills were more sensitive to Cd than hepatopancreas, although hepatopancreas was the main detoxification tissue for

ROS (Pan, Zhang, 2006). In the marine crab *Scylla serrata* naphthalene decreased GPx activity in hepatopancreas and haemolymph (Vijayavel, *et al.*, 2004) and a 30-day sublethal effect decreased GPx activity in the gonads (Vijayavel, *et al.*, 2005). In the crab *Carcinus maena*, hepatopancreas connective tissue and the apex of duct cells were immunostained with GPx antibodies (Orbea, *et al.*, 2000), until now there is no report on GPx activity in response to stress in *C. maenas*. It seems that crabs GPx respond to contaminants in a short period of time, but in response to long-term stress, GPx activity decreased. Lab-acclimated adult male grass shrimp *Palaemonetes pugio* responded similarly. Shrimps were exposed to empirically calculated 96-hr male-specific LC_{50} concentrations of fipronil (FP, a phenylpyrazole GABA disrupting pesticide), endosulfan (ES, a cyclodiene GABA disrupting pesticide), or Cd, as well as a control. GPx was significantly up-regulated by all these three treatments (Griffitt, *et al.*, 2006).

GPx has also been studied in freshwater prawns and crayfish. In the freshwater prawn *Macrobrachium rosenbergii* fed a diet supplemented with vitamin E lipid peroxidation was inhibited in hepatopancreas and gills. In these animals, GPx activity was significantly elevated in hepatopancreas but its activity remained unaltered in gills (Dandapat, *et al.*, 2000). In the freshwater crayfish *Astacus leptodactylus* GPx activity was significantly elevated in the hepatopancreas and muscle after 100, 150 and 200 mg kg(-1) vitamin E supplementation during feeding while its activity decreased in the ovaries (Barim, 2009). *A. leptodactylus* GPx activity was significantly elevated in hepatopancreas by 600 and 1200 mg kg (-1) of vitamin E supplementation in feed and its

activity remained unaltered in the gills and muscles (Beytut, *et al.*, 2009). These results suggest that other proteins besides GPx are involved in gill and muscle antioxidant response and that vitamin E does not substitute the antioxidant activity derived from Se dependent antioxidant proteins. During early larval development and metamorphosis *M. rosenbergii* appear to provoke high oxidative stress as suggested by high content of thiobarbituric acid reactive substances (TBARS). This may be due to direct exposure of the larvae to ambient oxygen in the water as well as their low antioxidant potential. As GPx did not exhibit specific pattern of changes in *M. rosenbergii* larval development (Dandapat, *et al.*, 2003), it is argued that some other antioxidant proteins are involved in H_2O_2 regulation as second messenger. A selenium dependent GPx cDNA was originally cloned from *M. rosenbergii* haemocytes and later detected in haemocytes, hepatopancreas, muscle, stomach, gill, intestine, eyestalk, heart, epidermis, lymph organ, ventral nerve cord, testis and ovary, indicting the broad cellular distribution of this protein. *M. rosenbergii* up-regulation of GPx activity and mRNA transcripts were involved with the protection against injection with the pathogen *Debaryomyces hansenii*- (Yeh, *et al.*, 2009). Both results indicate the GPx has an important function in H_2O_2 regulation in different tissues and in response to pathogens.

Several studies have investigated GPx activity in response to pathogens. In the shrimp *Palaemonetes argentinus* infected with the gill chamber parasite *Probopyrus ringueleti*, known for its capacity to cause host metabolic changes, including changes in oxygen consumption rates, no significant differences were detected in GPx activity (Neves, *et*

al., 2000). Most of the white spot syndrome virus (WSSV)-infected shrimps had GPx decreased activity after the infection. In *Fenneropenaeus indicus*, a significant reduction in the activity of GPx was detected in WSSV-infected shrimps compared to uninfected animals (Mohankumar, Ramasamy, 2006). Another example is the significant decrease of GPx activity in *Penaeus monodon* after WSSV infection by intramuscular injection (Mathew, *et al.*, 2007). However WSSV-resistant *Penaeus japonicus* had higher GPx expression, suggesting that antioxidants are essential components participating in the antiviral process (He, *et al.*, 2005). Bacteria effect on GPx activity of shrimp is variable. When *L. vannamei* was challenged with *Vibrio harveyi*, GPx was substantially down-regulated, indicating a that this may lead to accumulation of H_2O_2 to mount the attack to the pathogen (Wang, *et al.*). In contrast. when *L. vannamei* was fed with the probiotic *Bacillus subtillis* E20, an increase in survival rate was detected but no significant differences were found in GPx activity and, in that case, the increased resistance was shown to be mediated by immune modifications in phenoloxidase activity, phagocytic activity and clearance efficiency (Tseng, *et al.*, 2009). The responses appear to vary since *Pediococcus acidilactici* MA18/5M, used as prebiotic bacteria, significantly increased GPx activity and reduced the susceptibility of *Litopenaeus stylirostris* to the pathogen *Vibrio nigripulchritudo* (Castex, *et al.*, 2009). Besides responding to the host and pathogen species, GPx activity is also regulated by the diet and the environment. An example of diet regulation is the white shrimp *L. vannamei* fed a diet containing 2.0 g kg (-1) sodium alginate that induced a significant decrease in GPx activity, but an increase in phagocytic activity. The shrimp fed a diet containing sodium alginate at 0.5, 1.0 or 2.0

g kg (-1) had increased clearance efficiency of *Vibrio alginolyticus* (Cheng, *et al.*, 2005), suggesting that other H_2O_2 hydrolases are involved in increased immune resistance to *V. alginolyticus* infection. Conversely, the respiratory bursts of *L. vannamei* not feed with sodium alginate, increased significantly after a *V. alginolyticus* injection in order to kill the pathogen, and then induced the increase in GPx activity to protect cells against oxidative damage. However, GPx activity increased as a result of up-regulated expression of GPx mRNA which was induced by the increase in H_2O_2 (Liu, *et al.*, 2007). A selenium-dependent GPx has been cloned from *F. chinensis.* The alignment of the deduced GPx amino acid sequences with homologous proteins from other species showed that the essential residues for enzyme activity are highly conserved. RT-PCR analysis showed that the transcript of *F. chinensis* GPx increased in response to *Vibrio anguillarum* infection. GPx activity in gill tissues quickly increased at 6 h after *V. anguillarum* challenge and was maintained at relatively high levels from 6 to 24 h (Ren, *et al.*, 2009). The increase in GPx transcripts and activity in response to pathogens indicate that this Sel is an important enzyme in the oxidative burst response associated with an infection. Work associated with the increase in the aggressiveness of the oxidative bursts to the pathogens and the concomitant increase in antioxidant enzymes protection of the host is necessary in cultivable marine crustaceans.

In aquaculture conditions, especially at nursery stage, antibiotics are commonly used. Antibiotic molecules also influence the antioxidant metabolism in crustaceans. GPx is regulated by the antibiotic and antifungi saponin. GPx activity, phagocytic activity and clearance efficiency to *V. alginolyticus* in *L. vannamei* is enhanced by saponin (Su,

Chen, 2008). The study of non-environmentally hazardous antimicrobials in the enhancement of oxidative burst against pathogens should be an actively field of crustaceans mariculture research. Which antibiotics, antifungical and signal molecules are the more promising molecules in culture conditions for oxidative burst regulation?

Culture (pond) environment is crucial in the regulation of antioxidant enzymes like GPx. The available information on GPx and other Sels with peroxidase activity in cultivable marine crustaceans is still scarse. In *L. vannamei*, acidic (5.6) or alkaline (9.3) pH induced oxidative stress and activated the expression of GPx (Wang, *et al.*, 2009). Work is still necessary to understand the effects of environmental changes such as oxygen, temperature and salinity, as well as the marine crustaceans metabolic state including life stage, molting cycle, reproductive state, circadian and circanual state, stress conditions related to culture density on GPx expression and activity. The understanding of antioxidant Sels regulation such as GPx under environmental, pathogen and intrinsic related conditions may lead to better culture strategies which in turn result in higher production efficiency. Experimental designs like response surfaces, are useful to understand the interaction of these factors and may help us to propose strategies to modulate enzyme antioxidant activity in the appropriate times to respond to pathogens and environmental disturbances for better defenses and at the same time, minimize ROS- and reactive nitrogen species (RNS)-induced cellular damage.

Thioredoxin reductases (Trxr) are another group of important Sels. Mammalian Trxr contain Sec. The currently known invertebrate Trxr do not contain Sec in the active site.

