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ANÁLISE DA TRANSCRIÇÃO DE GENES NO CAMARÃO MARINHO *Litopenaeus vannamei* (CRUSTACEA: DECAPODA) EXPOSTO A ESTRESSORES AMBIENTAIS

ORIENTADOR: **Prof. Dr. Afonso Celso Dias Bainy**

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POR

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SUCESSO!

RESUMO

Litopenaeus vannamei é a espécie de camarão marinho mais cultivado no mundo e no Brasil. Essa espécie é considerada bastante resistente a variações ambientais adversas. Entretanto, parece ser bastante susceptível a patógenos de origem viral. Com o objetivo de estudar os efeitos de estressores ambientais e sua susceptibilidade a patógenos virais, camarões *L. vannamei* foram submetidos à condição hiposmótica do meio e desafiados com o vírus da mancha branca. Um total de quarenta e seis genes foi identificado pela técnica de hibridização subtrativa supressiva como diferencialmente transcritos em brânquias de *L. vannamei* submetidos ao estresse osmótico. Os transcritos identificados codificam proteínas envolvidas com processos de defesa, sinalização celular, transferência de elétrons, proliferação e diferenciação celular, apoptose, metabolismo intermediário, proteínas de citoesqueleto e atividade metallopeptidase. A partir do perfil transcricional obtido, alguns genes foram avaliados por PCR quantitativo em camarões submetidos ao estresse osmótico e ao patógeno viral. Foi observada uma indução significativa dos genes *da proteína nuclear 1 induzida por TGF-beta, proteína QM, ciclofilina, lectina-C, malato desidrogenase* e *duas ATPs sintase mitocondriais* nos camarões submetidos ao estresse osmótico. Um segundo estressor ambiental avaliado no camarão foi a hepatotoxina microcistina (MC). Essa toxina é sintetizada por cianobactérias e liberada no ambiente. Frequentemente é detectada em ambientes eutrofizados e em áreas de cultivo de camarão. A conjugação com glutationa reduzida (GSH) pela glutationa S-transferase (GST) e enzimas de defesa antioxidante, como a catalase (CAT), são importantes mecanismos de defesa contra a toxicidade bioquímica de MCs. Foi investigada a atividade das enzimas CAT e GST e os níveis de transcrição gênica de *CAT* e oito isoformas *GST* no hepatopâncreas do camarão após 48h de uma injeção intramuscular com 100 μg kg^{-1} de extrato tóxico de *Microcystis aeruginosa*. MC foi capaz de induzir significativamente três isoformas de *GST* (*ω, μ e MAPEG*) em 12, 2,8 e 1,8 vezes, respectivamente, e aumentou a atividade enzimática total da GST e CAT. A partir dos resultados obtidos, podemos sugerir que o estresse osmótico em *L. vannamei* parece alterar principalmente genes envolvidos em mecanismos de defesa e de energia celular, e a MC altera a transcrição e expressão de GSTs e CAT que parecem estar

envolvidas na biotransformação e eliminação da toxina e em processos de proteção celular. Esses estudos reforçam a importância de

caracterizar ainda mais o perfil transcricional e as regulações pós-transcricionais em *L. vannamei* exposto a estressores ambientais e a patógenos infecciosos para uma melhor compreensão dos principais mecanismos envolvidos nas defesas do camarão.

ABSTRACT

Litopenaeus vannamei is the most cultivated marine shrimp speices in Brazil and the world. The species is considered quite resistant to adverse environmental variations. Nevertheless, it appears quite susceptible to viral pathogens. To study the effects of environmental stressors and its susceptibility to viral pathogens, *L. vannamei* shrimp were submit to a hyposmotic environmental condition and challenged with the white spot virus. A total of 46 genes were identified using the suppression subtractive hybridization technique as differentially transcribed in penaeid *L. vannamei* submit to osmotic stress. The transcripts identified codify proteins involved with defense processes, cellular signaling, electron transfer, cellular proliferation and differentiation, apoptosis, intermediary metabolism, cytoskeleton proteins and metallopeptidase activity. Based on the transcriptional profile obtained, some genes were evaluated by quantitative PCR in shrimp submit to osmotic stress and viral pathogens. Significant induction of the genes of the *nuclear 1 protein were observed, induced by TGF-beta*, *QM protein*, *cyclofphylin*, *lectin-C*, *malate dehydrogenase* and *two ATPs synthase mitochondrial* in the shrimp submit to osmotic stress. A second environmental stressor evaluated in the shrimp was the microcystin (MC) hepatotoxin. This toxin is synthesized by cyanobacteria and liberated in the environment. It is frequently detected in eutrophic environments and in areas of shrimp cultivation. The conjugation with glutathione (GSH) reduced by the glutathione S-transferase (GST) and antioxidant defense enzymes, such as catalase (CAT) are important defense mechanisms against biochemical toxicity of MCs. The activity of the CAT and GST enzymes were studied and the levels of genic transcription of *CAT* and eight *GST* isoforms in the hepatopancreas of the shrimp after 48 hours of an intramuscular injection with 100 μg kg^{-1} of toxic extract of *Microcystis aeruginosa*. MC was capable of significantly inducing three isoforms of *GST* (*ω, μ e MAPEG*) by 12, 2.8 and 1.8 times respectively, and increased the total enzymatic activity of GST and CAT. The results obtained allow suggesting that osmotic stress in *L. vannamei* appears to mainly alter genes involved in defense mechanisms and cellular energy, and the MC alters the transcription and expression of GSTs and CAT that appear to be involved in the biotransformation and elimination of the toxin and in processes of

cellular protection. These studies reinforce the importance of the greater characterization of the transcriptional profile and the post-

transcriptional regulations in *L. vannamei* exposed to environmental stressors and infectious pathogens for a better understanding of the main mechanisms involved in the shrimp's defenses.