Drosophila has two Trxr, *Apis sp.* and *Anopheles sp.* have a single *Trxr* gene (Corona, Robinson, 2006). In *Drosophila*, *Trxr-1* encodes three splice variants that include one mitochondrial and two cytoplasmic forms (Missirlis, *et al.*, 2002). The functional significance of the second *Drosophila Trxr* gene (*Trxr2*) is unknown, but it encodes a protein with a potential mitochondrial targeting peptide. *Anopheles sp.* has a single *Trxr* gene, and, as in the *Drosophila* ortholog, has three splice variants coding for one mitochondrial and two cytoplasmic forms (Bauer, *et al.*, 2003). *Apis* also has a single *Trxr* gene. Full genome analysis revealed that, as in *D. melanogaster*, the enzyme glutathione reductase is absent in *Anopheles gambiae* and functionally substituted by the thioredoxin system (Bauer, Gromer, Urbani, Schnolzer, Schirmer, Muller, 2003). Trxr was reported from the tsetse fly *Glossina morsitans morsitans* and showed a response in the expression levels of genes during fly development, in different adult tissues, in the adult midgut through the digestive cycle and following trypanosome infection (Munks, *et al.*, 2005). A Trxr was reported from the Chinese mitten crab *Eriocheir sinensis*. Transcripts were identified in gill, gonad, hepatopancreas, muscle, heart and haemocytes. With *Listonella anguillarum* challenge, Trxr reached the maximum level at 6 h post-stimulation, and then dropped back to the original level gradually (Mu, *et al.*, 2009). A Trx was reported from *L. vannamei*, interestingly, this Trx contains besides the canonical active site CXXC disulfide motif, one Cys (C73) residue in the interface of a putative dimer previously reported for human Trx. It is mainly expressed in gills and pleopods and the variation of Trxr mRNA upon hypoxia and re-oxygenation is not statistically significant (Aispuro-Hernandez, *et al.*, 2008). Acidic (5.6) or alkaline (9.3)

pH induces oxidative stress and activates the expression of Trx in *L. vannamei* (Wang, Zhou, Wang, Tian, Zheng, Liu, Mai, Wang, 2009). As some correlations have been observed in crustacean genes with respect to mammalians, the question becomes, Is there another *L. vannamei* Trxr with Sec in the active site? Work has to be done to address this question.

Drosophila Selenoprotein D and B are involved in Selenoproteins synthesis pathway. Do marine crustaceans homologs exist?

Sel synthesis has been well studied in bacteria and requires the participation of four gene products (Böck A, 1991): selenocysteine synthase (*selA*), selenocysteine-specific elongation factor (*selB*), selenocystein-especific tRNA (*selC*) and selenophosphate synthetase (*selD*), an enzyme required for Sels synthesis. As originally cloned in flies, the highly conserved *selD* gene has been identified as *sps1* (*selD* in flies) (Alsina, Serras, Baguna, Corominas, 1998). The *patufet* gene (*ptuf*) was first studied by Alsina et al. (1998); this gene encodes the *Drosophila* homologue of selenophosphate synthetase (*sps1*) and is involved in Sel biosynthesis. Disruption of the *Drosophila ptuf* gene results in impairment of Sel biosynthesis, ROS burst and larval lethality (Alsina, Serras, Baguna, Corominas, 1998; Morey, *et al.*, 2003). Wild-type flies showed a highly dynamic pattern of *ptuf* mRNA expression during larval and pupal development (Alsina, *et al.*, 1999). Mutant organisms which have no Sel synthesis have lower levels of cell proliferation, a higher proportion of cells arrested in G2 as seen by cyclin B labeling and

increased levels of ROS. Those results suggested an important role of Sels in cell function and have shown a close correlation between *ptuf* expression and cell proliferation, the involvement of *ptuf* on the redox state of the cell and the effects of *ptuf* mutants on cell-cycle progression. All these effects are likely mediated through the synthesis and function of Sels. This supports a leading role for Sels in redox regulation and cell-cycle progression.

To study the function of these Sels in development and growth, a null mutation in the *D. melanogaster* (*selD*) gene (*selD(ptuf)*) was obtained (Serras, *et al.*, 2000). The *selD(ptuf)* loss-of-function mutation causes aberrant cell proliferation and differentiation patterns in the brain and imaginal discs, as deduced from genetic mosaics, patterns of gene expression and analysis of cell cycle markers. Therefore, the use of *Drosophila* imaginal discs and brain and in particular the *selD(ptuf)* mutation, provides a good model to investigate the role of Sels in the regulation of cell proliferation, growth and differentiation. Nervous system development studies are practically non-existent in marine crustaceans; discovery and silencing of SelD in marine crustaceans could lead to important knowledge on neuronal growth and development.

The alteration of the redox balance caused by (*selD(ptuf)*) mutation affects the Ras/MAPK signaling pathway (Morey, *et al.*, 2001). The *selD(ptuf)* mutation suppresses the phenotypes in the eye and the wing caused by hyperactivation of the Ras/MAPK cassette. The mutation also suppresses the *Drosophila* EGF receptor (DER) and

sevenless (Sev) receptor tyrosine kinases (RTKs), which signal in the eye and wing, respectively. No dominant interaction was observed with *selD(ptuf)* conditions in the Wnt, notch, insulin-Pi3K, and DPP signaling pathways. It seems that Sels selectively modulate the Ras/MAPK signaling pathway through their antioxidant function. This is further supported by the fact that a selenoprotein-independent increase in ROS caused by the catalase amorphic *Cat(n1)* allele also reduces Ras/MAPK signaling. (Shim, *et al.*, 2009) They presented the first evidence for the role of intracellular redox environment in signaling pathways in *Drosophila* as a whole organism. SelD regulates the intracellular glutamine by inhibiting glutamine synthetase expression and glutamine in elevated levels works as an intracellular signal (Shim, Kim, Jung, Lee, Xu, Carlson, Kim, Kim, Hatfield, Lee, 2009).

H_2O_2 is one of the most stable ROS and functions as a second messenger in signaling pathways. Certain Sels as GPx and Trxr are involved in redox balance through their peroxidase activity. There are no crustacean reports of *selD*, it is very likely that crustacean Sels need synthesis accessory proteins as SelD. The identification and study of physiological role of SelD-like proteins in crustaceans and Sels synthesis will help in the development of new strategies for the improvement of their redox metabolism.

Incorporation of Sec into Sels requires several gene products, such as the specialized elongation factor SelB and the tRNA(Sec). While the molecular actors have been discovered and their role elucidated in the eubacterial machinery, the data pointed to a

higher degree of complexity in archaea and eukaryotes (Fagegaltier, *et al.*, 2001). *Drosophila* SelB/eEFsec is not essential for viability, longevity or oxidative stress defense (Hirosawa-Takamori, *et al.*, 2004). The organisms lacking the SelB/eEFsec gene were viable and fertile and oxidative balance and the lifespan of these flies are not affected. Thus, Sels may have developed an insect-specific adoption of novel functions once the components of their redox regulating system became independent of Sels biosynthesis. The fact that Sels biosynthesis is maintained in flies suggests that following initial gene duplication events in ancestral organisms, Sels-coding genes may have adopted new and possibly important, but non-vital, functions. These processes may account for the continued requirement for Sels synthesis once the redox homeostasis system became independent of Sec-bearing enzymes during the course of insect evolution. No crustacean elongation factor as SelB have been discovered, nothing is known about the importance of Sels elongation factors in crustaceans redox homeostasis system and viability.

Embryogenesis related selenoproteins: BthD, H and K

In *Drosophila*, an *in silico* program that searches for Sec insertion sequence elements to detect Sels, followed by subsequent metabolic labeling with ^{75}Se and gene signature analyses, was used to detect Sel BthD (Martin-Romero, Kryukov, Lobanov, Carlson,

Lee, Gladyshev, Hatfield, 2001). BthD is expressed dynamically during *Drosophila* development (Kwon, *et al.*, 2003). High levels are detected in the adult ovary, and a large maternal contribution of BthD protein and RNA is given to early embryos. At late stages of embryogenesis, BthD accumulates in the developing salivary gland. RNAi studies revealed that BthD is required for proper salivary gland morphogenesis and the loss of BthD reduced animal viability. Such an expression profile argues against BthD having a general function in cellular metabolism. In tissues where it is expressed, BthD is localized in the cytoplasm and is not detected in the nucleus. BthD staining in SL2 cells co-localizes with that of a known Golgi marker. It is tempting to speculate, therefore, that BthD protein traffics through the Golgi to another compartment.

SelK is a human and mouse Sel homologue of *D. melanogaster* G-rich (Martin-Romero, Kryukov, Lobanov, Carlson, Lee, Gladyshev, Hatfield, 2001). dselH and dselK genes, were identified in *D. melanogaster*. Thereby, dselK is homologue to G-rich. Subcellular localization analysis using GFP-tagged G-rich showed that G-rich was localized in the Golgi apparatus. The fusion protein was co-localized with the Golgi marker proteins but not with endoplasmic reticulum (ER) (Chen, *et al.*, 2006). RNAi was used in *D. melanogaster* embryos and in Schneider S2 cells to inhibit expression of dselH and dselK. The inhibition of either dselH or dselK expression significantly reduces viability in embryos. dselH silencing decreases total antioxidant capacity in embryos and Schneider cells, and increases lipid peroxidation in cells. These studies suggest that the well-known role of Sels in vertebrate antioxidant defenses also extends to include

invertebrates (Morozova, *et al.*, 2003). No BthD, dselH or dselK homolog have been identified in crustaceans. Nothing is known about Sels role in crustacean embryogenesis, improvement of Sels-dependent redox signaling pathways and metabolism may lead to the increase of larvae viability and survival rate.