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Figure 4. Relative expression of gene transcripts from hepatopancreas of *L. vannamei* 48h-post inoculation with 100 μg kg^{-1} of a *M. aeruginosa* lysate. The data are expressed as expression ratio for each gene among MC-treated and control shrimp (n = 8 individual shrimp for each group). *Refers to significant differences (REST 2009 software Qiagen®; $p<0.05$) among up- or down-regulated genes. The target gene expression was normalized by the expression levels of the non-regulated housekeeping genes 18S ribosomal and ATP synthase (M34362 and EU194608, respectively). Legend: Glutathione-S-tranferase isoforms (GST classes: Ω*GST* (omega), *μGST* (mu), *δGST* (delta, three isoforms) *microssomal GSTs* (MAPEG, two isoforms) and *σGST* (sigma) and catalase (CAT) 90

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LISTA DE SIGLAS

ATPsy1 – ATP sintase da cadeia F (*ATP synthase F chain*)
ATPsy2 – precursor mitocondrial da ATP sintase subunidade 9 (*ATP synthase subunit 9 mitochondrial precursor*)
Ca^{2+} - cálcio
CAT - catalase
cDNA – DNA complementar
CDNB - 1-cloro-2,4-dinitrobenzeno
EST – etiqueta de sequência expressa (*expressed sequence tag*)
GSH – glutationa reduzida
GO – gene ontology
GST – glutationa *S*-transferase
H_2O_2 – peróxido de hidrogênio
K^+ - potássio
L8 – gene ribosomal L8 (*housekeeping gene*)
LEC-C – lectina tipo-C (*lectin-C/ CTL*)
MAPEG – proteína associada à membrana envolvida no metabolismo de eicosanoides e glutationa
MC - microcistina
MC-DLeu1-LR – microcistina variável D-leucina1
MDH – malato desidrogenase (*malate dehydrogenase 2-2 NAD*)
Mg^{2+} - magnésio
N:P – relação nitrogênio:fósforo
PPI – peptidilprolil tipo-ciclofilina (*cyclophilin-type peptidyl-prolyl*)
QM – proteína QM (*QM protein*)
ROS – espécies reativas de oxigênio
RST 9501 – cepa de cianobactéria *Microcystis aeruginosa* isolada da Lagoa dos Patos (região sul do Brasil, Dr. João Sarkis Yunes professor pesquisador da FURG/Brasil)
RT-qPCR – reação da transcriptase reversa seguida de reação em cadeia da polimerase (*reverse transcription quantitative PCR*)
TINP1 – porteína nuclear 1 induzível por TGF-beta (*TGF beta inducible nuclear protein 1*)
TRYP – serino-protease tipo tripsina (*trypsin-like serine proteinase*)
SSH – hibridização subtrativa supressiva (*suppression subtractive hybridization*)
WSSV – virus da síndrome da mancha branca (*white spot syndrome vírus*)
18S – gene ribosomal subunidade 18S (*housekeeping* gene)

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CONTEÚDO

1. INTRODUÇÃO GERAL

1.1. Condição osmótica do meio, perfil transcricional de genes de camarões L.vannamei e WSSV

A aqüicultura costeira difere de acordo com as pessoas envolvidas, recursos utilizados, métodos empregados e características do ambiente circundante (BARG, 1994). Grande parte da indústria mundial da atividade do cultivo de camarões marinhos está localizada em ambientes costeiros (FAO, 2010). A região costeira abrange um espaço com fronteiras abertas entre a terra, a atmosfera, os oceanos e os corpos de água doce. A estrutura de funcionamento destes quatro componentes é dinâmica e interdependente, apresentando comportamentos distintos conforme variações climáticas e ações do homem sobre a natureza (SEIFFERT *et al.*, 2001). Inúmeras são as atividades sócio-econômicas existentes na região costeira, dentre as quais destacamos o turismo, a pesca, o comércio, a agricultura, indústria e a mineração, além da pressão urbana existente (CLARK, 1992). Estimativas indicam que mais da metade da população mundial reside no ambiente costeiro (GREEN *et al.*, 1996).

Parte dos efluentes oriundos das diversas atividades humanas situadas a montante nos cursos de água das bacias hidrográficas tende a ser canalizado para o ambiente costeiro, que absorve, processa ou acumula essa carga de nutrientes e/ou poluentes (CLARK, 1992). Durante este processo de solubilização e assimilação, podem ocorrer alterações significativas nos parâmetros físicos e químicos de qualidade de água no ambiente aquático que circunda as fazendas de cultivo de camarões marinhos, como por exemplo, a concentração dos íons dissolvidos na água (FERREIRA *et al.*, 2011).

Os camarões marinhos migram continuamente durante seu ciclo de vida de acordo com a concentração iônica da água. Quando adultos, vivem em ambientes com maiores concentrações iônicas na água, regiões oceânicas. Durante as fases mais jovens, como as fases larvais até adultos juvenis, preferem águas com menor concentração iônica, regiões estuarinas (ANDREATTA; BELTRAME, 2004). Este tipo de comportamento favorece a possibilidade de cultivar estes organismos em regiões costeiras ou continentais, podendo-se, quando necessário, preparar, de forma artificial, a água de cultivo.

Atualmente, a espécie *Litopenaeus vannamei* é a mais cultivada no mundo e também no Brasil (FAO, 2010). Grande parte da atividade

da carcinicultura brasileira é realizada em regiões estuarinas, utilizando águas salobras ou com salinidade oscilando entre 10 e 20. Entretanto, também vem ganhando espaço o cultivo em ambientes continentais. Estima-se que a área de cultivo continental de camarões é de aproximadamente 2.000 hectares, sendo distribuída nos estados do Ceará, Paraíba, Rio Grande do Norte e Pernambuco (MAIA, 2011).

Segundo Boyd (1990), a salinidade é definida como a concentração total de íons dissolvidos na água. Água do mar é antes de tudo uma solução de NaCl. Na^+ e Cl^- são responsáveis por mais de 86% do teor de sal em massa. A ordem decrescente nas concentrações dos demais cátions é Mg^{2+}, Ca^{2+}, K^+ e Sr^{2+}. A concentração do ânion Cl^- é aproximadamente igual à soma das concentrações dos cátions. Os outros ânions ($SO4^{2-}$, HCO^{3-}, Br^-, F^-) são muito menos significativos no equilíbrio de carga de água do mar (PILSON, 1998). A principal diferença da água doce para água marinha é que o HCO^{3-} é o principal ânion e tem uma concentração muito maior do que Cl^-. O Ca^{2+} é o principal cátion na água doce, seguido de Na^+ e Mg^{2+}, em seguida, K^+.

O animal pode alcançar o eqüilíbrio osmótico por dois mecanismos distintos, osmoconformidade e osmorregulação. Organismos osmoconformadores minimizam as perdas de água e íons alterando a concentração osmótica entre o sangue (hemolinfa) e o meio em que estão imersos, até que igualem a este meio, permanecendo dessa forma isosmótico. Os organismos osmorreguladores utilizam suas próprias concentrações de íons independentes da concentração do meio, produzindo um contra fluxo de solutos em proporções iguais aos perdidos pela difusão (MANTEL; FARNER, 1983; VALENÇA; MENDES, 2003). Camarões peneídeos são considerados osmorreguladores podendo as concentrações isosmóticas variar entre 20 e 30 de acordo com a espécie (LIN *et al.*, 2000).

A energia consumida para manter um equilíbrio iônico interno da hemolinfa de camarões e, assim, possibilitar a mineralização do exoesqueleto, é influenciada pela composição da água do ambiente de cultivo. O camarão na fase juvenil tem um gasto energético considerável para manter sua homeostase interna. Este é ainda mais acentuado, se considerarmos que o intervalo de muda ou troca do exoesqueleto nesta fase, pode ocorrer a cada quatro ou dez dias (WICKINS, 1976). Um ambiente ionicamente adverso pode alterar a taxa de crescimento, principalmente pela redução da freqüência de muda (WICKINS, 1976). O Na^+ e Cl^- constituem cerca de 80 % do total de osmólitos. O Ca^{2+} parece ser acumulado enquanto o Mg^{2+} é hiporegulado na hemolinfa de

peneídeos dentro de toda a faixa de salinidade (0,5 a 50) em que eles sobrevivem (LIN *et al.*, 2000).

Segundo Maia (2011), houve um incremento em torno de 50% na produção mundial de camarões peneídeos cultivados em água doce entre os anos de 2005 a 2009. Os principais fatores que estimularam a interiorização da carcinicultura no mundo foram o incremento da demanda, o aumento dos preços globais e as falhas no sistema produtivo tradicional costeiro ocasionada principalmente pelas doenças. No Brasil, destacamos as doenças de origem viral (WSSV no sul e IMNV no nordeste) capazes de comprometer a viabilidade econômica da atividade (MAIA, 2011).

O vírus que causa a síndrome da mancha branca em camarões ou *white spot syndrome virus* (WSSV), é o agente patogênico cosmopolita que mais causou efeitos negativos aos cultivos de camarão no mundo, tendo afetado esta atividade econômica em mais de 30 países (TAPAY *et al.*, 1999; WANG *et al.*, 1999). No Brasil, o surgimento da enfermidade ocorreu no Estado de Santa Catarina em meados de 2005 (SEIFFERT *et al.*, 2005). De forma semelhante ao ocorrido em outros países, houve um grande impacto econômico local sendo registrada uma redução na produção de camarões superior a 90% entre os anos de 2004 e 2009 (COSTA *et al.*, 2010). Entretanto, esta enfermidade não paralisou totalmente a atividade em nenhum dos países que foram acometidos pela doença existindo atualmente diferentes formas de cultivo possíveis de serem realizadas com a presença do vírus (SEIFFERT, 2005).

O vírus da síndrome da mancha branca é de DNA dupla fita, envelopado, com partículas de virions simétricas no envelope, elipsóides a baciliformes semelhantes ao baculovírus (DURAND *et al.*, 1997). O vírus possui de 120-150 nm de diâmetro por 270-290 nm de extensão (INOUYE *et al.*, 1994; NAKANO *et al.*, 1994; WANG *et al.*, 1995). É um vírus envolto por nucleocapsídeo e seu genoma tem aproximadamente 300 kb (VAN HULTEN *et al.*, 2001; YANG *et al.*, 2001; CHENG *et al.*, 2002). Devido ao fato de ser um vírus completamente diferente, com características próprias, recentemente o Comitê Internacional em Taxonomia de Vírus aprovou uma proposta de inserção do vírus em uma nova família, *Nimaviridae*, gênero *Whispovirus* (LEU *et al.*, 2009). A rota de entrada e mecanismo de disseminação de WSSV entre os tecidos tem sido demonstrada recentemente (ESCOBEDO-BONILLA *et al.*, 2007). Brânquias e epitélio cuticular do intestino anterior em *L. vannamei* são portas de entrada após a inoculação oral de WSSV. Após a replicação primária

nestes tecidos, o vírus atravessa a membrana basal e atinge os seios associados à hemolinfa (Figura 1). Através da circulação da hemolinfa, o vírus infecta os órgãos internos, onde ocorre a replicação viral e a disseminação da infecção (ESCOBEDO-BONILLA *et al.*, 2007).

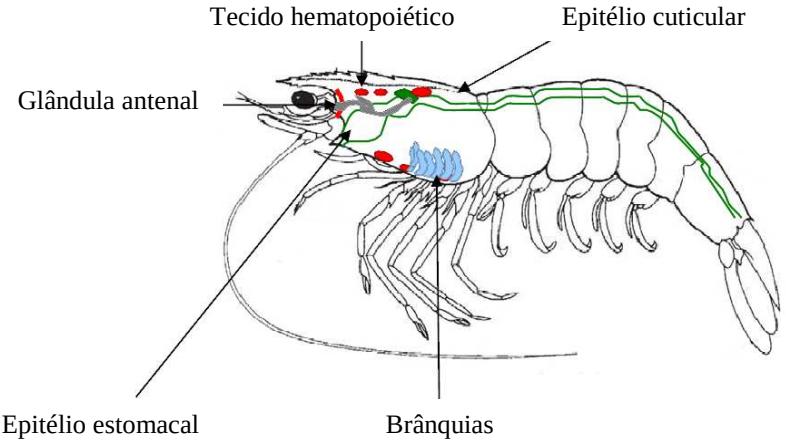

Figura 1. Principais tecidos-alvo de replicação de WSSV no camarão (Fonte: adaptado de RAHMAN, 2007)

WSSV possui vários crustáceos como portadores assintomáticos (LO *et al.*, 1996; FLEGEL, 1997). O vírus foi detectado em ovos dormentes de rotíferos provenientes dos sedimentos de viveiros de camarões (YAN *et al.*, 2004). Esse vírus foi identificado em diferentes regiões geográficas e alguns estudos têm identificado diferentes isolados geográficos do vírus, mas com similaridade de 99,3% na sua composição de nucleotídeos (ESCOBEDO-BONILLA *et al.*, 2008). Entretanto, existem regiões do genoma viral que apresentam variações de sequências significativas capazes de determinar a origem dos isolados e sua dispersão geográfica (DIEU *et al.*, 2004). Müller *et al.* (2010) fizeram a genotipagem de WSSV de isolados brasileiros e

compararam os resultados com isolados de outros países da América. Foi demonstrado nesse estudo que o vírus proveniente das duas regiões do país demostraram padrões diferentes nos marcadores genéticos avaliados. Ao analisar um marcador isoladamente (ORF 94), Santa Catarina, México, Nicarágua, Honduras e Panamá pareceram seguir o mesmo padrão molecular, com exceção de isolados provenientes da Bahia e EUA. Sugere-se que esta similaridade pode estar relacionada a uma similaridade de virulência de WSSV provenientes das regiões analisadas.

Os sinais clínicos da síndrome da mancha branca em camarões peneídeos são caracterizados por anorexia, letargia, nado errático na superfície da água do viveiro e o corpo com uma coloração avermelhada (MORALES-COVARRUBIAS, 2004). Na fase crônica da doença é possível observar pontos brancos pequenos na carapaça, na região do cefalotórax (Figura 2), menores que os descritos para a espécie *Penaeus monodon* (0,5 a 2,0 mm) (MORALES-COVARRUBIAS, 2004). Esses pontos brancos são depósitos de Ca^{2+} que também podem ser provocados por trocas ambientais relacionadas à concentração de Ca^{2+} e o pH do tanque (MORALES-COVARRUBIAS, 2004). O maior acúmulo de Ca^{2+} na cutícula pode ser explicado pelo fato de que em ambientes hipercápnicos (excesso de dióxido de carbono na hemolinfa) existe um maior ingresso de bicarbonato pelas brânquias para tamponar o pH da hemolinfa, o que permitiria a deposição de Ca^{2+} na cutícula do exoesqueleto. Entretanto, ainda não foi esclarecido porque o vírus da mancha branca induz a formação de depósitos de cálcio na cutícula.