Crustacean tissue differentially expressed selenoprotein: Selenoprotein M

The first marine invertebrate SelM to be identified was from *Suberites domuncula* (porifera) (Muller, Borejko, Brandt, Osinga, Ushijima, Hamer, Krasko, Cao, Muller, Schroder, 2005). Using differential display of transcripts, they demonstrated that, after a 72-h exposure of primmorphs to selenium, a gene coding for SelM was expressed. The deduced protein sequence of SelM (14 kDa) shows characteristic features of metazoan Sels. The catalytic site of *Suberites domuncula* SelM, Ser-Gly-Sel-Arg-Leu, is similar to the human Gly-Gly-Sel-Gln-Leu. The complete protein, with a calculated molecular mass of 13.9 kDa (123-amino-acid ORF) shares the highest sequence similarity with the 15 kDa SelM from humans (accession number NP_536355M) (Korotkov, *et al.*, 2002); and it has comparatively low similarity to the *D. melanogaster* putative protein. Therefore, the sponge molecule was termed selenoprotein M (SelM_SUBDO) and its cDNA *SDSelM*. In our recent study, *SelM* isolated from *L. vannamei* (Clavero-Salas, Sotelo-Mundo, Gollas-Galvan, Hernandez-Lopez, Peregrino-Uriarte, Muhlia-Almazan, Yepiz-Plascencia, 2007) was also found to be homologous to human SelM (Gromer, *et al.*, 2005), *Ixodes scapularis* SelM (Ribeiro, *et al.*, 2006) and *Tribolium castaneum* SelM

(GenBank accession XM_965244) suggesting a wide distribution of SelM. We also demonstrated that SelM expression in the gill of infected shrimp was transitorily reduced at 1, 3 and 12 h post-infection and increased at 6 and 24 h (Clavero-Salas, Sotelo-Mundo, Gollas-Galvan, Hernandez-Lopez, Peregrino-Uriarte, Muhlia-Almazan, Yepiz-Plascencia, 2007). This modulation is interesting and prompts its possible involvement in alleviating the oxidative stress caused by WSSV-infection. We also demonstrated that SelM was detected in gills, muscle, hepatopancreas and pleopods, with higher abundance in the hepatopancreas and gills. Peroxidase activity decreased upon silencing of SelM in gills, but no significant effect was detected in hepatopancreas. In contrast, total cell H_2O_2 concentration did not change in gills and hepatopancreas of silenced shrimp (García-Triana et al, 2010). The difference in expression during a disease and on different tissues indicates that SelM is involved in diverse regulatory responses. Which tissue-specific function is performed by SelM? Is SelM involved in H_2O_2 regulation as a second messenger in the different tissues? Is this function different when related to a pathogen response?

Unknown function selenoprotein: selenoprotein W

Selenoprotein W (SelW) is a small selenoprotein (85 to 88 amino acids) identified in different vertebrates, such as mice, rats, monkeys, humans, sheep, pigs, fish and

chickens. The biological function of SelW has not been definitely identified. Evidence has been obtained that it can serve as an antioxidant, in response to stress, in cell immunity, as specific target for methyl mercury, and has a thioredoxin-like function (Whanger, 2009). In marine crustaceans, SelW has only been reported in *P. monodon*, where yellow head virus (YHV) infected *P. monodon* showed a decreased expression of SelW (Chintapitaksakul, *et al.*, 2008), and we also have a small cDNA sequence for SelW in the shrimp *L. vannamei* (Yepiz-Plascencia et al., unpublished data). Do SelW and other novel Sels have the same functions as the vertebrate counterparts currently known? As SelW, it is possible that other Sels will be discovered and their metabolic roles should be elucidated to completely understand the selenoproteome function in marine crustaceans metabolism.

Toward novel selenoproteins research in marine crustaceans

Figure 1 shows a dendogram obtained with data available in GenBank for a conserved region of Sels sequences from invertebrates and two Trxr of *D. melanogaster*. Sels synthesis involves closely related proteins, *Drosophila* Sps2 is more related to *L. major*, *T. brucei* and *Drosophila* SelD. Crustaceans GPxs from *L. vannamei*, *M. rosenbergii* and *S. serrata* are closely related to *L. vannamei* Trx1. Antioxidant *D. melanogaster* Trxr1 and Trxr2 are also related. Embryogenesis related Sel like BthD and SelK are grouped together. Putative antioxidant Sels like *L. vannamei* and *S. domuncula* SelM are closely related.

Drosophila is the closer selenoproteome studied from invertebrates organism related to marine crustaceans. Since only few Sels appear to be encoded in the *Drosophila* genome (Martin-Romero, Kryukov, Lobanov, Carlson, Lee, Gladyshev, Hatfield, 2001), marine crustacean selenoproteome could be as relatively simple as that identified in *Drosophila*. Several Sels have been identified in invertebrates, but due to their importance in viability, development and physiology on experimental organisms as *Drosophila*, three of them are the most interesting to search for in the marine crustacean selenoproteome. The importance of SelD for cell function, cell proliferation and cell cycle progression in *Drosophila* has been demonstrated. It has also been shown that SelD is important for Sels synthesis and that its disruption results in impairment of Sels biosynthesis, ROS burst and lethality. Thus, it is very important to identify SelD in marine crustaceans to search for Sels biosynthesis pathways and possible control points in enzyme regulation. Sel BthD is expressed dynamically during *Drosophila* development and plays a general function in cellular metabolism and probably in protein secretion or processing. Elimination of BthD reduces animal viability. SelK discovery and study should be also an important study theme. Since this Sel is important in *Drosophila* viability it might be important in marine crustacean metabolism, it could also share the same physiological and developmental functions, but several studies should be done to test this prediction.

The identification and characterization of Sels in marine crustaceans is a recent field of research. Studies are still necessary in the combined effect of environment, physiological status and pathogen challenges to elucidate the role of selenoproteome in marine

crustaceans. That knowledge will lead crustacean culture industry to improve culture strategies, crustacean health and profits.

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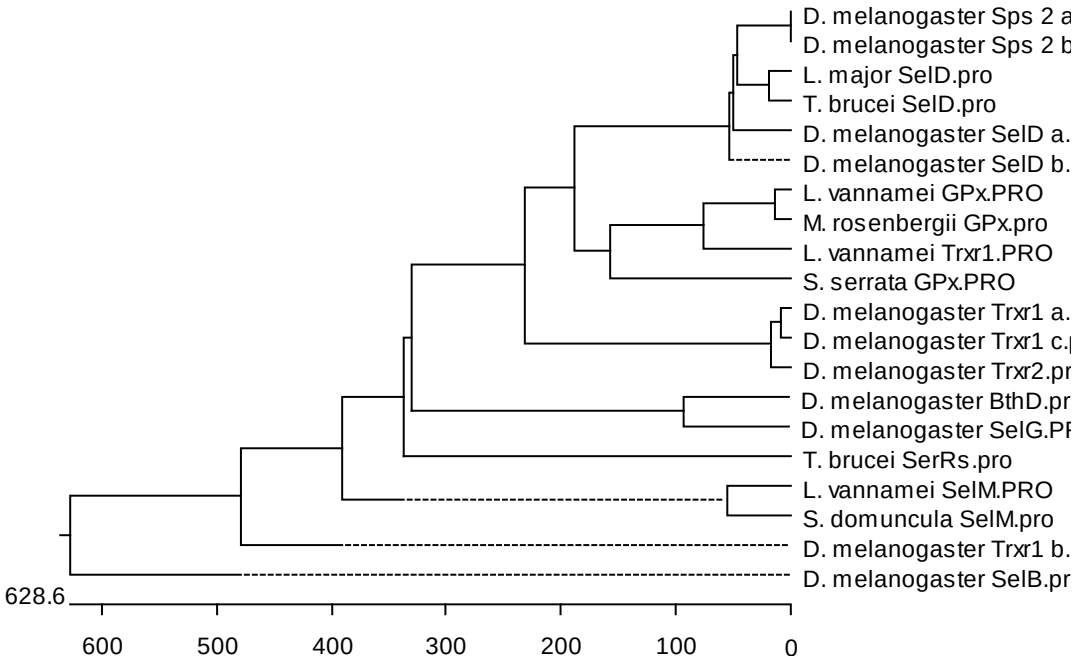
Figure legends

Figure 1. Invertebrate Selenoproteins and two Trxr of *D. melanogaster* dendogram elaborated with MegAlign 4.05 (DNASTAR Inc.). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events

Fig 1.