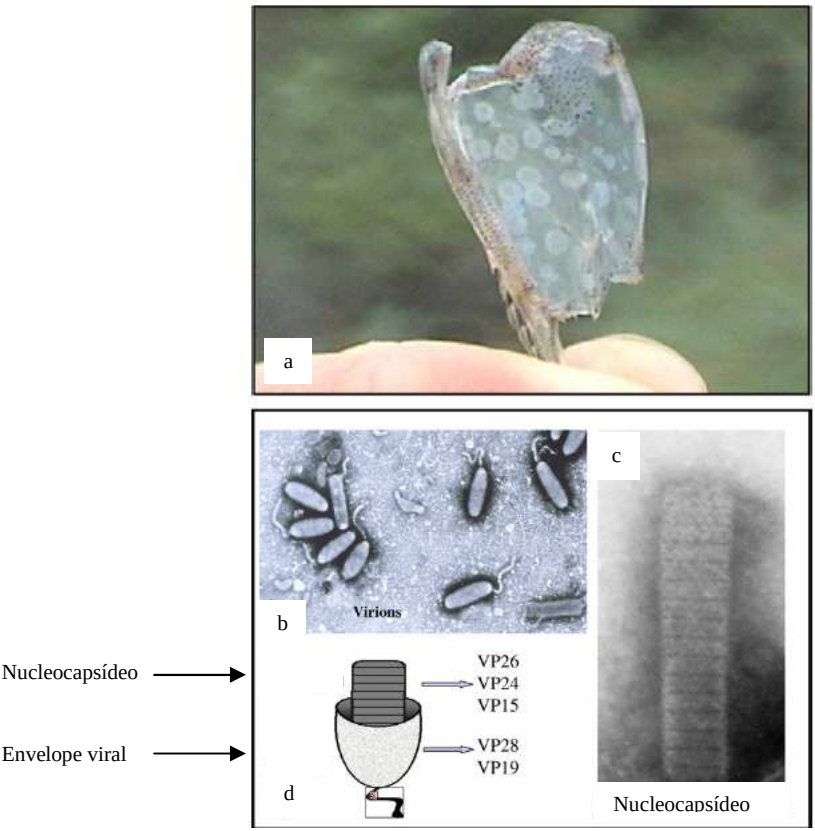

Figura 2. WSSV (a) carapaça de *Litopenaeus vannamei* em fase juvenil que apresenta pontos brancos típicos na parte interna da carapaça devido à infecção viral; (b) micrografia de transmissão eletrônica (TEM) de virions de WSSV semi-purificados a partir da hemolinfa de juvenis infectados; (c) TEM de um nucleocapsídeo mostrando o arranjo de 90º de subunidades do capsídeo na sua superfície; (d) esquema demonstrando um corte transversal da cobertura externa do envelope, o nucleocapsídeo interno e as principais proteínas associadas (Fonte: adaptado de LIGHTNER, 2011).

O agente patogênico da síndrome está bem caracterizado, entretanto o conhecimento da interação hospedeiro-patógeno-ambiente e sua correlação com a expressão gênica dos camarões é muito insipiente.

Segundo Sniezko (1973), um desequilíbrio nessa interação pode desencadear ou dificultar o estabelecimento e a transmissão de doenças (Figura 3). Existem inúmeras evidências de que a incidência de doenças em camarões é exacerbada por oscilações bruscas em diferentes parâmetros de qualidade de água (TSAI *et al.*, 1993; GUAN *et al.*, 2003). Estudos recentes têm relacionado parâmetros de qualidade de água com o surgimento da síndrome, tais como temperatura, salinidade, oxigênio, hipertrofização, chuvas, entre outros (WANG; CHEN, 2006; GRANJA *et al.*, 2006). Alterações na salinidade têm sido associadas com uma maior susceptibilidade do camarão às doenças virais pela interferência dessa condição nas respostas imunes do camarão (WANG; CHEN, 2006; JOSEPH; PHILIP, 2007).

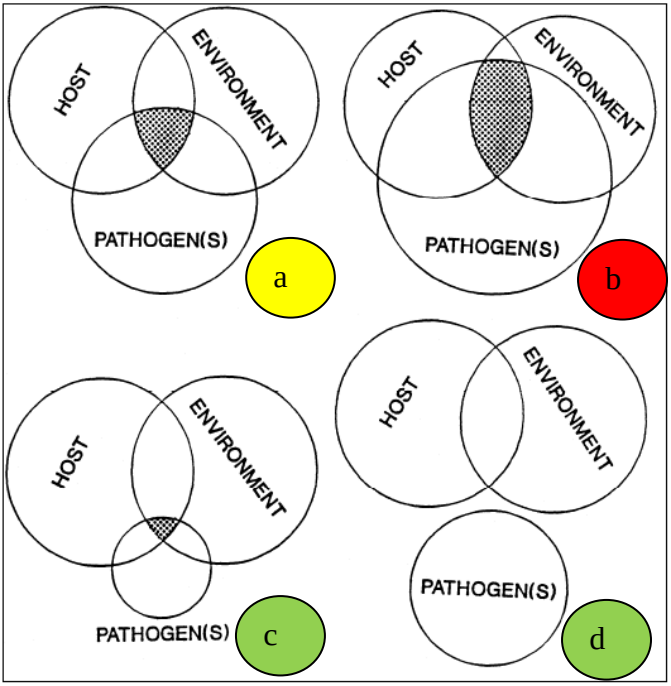

Figura 3. Conceito de interação hospedeiro-patógeno-ambiente, quando o tamanho de um dos componentes é aumentado, a severidade da doença aumenta; a) interação harmônica entre os fatores, com o patógeno presente sem causar enfermidade; b) Efeito de um patógeno altamente virulento; c) Efeito de um patógeno pouco virulento; d) Exclusão do patógeno (Fonte: adaptado de LIGHTNER e REDMAN, 1998).

Durante as duas últimas décadas, vários métodos de alto-desempenho têm sido estabelecidos para o entendimento de como as condições ambientais afetam a expressão de genes. Isto inclui a análise de ESTs (seqüências alvo expressas), hibridização subtrativa supressiva (SSH), hibridização diferencial, análise serial de expressão de genes, microarranjos e eletroforese bidimensional (2-DE). Na área de imunologia de camarão, análises de EST têm sido utilizadas para várias espécies, incluindo *L. vannamei*, *L. setiferus*, *Penaeus monodon*, *Marsupenaeus japonicus* e *Fenneropenaeus chinensis* (GROSS *et al.*, 2001; ROJTINNAKORN *et al.*, 2002; de LORGERIL *et al.*, 2005; TASSANAKAJON *et al.*, 2006; DONG; XIANG, 2007; LEU *et al.* 2007; MÜLLER, 2009; PONGSOMBOON *et al.*, 2011). As ESTs são derivadas de bibliotecas de cDNA e clones de SSH construídos a partir de vários tecidos, tais como pós-larvas, hepatopâncreas, hemócitos, brânquias e órgão linfóide. ESTs de camarão propiciam uma primeira observação para descrever a expressão diferencial no nível transcricional em diferentes condições experimentais.

Nos últimos anos, a atenção nas interações patógeno-hospedeiro tem aumentado, particularmente, na resposta imune contra invasores. Recentemente, vários microarranjos de camarão têm sido construídos a partir de estudos utilizando diferentes órgãos e espécies. O primeiro microarranjo de cDNA de *L. vannamei* continha 2.469 ESTs da biblioteca de cDNA padrão e biblioteca de SSH de três tecidos sob diferentes estímulos (ROBALINO *et al.*, 2007). Um grande percentual (47% a 72%) de genes transcritos diferencialmente em diferentes pesquisas não identificaram similaridade significativa com qualquer proteína de outros organismos e carecem de identificação de domínios confiáveis (DHAR *et al.* 2003; WANG *et al.* 2006; de la VEGA *et al.* 2007; ROBALINO *et al.* 2007; WONGPANYA *et al.* 2007; FAGUTAO *et al.* 2008; PONGSOMBOON *et al.* 2008; WANG *et al.* 2008). De acordo com Aoki *et al.* (2011), estudos com camarão têm demonstrado um alto percentual de seqüências sem similaridade sugerindo que alguns desses genes podem estar relacionados às respostas imunes, mas que não foram identificados porque não tem, até o momento, sua função definida.

A SSH combina PCR supressiva com etapas de normalização e subtração (DIATCHENKO *et al.*, 1996), e consiste na síntese de cDNA a partir de duas amostras de tecidos (Figura 4). O cDNA alvo é denominado *tester* e o controle é o cDNA *driver*. Ambos cDNAs são digeridos com uma enzima de restrição. O *tester* é subdividido em dois e cada parte é ligada a cada um dos adaptadores. A partir da ligação são

realizadas duas hibridizações para o enriquecimento de sequências expressas diferencialmente. Em seguida, os cDNAs expressos diferencialmente são amplificados por PCR. Dois ciclos de amplificação também são utilizados com o objetivo de reduzir o produto de amplificações inespecíficas. O próximo passo é a clonagem de cDNAs para identificação dos transcritos através de sequenciamento. A técnica apresenta vantagens como a detecção de genes diferencialmente transcritos pouco abundantes, supressão da amplificação de genes altamente transcritos, identificação de genes sem conhecimento prévio de suas sequências e uso de técnicas comuns de biologia molecular.

Estudos recentes têm utilizado esse sistema para identificar genes que podem estar envolvidos em mecanismos de defesa do camarão e posteriormente avaliar sua transcrição (HE *et al.*, 2004; HE *et al.*, 2005; de la VEGA *et al.*, 2007; REYES *et al.*, 2007; NAYAK *et al.*, 2010). Pan *et al.* (2005) construíram uma biblioteca subtrativa utilizando a técnica de SSH em amostras de hepatopâncreas de camarão e identificaram trinta e um genes diferencialmente transcritos. A partir desses, dez genes foram aleatoriamente selecionados para avaliação de seu nível de transcrição por RT-PCR semi-quantitativo e cinco genes foram avaliados por *Northern Blotting*. Os autores verificaram que houve aumento da transcrição de todos os genes selecionados, o que sugeriu que a transcrição da maioria dos trinta e um genes poderia estar aumentada nos camarões resistentes ao vírus se comparados com camarões normais e que estariam envolvidos na resposta de defesa contra a infecção viral.

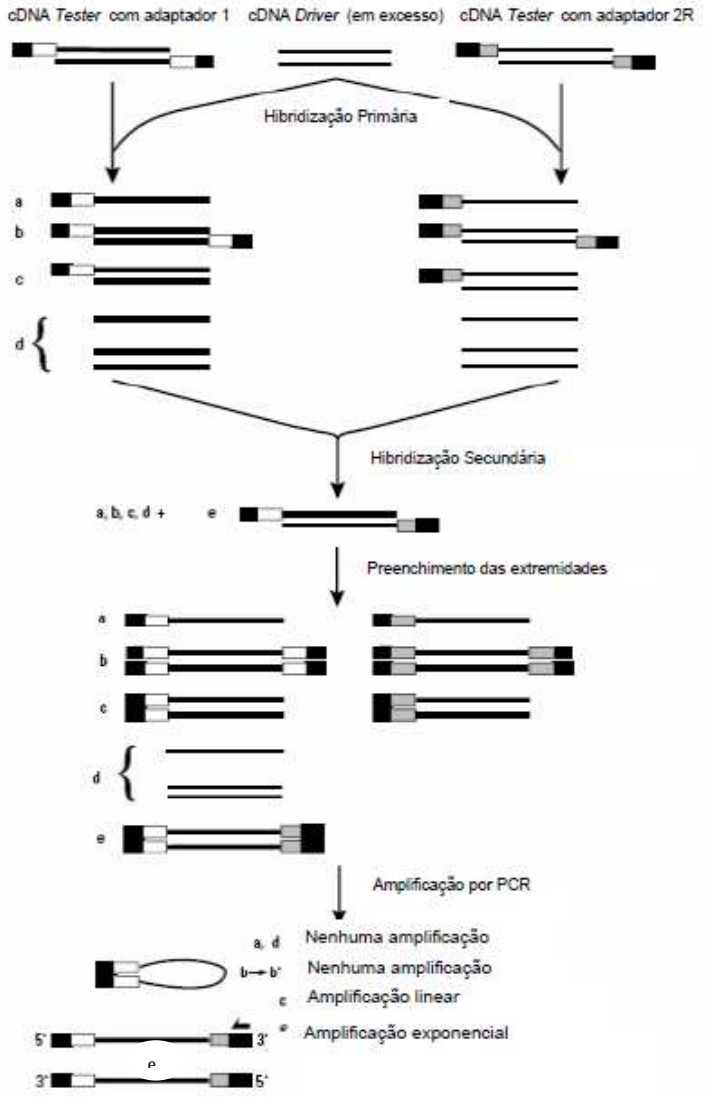

Figura 4. Esquema do método SSH. As linhas sólidas representam os cDNAs digeridos com *Rsa1* "*tester*" ou "*driver*". As caixas sólidas representam a região externa dos adaptadores Ad1 e Ad2R. As caixas claras representam a parte interna dos Ad1 e correspondem ao iniciador da *Nested* PCR, PN1. As caixas sombreadas representam a parte interna de Ad2R e corresponde ao iniciador de *Nested* PCR, PN2 (Fonte: adaptado de DIATCHENKO *et al.*, 1996).