CAPÍTULO 2

Importancia de cMnSOD en el metabolismo REDOX de *L. vannamei*

Hypoxia, reoxygenation and cytosolic manganese superoxide dismutase (cMnSOD) silencing in *Litopenaeus vannamei*: effects on cMnSOD transcripts, superoxide dismutase activity and superoxide anion production capacity

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Abstract

The effects of silencing cytosolic manganese superoxide dismutase (cMnSOD), an enzyme involved in the antioxidant defense, were analyzed in Whiteleg shrimp, *Litopenaeus vannamei*, adults. Shrimp were intramuscularly injected with long dsRNAs corresponding to the N-terminal portion of the cMnSOD and held under normoxic conditions for 24 h. Another group of shrimp was exposed to hypoxia for 6 h followed by reoxygenation for 1 h. Shrimp injected with long dsRNAs had lower cMnSOD transcripts in gills and hepatopancreas. In the cMnSOD silenced shrimp, superoxide dismutase (SOD) activity decreased in gills but not in hepatopancreas. Shrimp subjected to hypoxia had lower cMnSOD transcripts and SOD activity in gills and hepatopancreas; the production of superoxide anion ($O_2^{\cdot-}$) by haemocytes was also lower in this group. Reoxygenation reverted the effect of hypoxia increasing the levels of cMnSOD transcripts, SOD activity and the production of $O_2^{\cdot-}$. These results suggest that cMnSOD contributes significantly to the SOD activity in gills and hepatopancreas and indicate its importance in the redox system regulation for *L. vannamei*.

Keywords: cytosolic manganese superoxide dismutase (cMnSOD), dsRNA, hypoxia, *Litopenaeus vannamei*, superoxide anion, superoxide dismutase activity.

1. Introduction

Cytosolic manganese superoxide dismutase (cMnSOD) is an antioxidant enzyme that dismutates superoxide anion ($O_2^{\cdot -}$) into a hydrogen peroxide molecule and oxygen, protecting against cellular and genetic damage generated by $O_2^{\cdot -}$. Very little information is available about crustacean cMnSODs physiology. The first crustacean cMnSOD was reported in the blue crab, *Callinectes sapidus* [1], followed by the characterization in the shrimp *Litopenaeus vannamei* [2, 3] and the prawn *Macrobrachium rosenbergii* [4]. cMSOD from *L. vannamei* is transiently induced in hemocytes after infection with the white spot syndrome virus (WSSV) reaching the highest levels 1 h post-infection and then decreasing as the viral infection progressed to levels significantly lower than uninfected controls by 12 h post-infection [2]. Interestingly, in this case, the expression of a viral gene rises when the cMnSOD levels begin to decline, suggesting accumulation of ROS by decreasing cMnSOD activity in an attempt to fight viral infection. cMnSOD is also affected by environmental factors. When *L. vannamei* is exposed to pH 5.6 and 9.3, the gene expression of cMnSOD was induced [5]. However, nothing is known about the cMnSOD role against other stress factors, such as hypoxia and reoxygenation, in shrimp.

Low environmental O_2 concentration (hypoxia) or the subsequent increase in O_2 concentration (reoxygenation) are physiological stressors to most multicellular aerobic organisms. Reoxygenation of hypoxic tissues restores the energy potential and results in generation of reactive oxygen species (ROS) [6]. Modulation of ROS is vital in all known organisms. ROS are important regulators of metabolism, but their accumulation can be harmful for many cellular components. In *Litopenaeus vannamei*, $O_2^{\cdot -}$ production increased while antioxidant capacity decreased in hepatopancreas at the first hours of reoxygenation, this could translate into tissue damage, which may significantly

jeopardize the commercial aquaculture product [7]. Hypoxia decreases *L. vannamei* immune response [8, 9], and increases the risk of infection by opportunistic pathogens [10-13] and of oxidative stress [7, 14].

Superoxide dismutases (SODs) play key roles in the degradation of $O_2^{\cdot -}$. SOD activity has been studied in *L. vannamei* to evaluate its role in metabolism and in the response to stress. Important changes in SOD activity have been detected in environmental stressful conditions. Temperature affects SOD activity in *L. vannamei*, when exposed during 24 h to 32^{o}C, SOD activity decreased significantly [15]. Salinity also affected SOD activity. When *L. vannamei* is exposed to a very low salinity (3 ppt) SOD activity increases [16]. Interestingly, in a different experiment, exposure to low salinities (25, 20 and 15 ppt) followed by a challenge with *Vibrio alginolyticus* the SOD activity decreased 28% [17]. Finally, pH has an effect over SOD activity, *L. vannamei* transferred to pH 6.4 and also to pH 10.1 had significant lower SOD activity after 6 to 24 hours and pH 10.1 decreased SOD activity after 72 h [18]. To our knowledge, there are no reports about the effect of hypoxia and reoxygenation or on the effects of cMnSOD silencing in normoxic conditions on the SOD activity in *L. vannamei*.

In non-model organisms such as shrimp, silencing by RNA interference (RNAi) is an alternative to understand gene function, opening an opportunity and strategy when knowledge and methodology for classical genetics are lacking. To date, there is no information about cMnSOD silencing in crustaceans. Gene expression silencing was first shown in *L. vannamei* by successfully decreasing the effects of virus infection by [19] and was also shown to be gene-specific [20-25].

Until now, nothing is known about the role of cMnSOD and SOD activity in response to the stress generated by hypoxia and reoxygenation in shrimp. The aim of this work was to assess the importance of cMnSOD in the metabolic response of *L.vannamei* whether by silencing cMnSOD in normoxic conditions or by exposure to hypoxia and reoxygenation challenge.

2. Materials and Methods

2.1 Animals

For the cMnSOD silencing experiment, adult shrimps (average weight 15 g) *L. vannamei*, at intermolt stage, were donated from the University of Sonora, DICTUS, Kino Bay Unit. For the hypoxia and reoxygenation experiment, white shrimp *L. vannamei* (average weight 15 g) were donated by the Selecta farm at Kino Bay, Sonora. Shrimps were acclimated for 14 days at 28 °C, 37 psu, constant aeration (6 mg/L dissolved oxygen) and fed *ad libitum* twice daily with commercial feed (Camaronina 35®, Agribrands Purina, Mexico). One-third of the water volume was changed daily, and uneaten food particles and feces were removed daily. Randomly selected healthy intermolt shrimp were placed in separate aquaria. Experiments involving animals were performed following institutional, national and international animal care and use guidelines.

2.2 Total RNA preparation, first strand cDNA synthesis and cMnSOD mRNA detection

Gills, muscle and hepatopancreas were dissected from adult shrimp, immediately frozen in liquid nitrogen and kept at -80 °C until used. Total RNA was extracted by using Trizol® according to instructions of the manufacturer. The reverse transcription (RT) step was performed using Quantitect Reverse transcription (Qiagen)® according to the manufacturer instructions. For this, 1 μg of total RNA was reverse transcribed using oligo dT (12–18). For a 20 μL final volume PCR reaction, the following were added: 18 μL of Platinum® PCR Supermix (Invitrogen), 0.5 μL of each primer (20 μM) and 1 μL of cDNA (equivalent to 50 ng of total RNA). Detection of the cMnSOD transcripts was done using the primers AbcMnSODF (5'-GCTGAGGCAAAGGAAGCTTAC-3') and AbcMnSODR (5'- CTGAGCAACACCAGCCTGC-3') designed based on our previously reported sequence (positions 54-234 of the sequence, GenBank accession no. **DQ005531**) and corresponding to the N-terminal portion of the protein that is

completely different to the mitochondrial MnSOD [2]. These primers amplify a 180 bp fragment and were used under the following conditions: 95 °C, 1 min, 95 °C, 30 s, 61 °C, 1 min, 68 °C, 1 min (31 cycles); 72 °C, 10 min, and kept at 4 °C until used. A ribosomal protein L8 producing a 428 bp fragment, (positions 72-500 of the sequence, GenBank accession no. **DQ316258**) was amplified side by side for comparisons using the forward and reverse primers (5'-GAAGAGGATCGGTGTTCAAGT-3') and (5'-CTCCTGAAGGAAGCTTTACAC-3'), respectively, and using the conditions mentioned before. Positive and negative controls were included. The PCR products were analyzed by agarose gel electrophoresis.