Em surtos da infecção pelo vírus da mancha branca observa-se que um pequeno número de camarões de cultivo sobrevive à doença. Uma das hipóteses é que esses indivíduos sejam mais resistentes sobrevivendo ao surto infeccioso. Existem poucos estudos sobre a influência da transcrição gênica na resistência de camarões. Dentro desse contexto, torna-se importante avaliar a abundâncias e classes de transcritos envolvidos em respostas de defesa e resistência de camarões em relação a alterações ambientais e a susceptibilidade dos indivíduos a infecções virais.

Com o objetivo de identificar transcritos diferencialmente expressos em camarões expostos a um estresse osmótico no meio, brânquias de *L. vannamei* foram coletadas em dois intervalos de exposição desafiados ou não pelo vírus patogênico WSSV.

A resposta de transcrição destes genes pode auxiliar no entendimento de como crustáceos respondem ao estresse osmótico e verificar se há alteração na transcrição de genes na presença do agente infeccioso.

1.2. Hepatotoxina microcistina (MC) e seu efeito sobre a expressão gênica de glutationa S-transferases (GSTs)

Um grande número de espécies de cianobactérias são produtoras de potentes hepatotoxinas ou neurotoxinas. Pesquisadores tem demonstrado que essas toxinas têm um impacto na saúde humana e de organismos aquáticos (HALLEGEFF, 1993; CODD, 1995; FALCONER *et al.*, 1999; CARMICHAEL, 2001; PITOIS *et al*, 2001). Organismos aquáticos estão expostos no seu ambiente natural ou em condições de cultivo a eventos de formação de florações de cianobactérias tóxicas, consequência de um desequilíbrio das relações N:P comumente encontrado em águas eutrofizadas (Figura 5) (YUNES *et al,*. 1996). A exposição dos organismos à cianobactérias pode ocorrer via ingestão das células ou por bioacumulação ou difusão das toxinas liberadas na água, principalmente após a lise destas células (PFLUGMACHER *et al.*, 2005).

O risco potencial do efeito de cianobactérias sobre camarões surgiu na década de 1970, quando Lightner (1978) suspeitou que as cianobactérias marinhas pudessem estar causando mortalidade de camarões. Por causa das águas rasas e o aumento de nutrientes nos tanques de cultivo de camarão, florações de cianobactérias ocorrem com frequência (PFLUGMACHER *et al.*, 2005). Sugeriu-se que várias

espécies de cianobactérias, como *Microcystis, Nodularia, Lyngbya* e *Oscillatoria*, poderiam estar liberando as toxinas no meio. Essas toxinas estariam sendo bioacumuladas nos organismos contribuindo para os efeitos observados na saúde dos animais (SMITH, 1996). Estudos indicam que apenas quantidades muito pequenas de hepatotoxinas acumulam nos camarões cultivados (KANKAANPAA *et al.*, 2005).

Algumas cianobactérias podem produzir uma variedade de toxinas, das quais as microcistinas (MCs) são as mais amplamente distribuídas (CARMICHAEL, 1994). MCs são heptapeptídeos cíclicos pequenos compostos de vários aminoácidos e possuem a estrutura geral (-D-Ala-L-X-eritro-b-metil-D-isoAsp-L-Y-Adda-D-iso-Glu-N-metildehidro-Ala) (Figura 5), onde X e Y são dois L-aminoácidos variáveis. Combinações diferentes de dois aminoácidos dão origem a muitas variantes de MC. MCs são produzidas a partir de várias espécies de cianobactérias, como dos gêneros *Microcystis (M. aeruginosa, M.wesenbergii, M. viridis)* (Figura 5), *Oscillatoria (O.agardhii, O.rubescens, O.tenuis), Anabaena, Haphalosiphon, Aphanocapsa, Cyanobium, Arthrospira, Limnothrix, Phormidium, Hapalosiphon, Nostoc, Anabaenopsis* e *Synechocystis* (ZEGURA *et al.*, 2011). Cerca de 60 variantes estruturais de MCs foram caracterizadas a partir de florações e cepas isoladas de cianobactérias (SIVONEN; JONES, 1999). Embora muitas cepas produzam diversas MCs simultaneamente, geralmente apenas uma ou duas delas são dominantes a partir de uma única cepa (SIVONEN; JONES, 1999). Variações qualitativas nas MCs são mais freqüentes entre as cepas de *Anabaena*, mas também em *Microcystis* (SIVONEN *et al.*, 1995).

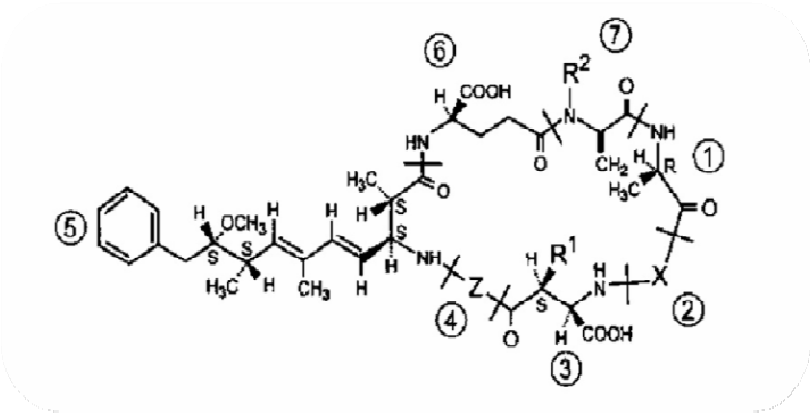

Figura 5. Estrutura geral das microcistinas (MCs) ciclo-(D-Ala[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-Glu[6]-Mdha[7]), heptapeptídeo hepatotoxinas de cianobactérias, mostrando as variações mais frequentemente encontradas, X e Z são L-aminoácidos variáveis (em MC-LR, X=L-leucina (L) e Z= L-arginina (R)). 1: D-alanina; 2: L-leucina; 3: ácido β-metilaspártico; 4: L-arginina; 5: ácido 3-amino-9-metoxi-2,6,8-trimetil-10-fenildeca-4,6-dienóico; 6: ácido D-glutâmico; 7: N-metildihidroalanina (Fonte: adaptado de SIVONEN e JONES (1999)).