2.3 Synthesis of dsRNA

Two DNA templates were used to produce separately each single-stranded RNA (ssRNA) by *in vitro* transcription for each strand of the cMnSOD. Also, ssRNA was synthesized from a clone for a mango ethylene receptor (ER) that was used as unrelated negative double-stranded RNA (dsRNA) control. The DNA templates were first cloned in the pGEM T Easy Vector (Promega®) in both orientations. A 180 bp fragment of the coding sequence of cMnSOD (positions 54-234 of the sequence, GenBank accession no. **DQ005531**) was obtained with the primers AbcMnSODF (5'-GCTGAGGCAAAGGAAGCTTAC-3') and AbcMnSODR (5'-CTGAGCAACACCAGCCTGC-3'), while an 850 pb of the ER clone was used (clone graciously donated by Dr. Islas-Osuna, unpublished data). The clones obtained in pGEM T Easy contain a T7 promoter adjacent to the inserted DNA fragments. The T7 primer was combined with specific primers to obtain from each clone a PCR product containing the T7 promoter. ssRNA was transcribed *in vitro* from the PCR products templates using T7 phage RNA polymerase RiboMAX (Promega®). Then the DNA template was degraded with DNase I using 1 U/µg of template DNA. The *in vitro* synthesized RNAs were purified according to the manufacturer protocol. Equal amounts of the two complementary RNAs (cRNA) strands were mixed and annealed by incubation at 80°C for 10 min and slowly cooled to room temperature for 50 min. The formation of dsRNA

was detected by the changes in migration of the dsRNA vs the ssRNA by agarose gel electrophoresis.

2.4 Knockdown assay for cMnSOD

L. vannamei adults at intermolt stage were placed in 50 L glass aquarium with seawater (37 psu), temperature controlled (28°C) and constant aeration. Shrimp were randomly divided into seven groups. The following treatments were included: non-injected (NI), injected with shrimp saline solution (ISS, 400 mM NaCl, 20 mM Tris, pH, 7.5), injected with 20 µg of dsRNA mango ethylene receptor ER as exogenous unrelated control, and injected with 5, 10, 20 or 40 µg cMnSOD dsRNA ; prior to injection, all the dsRNA were resuspended in shrimp saline solution. The shrimp were injected intramuscularly through the dorsal area of the second abdominal segment. Samples were collected 24 hours after injection of the dsRNA, immediately frozen on liquid nitrogen and stored at -80 °C until analyzed.

2.5 Hypoxia and reoxygenation assay

L. vannamei adults at intermolt stage were placed in 150 L glass fiber aquarium with seawater (37 psu), temperature controlled (28°C) and constant O_2 concentration (6 mg O_2/L). Shrimp were randomly divided into three groups: Normoxia (Nor, 6 mg O_2/L) as control, hypoxia for 6 h (Hyp6, 1.5 mg O_2/L), hypoxia for 6 h followed by reoxygenation 1 h (Reo6, 6 mg O_2/L). Gills and hepatopancreas were collected at the end of the hypoxia or reoxygenation treatment, frozen on liquid nitrogen and stored at -80 °C.

2.6 qRT-PCR cMnSOD transcripts determination for silencing or hypoxia and reoxygenation assays

cMnSOD and ribosomal protein L8 transcripts levels were detected by real time quantitative RT-PCR (qRT-PCR) in gills and hepatopancreas. Two separate cDNA reactions and two PCR reactions for each individual shrimp and tissue were done (n = 8)

for qRT-PCR on an iQ5 Real-Time PCR Detection System (Bio-Rad) in 20 µL final volume reaction containing 10 µL of iQ SYBR Green Supermix (Bio-Rad), 8 µL of H_2O, 0.5 µL of each primer (20 µM), and 1 µL of cDNA (equivalent to 50 ng of total RNA). A fragment of 382 bp for cMnSOD was obtained using the primers AbcMnSODF and cMnSODRa1(5'-TCTTCGTAGCGGCAATTAGG-3') under the following conditions: 95 °C, 5 min, 95 °C, 30 s, 60 °C, 35 s, 75 °C, 55 s (40 cycles). A single fluorescence measurement and a final melting curve program decreasing 0.3 °C each 20 s from 95 °C to 60 °C was run to discard unspecific amplifications. The L8 cDNA (positions 333-500 of the sequence, GenBank accession no. **DQ316258**) was amplified side by side for comparisons using the L8F2 (5'-TAGGCAATGTCATCCCCATT-3') and L8R2 (5'-TCCTGAAGGGAGCTTTACACG-3') primers, producing a fragment of 167 bp and under the same conditions. Positive and negative controls were included. Standard curves of cMnSOD and L8 were run to determine the efficiency of amplification using dilutions from $5x10^{-3}$ to $5x10^{-8}$ ng/µl of PCR fragments. For each measurement, expression levels (ng/µl) were normalized to L8 and expressed as relatives values (cMnSOD/L8).

2.7 Superoxide dismutase activity

SOD activity was determined employing xanthine and xanthine oxidase (XO) to generate $O_2^{\cdot -}$ which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity is measured by the degree of inhibition of this reaction. SOD activity was determined with the RANSOD kit (RANDOX) with the following modifications. Hepatopancreas and gills (30 mg, n = 8) were homogenized in 100 µl of phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 7) and the aqueous extract separated by centrifugation at 12,000 g for 12 min at 4°C. Then, in a 96 wells microplate, 5 µL of the aqueous extract from gills or hepatopancreas were mixed with 85 µL of R1 mixed xantine substrate (0.05 mmol/l xantine, 0.025

mmol/l I.N.T., CAPS 40 mmol/l, EDTA 0.94 mmol/l, pH 10.2) and then 12.5 µL of R2 XO (XO 80 U/l) were added. This reaction was immediately mixed and the absorbance was recorded at 490 nm after 30 s in a microplate reader. The activity was expressed as the difference in absorbance after 3 minutes of reaction per mg of protein. Protein concentration was determined using the BCA Protein Assay Kit (Thermo scientific) according to the manufacturer instructions. Specific activity unit was defined as the amount of enzyme that causes an absorbance change of 0.001/min/mg protein.

2.8 Superoxide anion production capacity

The $O_2^{\cdot -}$ production capacity was determined using a spectrophotometric nitroblue tetrazolium (NBT) reduction assay [26]. Briefly, haemolymph (200 µl, n = 8) was extracted with 200 µl of shrimp isotonic anticoagulant solution. 50 µl of haemolymph and 50 µl of Hank´s solution were added to a microplate well and the hemocytes stimulated with 50 µl of laminarin (2 mg/ml) and basal non stimulated treatments were prepared. Finally 50 µl of NBT (0.3%) were added. Samples were incubated 30 min at 37^{o} C, washed once with 200 µl of methanol (100%), twice with 200 µl of 70% methanol and dried; then 120 µl of KOH (2 M) and 140 µl of DMSO were added. The absorbance at 655 nm was measured in a microplate reader. Stimulated vs basal samples were used to determine $O_2^{\cdot -}$ production capacity.

2.9 Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple comparison tests (α= 0.01) were applied to the data. The NCSS and PASS (2001) Statistical Systems software was used.

3. Results

3.1 Silencing of cMnSOD reduced transcript differentially in gills and hepatopancreas

We have previously reported transient induction of cMnSOD after WSSV infection in hemocytes, although the expression in other tissues was not investigated. In this study

we addressed the effect of silencing by dsRNA. cMnSOD silencing was detected in all tissues tested (gills, muscle and hepatopancreas) with different relative silencing effects (Fig. 1). Judging from the intensity of the bands, higher cMnSOD silencing was detected in gills and hepatopancreas, followed by muscle.

Intramuscular injection of cMnSOD dsRNA appeared to knock down most of the transcripts in gills after 24 h of treatment (Fig. 2). No differences were detected between NI, ISS, ER and 5 µg intramuscularly injected shrimp (α =0.01). In the shrimp injected with 10, 20 and 40 µg of cMnSOD dsRNA, gills cMnSOD transcript levels were lower than in NI, ISS, ER and 5 µg (α =0.01). As dsRNA doses increased, a decrease in cMnSOD transcripts was detected (Fig. 2). Injection of 10 and 20 µg dsRNA reduced cMnSOD transcripts 85% of the NI expression and at 40 µg dsRNA, silencing was of 100%. cMnSOD transcripts in hepatopancreas in non-injected shrimp were almost 10-fold higher than in gills. In hepatopancreas, intramuscular injection of 5, 10, 20 and 40 µg of dsRNA induced cMnSOD silencing (Fig. 2). Handling of shrimps did not generate differences in expression in NI compared to ISS and ER (α=0.01). cMnSOD transcripts were lower in 5, 10, 20 and 40 µg injected shrimps than in NI, ISS and ER injected shrimps (α =0.01). At 5, 10 and 20 µg dsRNA, relative expression decreased 6-fold of the ISS expression and at 40 µg relative expression was reduced 8-fold of the ISS expression.

3.2 Silencing of cMnSOD and effect in superoxide dismutase activity

SOD activity in gills was not significantly different between NI, ISS and ER (α =0.01) (Fig 3). Also, SOD activity detected in NI, ISS and ER are statistically different (α =0.01) to the shrimp injected with 5, 10, 20 and 40 µg. After injection of 5, 10, 20 and 40 µg dsRNA treatments (α=0.01), no differences in the response to the amount of the injected dsRNA were detected. cMnSOD dsRNA treatments decreased SOD activity

55% respect to RE in gills. In contrast, in hepatopancreas, no significant differences in SOD activity were detected among controls and treatments (α =0.01) (Fig. 3). Also, there are no significant differences between gills and hepatopancreas for both controls and treatments (α =0.01).

3.3 Hypoxia reduces and reoxygenation increases cMnSOD transcripts differentially in gills and hepatopancreas

We have previously reported transient induction of cMnSOD mRNA after WSSV infection [2]. In this study, we investigated the expression of cMnSOD during normal conditions and also, the effect of hypoxia and reoxygenation. In all conditions, cMnSOD expression was greater in hepatopancreas that gills (Fig. 4). Hyp6 affected cMnSOD expression in gills and hepatopancreas (Fig. 4). No differences were detected between Nor and Reo6 in gills and hepatopancreas (α =0.01). In both tissues, Hyp6 decreased ~7 fold cMnSOD transcripts and Reo6 returned transcripts to Nor conditions.

3.4 Hypoxia and reoxygenation effects in superoxide dismutase activity and superoxide anion production capacity

SOD activity in gills was significantly different between Nor, Hyp6 and Reo6 (α =0.01) (Fig 5). Hyp6 treatment decreased SOD activity 2 fold with respect to Nor in gills and hepatopancreas. cMnSOD expression value was higher in gills Reo6 that in all the treatments. There were no significant differences between gills and hepatopancreas in Nor treatment. Also, there were no differences between gills and hepatopancreas in Hyp6 treatment. Finally, there were no differences between hepatopancreas Reo6 and Nor treatments (α =0.01).

Differences in granular hemocyte $O_2^{\cdot -}$ production capacity were detected. Significant differences (α =0.01) were detected between Nor, Hyp6 and Reo6 treatments (α =0.01) (Fig 6). $O_2^{\cdot -}$ production capacity related to granular hemocytes was 16.6 x 10^{-7} in normoxia, decreased in hypoxia to 0.38 x 10^{-7} and increased in reoxygenation at 48 x 10^{-}

^{7} (Fig 6). Hence, $O_2^{\cdot -}$ production capacity related to granular hemocyte count, decreased ~43-fold from normoxia to hypoxia and increased ~126-fold from hypoxia to reoxygenation, reaching higher levels than in normoxia.

4. Discussion

RNAi is defined as the gene-silencing effect mediated by dsRNA. TTo our knowledge, this is the first cMnSOD silencing report in crustaceans. cMnSOD silencing analyzed by RT-PCR indicated that the silencing effect is more profuse in gills and hepatopancreas that in muscle. The major silencing effect of cMnSOD in gills and hepatopancreas might be related to the differences in siRNA receptors in those tissues [23, 27].

From the data of cMnSOD transcript abundance in the NI, ISS and ER treatments (Fig 2), it is clear that hepatopancreas has higher amounts of cMnSOD transcripts than gills. cMnSOD silencing was studied in gills and hepatopancreas by qRT-PCR. cMnSOD long dsRNA was produced and used in the RNAi experiments. Injection of dsRNA significantly reduced the mRNA (α =0.01) compared to the negative controls (NI, ISS or ER). When 10, 20 or 40 µg of cMnSOD dsRNA were used, similar results were obtained. In *L. schmitti* injection of 20 µg of dsRNA shrimp (10 g) was sufficient to silence the CHH gene [28], while 3-5 µg dsRNA was used in 23-28 g *Metapenaeus ensis* shrimps to silence the gonad stimulating hormone [29] and 1 and 10 µg dsRNA of transglutaminase and clotting protein, respectively, in *P. monodon* [23]. In our previous study, Selenoprotein M (SelM) dsRNA injected was between 5 and 40 µg for each shrimp (15 g). From 10 to 40 µg of SelM dsRNA successfully reduced the SelM transcripts in gills and hepatopancreas, indicating an appropriate SelM RNAi silencing activation [27]. In the present study, the different amounts of cMnSOD dsRNA injected were between 5 and 40 µg for each shrimp (15 g). Only 10, 20 and 40 µg dsRNA cMnSOD successfully reduced the cMnSOD transcripts suggesting an appropriate cMnSOD RNAi silencing activation. When 20 µg of dsRNA were injected, the

transcript decrease was 83% in hepatopancreas and 87% in gills 24 h after injection; therefore, it appears that 20 µg is an adequate quantity to silence *L. vannamei* (15 g) cMnSOD transcripts. It was also evident that the cMnSOD dsRNAs effect is very stable 24 h after injection. No apparent (visual) harmful effects were detected in the shrimp injected with the cMnSOD dsRNA during the experiments; the animals continued behaving as prior to the injection. Silencing effect was different among tissues in shrimps. 5 µg injected shrimp did not decrease the transcript level in gills and decreased 83% transcript levels in hepatopancreas. As far as we know, there are only two reports of differential tissue-specific silencing by dsRNA in crustaceans. In our previous study with *L. vannamei*, 24 h after injection SelM silencing was obtained with 5 µg of dsRNA only in gills, but not in hepatopancreas [27]. In the shrimp *P. monodon*, the clotting protein transcript was silenced only in gills and heart one day post-injection, and no silencing was detected in hemocytes, hepatopancreas, intestine and lymphoid organ during that period of time [23]. cMnSOD and SelM silencing response detection with 5 µg of dsRNA might need more to time, as much as 7 days [23]. These results support the idea that differential tissue specific silencing is intrinsically related to the siRNA specific sequence. Is this siRNA specific sequence response primarily related to membrane receptors of the tissues? Several ligands are reported to deliver siRNA into the mammalians cell cytoplasm [30]. The search of siRNA delivery molecules into cytoplasm is a challenging and interesting research field.

In NI, ISS and ER controls, SOD activity was similar in hepatopancreas and gills. The effect of temperature [15], salinity [16, 17],and pH [18] have been reported for *L. vannamei*. There are no reports of SOD activity changes after silencing any antioxidant proteins in crustaceans. An ample silencing effect was detected in gills, where a decrease of 50% was shown in all the cMnSOD silenced treatments, indicating that cMnSOD is an important factor for the antioxidant response in gills. The results agree with the mRNA and activity decrease of a MnSOD silenced in the rot fungus *Phanerochaete chrysosporium* [31]. Although the abundance of cMnSOD transcripts is higher in

hepatopancreas compared to all the tissues studied, cMnSOD silencing does not have effect in hepatopancreas SOD activity, and since the assay measures total SOD activity, other SODs may be more important in this tissue.

The effect of hypoxia and reoxygenation on cMnSOD expression was studied in gills and hepatopancreas. In both tissues, Hyp6 and Reo6 affected the levels of mRNA (α =0.01). Relatively short stress times, such as 6 h of hypoxia, and the stress generated by hypoxia for 6 h followed by reoxygenation for 1 h regulate cMnSOD expression. Hyp6 treatment decreased cMnSOD mRNA significantly (α =0.01) and Reo6 treatment increased it to the same levels as recorded in normoxia (Nor). There are no reports related to the effect of hypoxia and reoxygenation in cMnSOD expression. We have previously reported cMnSOD differential expression in hemocytes during WSSV infection [2] and tissue differential expression in normoxia conditions, with greatest expression in hepatopancreas [3]. Also cMnSOD is highly expressed in haemolymph related organ [32] and increases with beta-1,3-glucan treatments [33]. Which transcription factors regulate the cMnSOD differential expression in *L. vannamei*? Are the pathogen activated cMnSOD transcription factors the same as environmental stress activated factors? In hypoxic conditions, the Nuclear factor kappaB (NF-kappaB), CCAAT-enhancer-binding protein (C/EBP) and nuclear factor 1 (NF-I) have been recognized as MnSOD expression regulators of mammalian fibroblast cell lines [34]. The identification and characterization of shrimp cMnSOD transcription factors regulators is an open interesting question.