A biossíntese dessas toxinas não é ribossomal e sim realizada por um amplo complexo multienzimático que inclui peptídeo sintetases, polipeptídeo sintetases e outras enzimas (TILLETT *et al.*, 2000; MOFFITT; NEILAN, 2000). Quando as cianobactérias estão em fase de multiplicação, a maioria das MCs estão localizadas dentro da célula, e muito poucas toxinas extracelulares são produzidas. Embora as MCs sejam quimicamente estáveis nos corpos de água, sua degradação microbiana pode ser rápida (SIVONEN; JONES, 1999). Baseando-se em estudos de toxicidade, a variante MC-LR (MW=995,2) é considerada uma das mais potentes toxinas cianobacterianas (ZEGURA *et al.*, 2011). As MCs são hidrofílicas, penetrando muito pouco de forma passiva pelas membranas celulares e, portanto, necessitam da captação via transporte ativo. O sistema de transporte multiespecífico para ácidos biliares tem sido descrito como os transportadores de MCs em fígado de camundongos (RUNNEGAR *et al.*, 1981). Esse é o motivo do fígado ser o principal órgão alvo. Entretanto, esses transportadores não são expressos somente no fígado, mas também no trato gastrointestinal, rim,

cérebro e há evidências que ultrapassem a barreira hemato-encefálica (RUNNEGAR *et al.*, 1981; FISHER *et al.*, 2005).

MC-LC são inibidores específicos de proteínas eucarióticas serino-treonina fosfatases 1 e 2A (PPI e PP2A) *in vitro* e *in vivo* (RUNNEGAR *et al.*, 1993). MC-LR ligam-se de forma covalente as proteíno-fosfatases (BAGU *et al.*, 1997). A conseqüência da inibição dessas proteínas é a hiperfosforilação de proteínas do citoesqueleto e assim, o bloqueio de muitos processos celulares, alteração e rearranjo do citoesqueleto, perda de adesões intercelulares nos desmossomos e, consequentemente, rompimento da arquitetura hepática (vide revisão em ZEGURA *et al.*, 2011). Também foi demonstrado que a indução de estresse oxidativo é um processo envolvido na hepatotoxicidade de MCs (BOUAICHA; MAATOUK, 2004). A exposição a baixas concentrações de MCs podem promover disfunção intestinal e hepática, bem como tumores hepáticos, e em altas concentrações podem causar hemorragia no fígado, necrose e choque hipovolêmico (citado por SUN *et al.*, 2008).

Em ambientes aquáticos, ainda não foi elucidado qual o destino destas toxinas e quais os efeitos crônicos que podem estar associados a esta exposição. Entretanto, sugere-se que os efeitos crônicos da toxina podem aumentar a suscetibilidade dos organismos a doenças (PFLUGMACHER *et al.*, 2005). Estudos em muitos organismos aquáticos, como *Artemia salina*, peixes e mexilhões, tem sugerido a formação de conjugados de MCYST-glutationa e MCYST-cisteína em fígado de animais expostos a esta toxina, originando um composto mais polar, que seria mais facilmente eliminado pelo organismo (PFLUGMACHER *et al.*, 1998). A formação do conjugado de glutationa (GSH) com MC-LR é sintetizada enzimaticamente por meio da glutationa-S-transferase solúvel (GSTs), e foi relatada sua ação nos processos de desintoxicação em diferentes organismos aquáticos como plantas (*Ceratophyllum demersum*), invertebrados (*Dreissena polymorpha, Daphnia magna*), ovas de peixes e peixes (*Danio rerio*) (PFLUGMACHER *et al.*, 1998). Acredita-se que a formação desse conjugado MC-GSH seja a primeira etapa no processo de detoxificação de cianotoxinas em organismos aquáticos. (PFLUGMACHER *et al.*, 1998; BEATTIE *et al.*, 2003).

Essa conjugação é mediada pelo sistema da Glutationa S-transferases microssomal e citosólica (GSTs). As Glutationa *S*-transferases (GSTs) (E.C. 2.5.1.18) compreendem uma família multifuncional de enzimas envolvidas nas reações de detoxificação de fase II de xenobióticos, toxinas e substratos endógenos incluindo os produtos tóxicos de dano tecidual ((PFLUGMACHER *et al.*, 2005). De

acordo com suas seqüências gênicas, propriedades químicas, físicas e imunológicas, em mamíferos, as GSTs têm sido classificadas em três principais famílias: GSTs citosólicas (incluindo sete classes nomeadas *alfa, pi, um, theta, sigma, omega* e *zeta*), GSTs mitocondriais (*kappa* classe) e GTS microssomais (atualmente designada MAPEG) (HAYES *et al.*, 2005). Estas classes diferem em sua expressão tecido-específica e distribuição no interior dos tecidos. Todas GSTs citosólicas tem sido encontradas como sendo homo- ou heterodiméricas (dentro da mesma classe) com um peso molecular de aproximadamente 50 kDa (MANNERVIK, 1985). *Alpha* GSTs são altamente expressas no fígado, rins, testículos e glândulas supra-renais de animais vertebrados. GSTs *mu* foram encontradas em altas concentrações no cérebro, músculos, fígado, rim e pulmão (HAYES; MANTLE, 1986; HAYES *et al.*, 1987). Em *L. vannamei*, GST *mu* foi analisada por PCR quantitativo e os transcritos foram detectados principalmente no hepatopâncreas e brânquias, mas também nos hemócitos e músculo, porém não foi identificada nos pleópodes (CONTRERAS-VERGARA *et al.*, 2004).

GSTs da classe *pi* são enzimas altamente ácidas, amplamente distribuídas, exceto no fígado adulto onde estão localizadas no epitélio (SATO *et al.*, 1984; SATO *et al.*, 1985). GSTs da classe *theta* foram descritas como sendo expressas somente no fígado (HIRATSUKA *et al.*, 1990; MEYER *et al.*, 1991). A atividade geral de GST é determinada usando-se o CDNB (1-cloro-2,4-dinitrobenzeno), um substrato sintético e não específico. As GST da classe alfa possuem maior atividade frente ao hidroperóxido de cumeno (CHP), as GST da classe mi tem maior atividade para o 1,2-dicloro-4-nitrobenzeno (DCNB) e as da classe *pi* possuem maior atividade frente ao ácido etacrínico (ETHA). Já as da classe teta possuem maior atividade frente ao 1,2-epoxi-3-p-nitrofenoxipropano (EPNP) (MEYER *et al.*, 1991; EGAAS *et al.*, 1999).

Com o objetivo de identificar isoformas de Glutationa *S*-transferases (GST) envolvidas na detoxificação de microcistinas no camarão marinho *Litopenaeus vannamei* foi inoculada a microcistina [D-Leu^{1}]MC-LR em um nível sub-letal. Após 48h, os órgãos foram coletados para a extração do RNA total e transcrição reversa de cDNAs. Foram desenhados iniciadores a partir de ESTs disponíveis no banco de dados correspondentes a diferentes isoformas de GST, isso com o objetivo de analisar por PCR em tempo real os níveis de transcrição das isoformas nos camarões inoculados com a toxina.