In normoxic conditions, SOD activity was similar in hepatopancreas and gills. Gill SOD activity is similar to the activity reported for *F. chinensis* but lower that reported in hepatopancreas [35]. In agreement with cMnSOD expression, gills and hepatopancreas SOD activity was higher (α =0.01) in Reo6 than Hyp6 treatment. The analysis of cMnSOD silencing and the effect of hypoxia 6 h and reoxygenation 1 h suggest cMnSOD is an important protein contributing to gill and hepatopancreas total SOD

activity. Shrimp SOD activity is influenced by several environmental stress factors. When *L. vannamei* is exposed during 24 h to 32^{o} C, SOD activity decreased significantly [15]. Very low salinity (3 ppt) increases SOD activity [16]. pH 6.4 and 10.1 significantly decreased SOD activity over 6 to 24 hours and pH 10.1 decreased SOD activity after 72 h [18]. We have shown that cMnSOD expression and its participation in SOD activity are important in the response to hypoxia and reoxygenation generated stress. Granular haemocyte O_2.- production capacity decreased in Hyp6 and increased in Reo6 to levels higher that in Nor control (α =0.01). Our data are concordant with previous reports [7].

cMnSOD is differentially expressed in shrimp tissues and its silencing is differentially regulated in gills and hepatopancreas. Silencing affects SOD activity also in a tissue-specific manner. Those changes seem to be related to the regulation of the stationary state in shrimp physiology. Correlated variations in cMnSOD transcripts and SOD activity in gills and hepatopancreas, and the concomitant variation of O_2.- production capacity in haemocytes in response to oxygen variation conditions points to a crucial role of this antioxidant enzyme. Our data support cMnSOD as a key enzyme in the responses to periodical fluctuations of oxygen concentration as normally occurs in crustaceans such as shrimp. Future work will uncover the complete role of cMnSOD in the marine crustaceans redox system.

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Captions to figures

Figure 1. Detection of cytosolic manganese superoxide dismutase (cMnSOD) silencing by RT–PCR in gills, hepatopancreas and muscle. RT–PCR products of cMnSOD were analyzed in a 1.5% agarose gel and compared to ribosomal protein L8. Lanes 1, 2 and 3 are for non injected (NI), injected with saline solution (ISS) and cMnSOD 5 µg dsRNA injected (5 µg) in gills respectively. Lanes 4, 5 and 6 for NI, ISS and 5 µg in hepatopancreas. Lanes 7, 8 and 9 for NI, ISS and 5 µg in muscle. Ribosomal protein L8 mRNA levels were also determined by RT–PCR and used for comparisons.

Figure 2. Expression of cytosolic manganese superoxide dismutase (cMnSOD) relative to L8 in gills and hepatopancreas of dsRNA-injected and control shrimp by real time qRT-PCR. Non injected (NI), injected with saline solution (ISS), injected with 20 µg of dsRNA mango ethylene receptor (ER), injected with 5 µg of dsRNA (5 µg), injected with 10 µg of dsRNA (10 µg), injected with 20 µg of dsRNA (20 µg) and injected with 40 µg of dsRNA (40 µg). Levels of transcripts were measured in duplicates for every individual shrimp. Bars represent mean ± standard deviations (n=8). Different letters denote significant differences (ANOVA $p<0.01$).

Figure 3. Superoxide dismutase activity (U/mg protein) in gills and hepatopancreas of dsRNA-injected and control shrimps. Non injected (NI), injected with saline solution (ISS), injected with 20 µg of dsRNA mango ethylene receptor (ER), injected with 5 µg of dsRNA (5 µg), injected with 10 µg of dsRNA (10 µg), injected with 20 µg of dsRNA (20 µg) and injected with 40 µg of dsRNA (40 µg). Levels of superoxide dismutase activity were measured by duiplicate for every shrimp. Bars represent mean ± standard deviations (n=8). Different letters denote significant differences (ANOVA $p<0.01$).

Figure 4. Expression of cytosolic manganese superoxide dismutase (cMnSOD) relative to L8 in gills and hepatopancreas of hypoxia and reoxygenation treatments. Normoxia (Nor), hypoxia 6 h (Hyp6) and hypoxia 6 h and reoxygenation 1 h (Reo6) treatments are shown. Levels of transcripts were measured in duplicates for every individual shrimp. Bars represent mean ± standard deviations (n=8). Different letters denote significant differences (ANOVA $p<0.01$).

Figure 5. Superoxide dismutase activity (U/mg protein) in gills and hepatopancreas of hypoxia and reoxygenation treatments. Normoxia (Nor), hypoxia 6 h (Hyp6) and hypoxia 6 h and reoxygenation 1 h (Reo6) treatments are shown. Levels of superoxide dismutase activity were measured by duplicate for every shrimp. Bars represent mean ± standard deviations (n=8). Different letters denote significant differences (ANOVA $p<0.01$).

Figure 6. Superoxide anion production capacity in haemocytes normalized to granular hemocytes count (superoxide anion production/granular haemocytes 10^{-7}). Normoxia (Nor), hypoxia 6 h (Hyp6) and hypoxia 6 h and reoxygenation 1 h (Reo6) treatments are shown. Bars represent mean ± standard deviations (n=8). Different letters denote significant differences (ANOVA $p<0.01$).

Fig.1

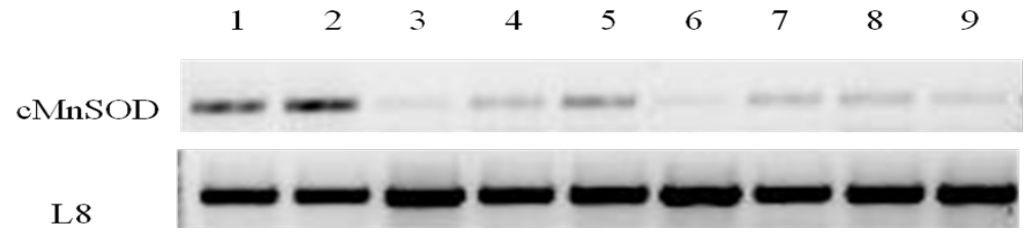

1
2
3
4
5
6
7
8
9
cMnSOD
L8

Fig.2

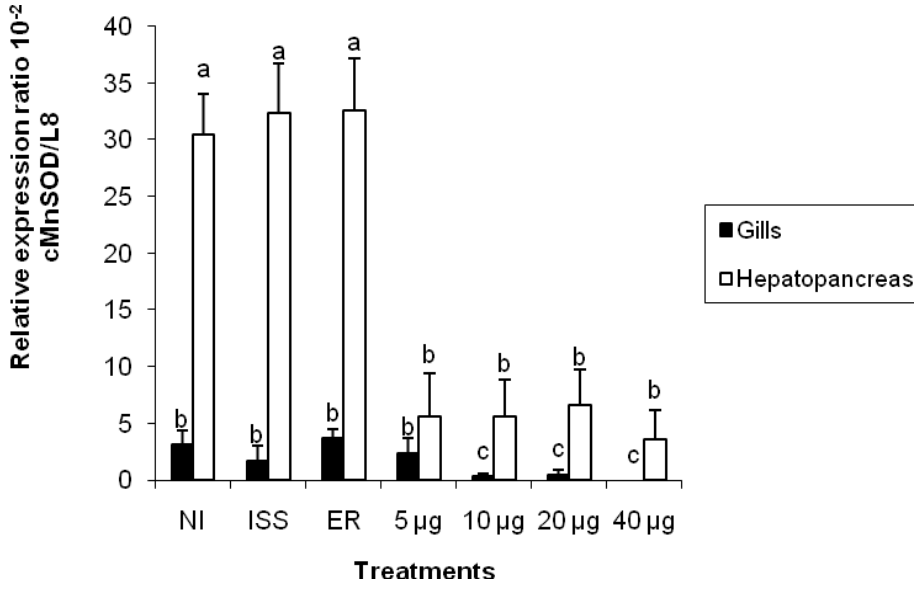

Relative expression ratio 10-2
cMnSOD/L8
40
35
30
25
20
15
10
5
0
a
a
a
b
b
b
b
b
b
b
c
c
c
b
NI
ISS
ER
5 µg
10 µg
20 µg
40 µg
Treatments
Gills
Hepatopancreas

Fig.3

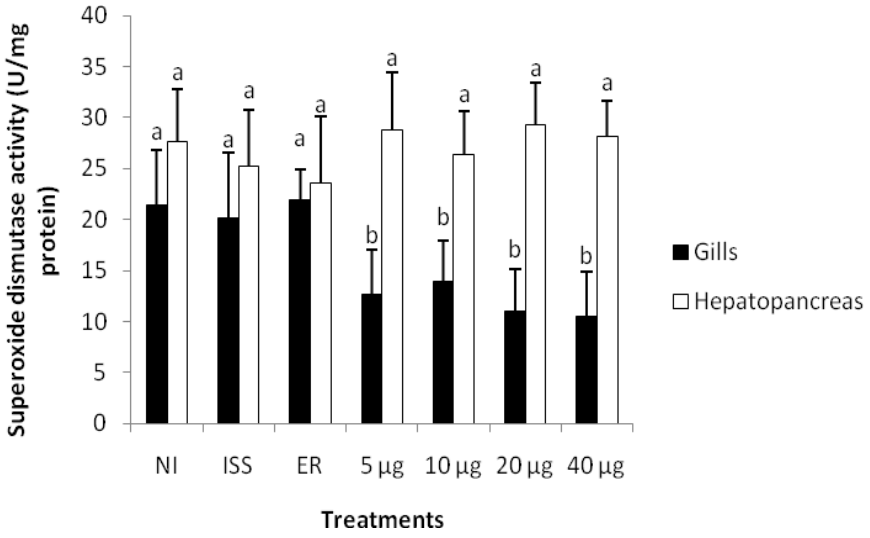

Superoxide dismutase activity (U/mg protein)
40
35
30
25
20
15
10
5
0
a
a
a
a
a
a
b
a
b
a
b
a
b
a
NI
ISS
ER
5 µg
10 µg
20 µg
40 µg
Treatments
Gills
Hepatopancreas

Fig.4

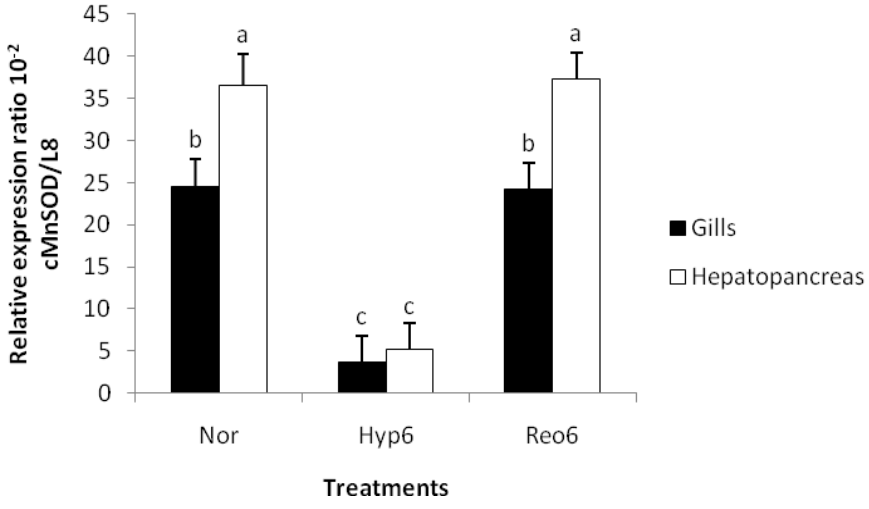

Relative expression ratio 10-2 cMnSOD/L8
45
40
35
30
25
20
15
10
5
0
b
a
c
c
b
a
Nor
Hyp6
Reo6
Treatments
Gills
Hepatopancreas

Fig.5

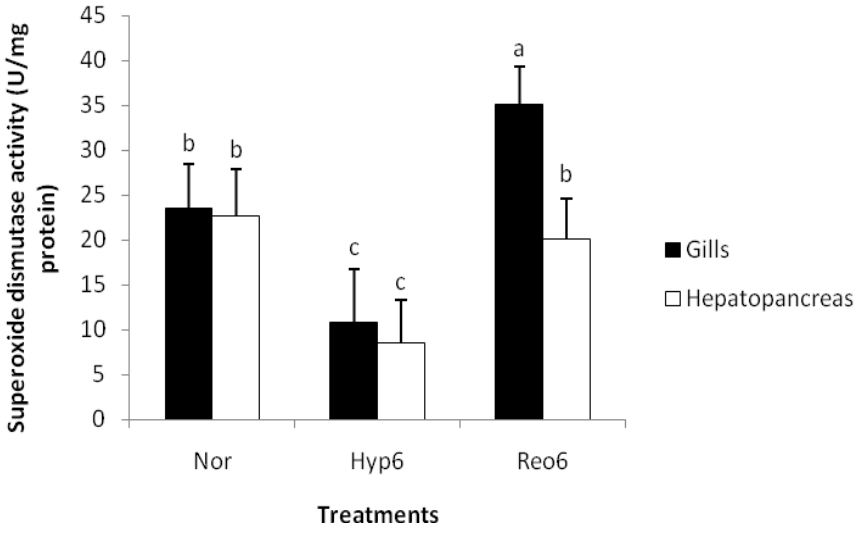
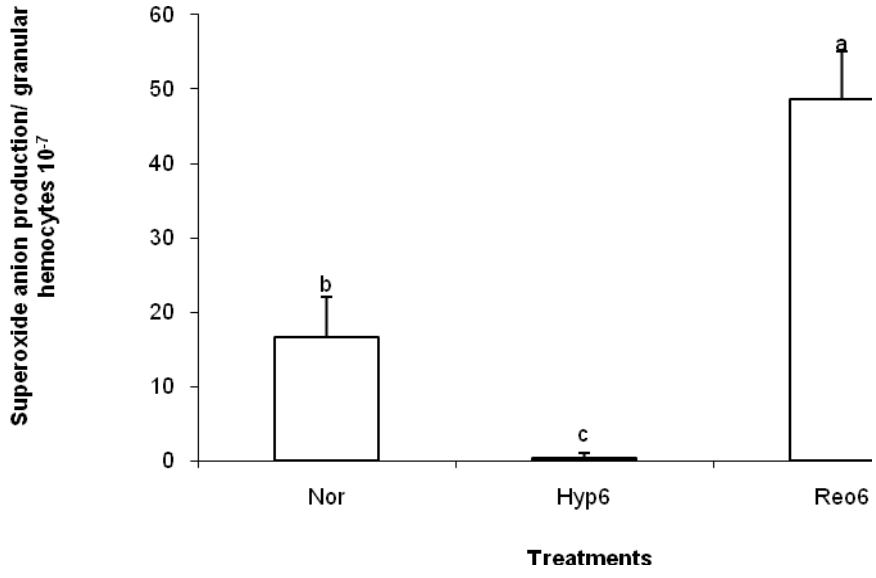

Superoxide dismutase activity (U/mg protein)
45
40
35
30
25
20
15
10
5
0
b
b
c
c
a
b
Nor
Hyp6
Reo6
Treatments
Gills
Hepatopancreas

Fig.6


Superoxide anion production/ granular hemocytes 10-7
60
50
40
30
20
10
0
b
c
a
Nor
Hyp6
Reo6
Treatments

CAPÍTULO 3

Conclusiones

La integración de los resultados obtenidos en esta tesis permitió contrastar la hipótesis propuesta. En el primer artículo del Capítulo 1 se determina que SelM se expresa de manera diferencial en tejidos de *L. vannamei* en condiciones de normoxia. En el primer artículo del Capítulo 1 y en el artículo del Capítulo 2 se determinó que SelM y cMnSOD se expresan de manera diferencial y que su silenciamiento también es regulado de manera diferencial en branquias y hepatopáncreas. También se establece que en branquias, el silenciamiento de SelM y cMnSOD está correlacionado con la disminución en la actividad de peroxidasas y SOD, respectivamente. En hepatopáncreas no hay disminución en las actividades de peroxidasas y SODs cuando son silenciados, lo cual sugiere que otras peroxidasas y SODs son más importantes para la determinación de las correspondientes actividades en ese tejido.

En el segundo artículo del Capítulo 1 se establece que en condiciones de hipoxia y reoxigenación, el aumento de transcritos de SelM en branquias concuerda con el incremento de actividad de peroxidasas, y a su vez, no se detectan cambios en la concentración de H_2O_2 en los diferentes tratamientos. Interesantemente, en el artículo del Capítulo 2 los transcritos de cMnSOD, tanto en branquias como en hepatopáncreas, disminuyen en hipoxia y aumentan en reoxigenación y esto también concuerda con la actividad de SOD en dichos tejidos y con las variaciones en la capacidad de producir $O_2^{\cdot -}$ en hemocitos. De manera tal, que aunque diferentes en la respuesta a la hipoxia y reoxigenación, SelM y cMnSOD pueden ser correlacionadas con la actividad de peroxidasas y SOD, respectivamente, en branquias, lo cual implica que tanto SelM como cMnSOD son importantes en la regulación del sistema REDOX de *L. vannamei*.

Es importante mencionar, que en el segundo artículo del Capítulo 1 la hipoxia y reoxigenación disminuyen la actividad de peroxidasas y aumentan la concentración de H_2O_2 en hepatopáncreas, esto podría estar relacionado a la utilización del H_2O_2 como segundo mensajero en la respuesta antioxidante a

variaciones ambientales de la concentración de oxígeno, demostrando la importancia de la regulación del H_2O_2 en respuesta a cambios en el medio.

El efecto del silenciamiento de SelM y cMnSOD en las actividades de peroxidasas y SOD, así como las variaciones de su expresión, actividades de peroxidasas, SOD, concentración de H_2O_2 y la capacidad de producir $O_2^{\cdot -}$ en hipoxia y reoxigenación, indican que estas enzimas son clave en la regulación del estrés oxidativo en branquias. En hepatopáncreas, al parecer tienen funciones diferentes, cMnSOD tiene inferencia en la regulación del estrés oxidativo y SelM podría ser reguladora de segundos mensajeros. Por lo tanto, las dos proteínas son de gran importancia en la regulación del metabolismo REDOX de *L. vannamei*.