A resposta de transcrição destes genes pode auxiliar no entendimento de como crustáceos metabolizam e eliminam a toxina do organismo.

2. OBJETIVOS GERAIS

- Identificar e avaliar a expressão de transcritos diferencialmente expressos na brânquia do camarão *litopenaeus vannamei* exposto a estresse osmótico e desafiado com wssv;

- Verificar a expressão e atividade de isoformas de glutationa *s*-transferase e da catalase no camarão *Litopenaeus vannamei* inoculado com cepa tóxica de *Microcystis aeruginosa*.

3. OBJETIVOS ESPECÍFICOS

CAPÍTULO 1:

1. Estabelecer as concentrações iônicas da água do meio e da hemolinfa dos camarões nas salinidades 10 e 20;
2. Identificar sequências similares de transcritos diferencialmente expressos (induzidos/ reprimidos);
3. Analisar a expressão relativa por PCR em tempo real de seqüências selecionadas a partir da SSH em brânquias de camarões infectados com WSSV e expostos a estresse osmótico.

CAPÍTULO 2:

1. Avaliar a transcrição relativa de oito novas isoformas de *GST* e da *CAT* através de PCR em tempo real no hepatopâncreas de camarões inoculados com MC;
2. Analisar a atividade enzimática de GST total e da CAT.

4. CAPÍTULO 1

Identification of differentially transcribed genes in shrimp *Litopenaeus vannamei* exposed to osmotic stress and challenged with WSSV virus

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Running title: Gene transcription in WSSV-infected hyposmotic shrimp

Keywords: SSH, gill, *Litopenaeus vannamei*, osmotic stress, WSSV, qPCR.

Abstract

The effects of hyposmotic stress and white spot syndrome virus (WSSV) challenge in the gene transcription levels was studied in the marine shrimp *Litopenaeus vannamei*. Messenger RNA from gills of shrimp submitted to osmotic stress was isolated in order to identify genes differentially transcribed through the suppressive subtractive hybridization (SSH) method. Two subtractive libraries forward and two reverse were constructed to identify up and down-regulated genes under these conditions. About 192 clones were sequenced, of which 46 genes were identified. These genes encode proteins corresponding to a wide range of biological roles, including defense, cell signaling, electron transfer, cell proliferation and differentiation, apoptosis, intermediary metabolism, cytoskeleton and digestion. Among the identified genes, 19 were up-regulated and 27 were down-regulated in the animals kept at a lower ion concentration. We evaluated the transcription levels of eight genes by qPCR in shrimp submitted to hyposmotic conditions with and without WSSV challenge. The SSH enabled the identification of genes that are influenced by hyposmotic stress. A significant up-regulation were observed in *lectin-C*, *QM*, *TGF beta inducible nuclear protein 1*, *ciclophilin*, *malate dehydrogenase*, *mitochondrial ATP synthase F chain* and *ATP synthase subunit 9 precursor* transcripts. However, the transcription of these genes in *L. vannamei* was not affected by WSSV infection both at isosmotic and hyposmotic conditions.

Introduction

The white spot syndrome virus (WSSV) is the most cosmopolitan pathogen to shrimp and has affected negatively the shrimp farming industry in more than 30 countries (Tapay et al. 1999; Wang et al. 1999). Although considerable progress has been made in the characterization of WSSV, the understanding of the shrimp defense system in response against viral infection has still several mechanisms to be elucidated (Pan et al. 2005). In the south region of Brazil, this disease was diagnosed in 2005 and caused a massive decrease on shrimp production (EPAGRI 2010).

Different management strategies on shrimp farming can be used to minimize the impacts caused by the WSSV (Seiffert 2005). Strong evidences support the hypothesis that the incidence of diseases can be minimized or exacerbated by changes on water quality parameters, such as salinity, temperature, oxygen, hardness among others (Tsai et al. 1993; Kautsky et al. 2000; Vidal et al. 2001; Guan et al. 2003; Yu et al. 2003; Liu et al. 2006; Peinado-Guevara and López-Meyer 2006; Rahman et al. 2006; Ruiz-Velazco et al. 2010; Tendencia et al. 2010). Increased survival of WSSV-infected shrimp was observed when they were kept at higher temperatures (Vidal et al. 2001; Guan et al. 2003; Jiravanichpaisal et al. 2004, 2006; Rahman et al. 2006). Some studies have associated changes on salinity and alkalinity with the appearance of WSSV infection (de la Vega et al. 2007; Costa et al. 2010; Ruiz-Velazco et al. 2010; Tendencia et al. 2010).

Salinity is one of the most important abiotic factors which affects growth and survival of marine and estuarine organisms and has complex and multifaceted biological effects (Péqueux 1995; Kumlu et al. 1999; Fielder et al. 2001; Atwood et al. 2003; Saoud et al. 2003; Davis et al. 2005; Buranajitpirom et al. 2010). Penaeid can be found worldwide in areas with a wide range of salinity, from hyposmotic conditions (0.5) to hyperosmotic (40) (Bray et al. 1994; Samocha et al. 1998, 2002; McGraw et al. 2002). In general, penaeid shrimps exhibit hyperosmotic regulation to seawater at salinities below the isosmotic concentrations and hyposmotic regulation to those above, with the isosmotic concentrations at 20-30 (Castille and Lawrence 1981). Fluctuations in salinity and decrease in water alkalinity enable unfavorable conditions to shrimp growth in nurseries due to ions deficiency (Atwood et al. 2003; Decamp et al. 2003; Saoud et al. 2003). Among the eleven major ions in seawater, Mg^{2+}, Ca^{2+} and K^{+} are the three limiting factors for the cultivation of penaeid. Mg^{2+} plays a key role in the lipids, proteins,

carbohydrates metabolism and serves as a cofactor in a large number of metabolic and enzymatic reactions (Davis and Lawrence 1997). Ca^{2+} is an important ion in osmoregulation, blood clotting, muscle contraction, nerve transmission, enzymatic activity and participates hardening the exoskeleton in the molting process in crustaceans (Davis and Gatlin 1996). Most of aquatic species can absorb Ca^{2+} directly from the surrounding condition to meet their Ca^{2+} requirement (Deshimaru and Yone 1978; Coote et al. 1996; Davis and Gatlin 1996). K^{+} is the main intracellular cation and it is also important in the activation of Na^{+}-K^{+}-ATPase, which is a key component in the regulation of extracellular volume (Mantel and Farmer 1983).

Little information is available about the effect of osmotic condition on the gene transcription pattern in shrimp and whether these changes might be associated with the WSSV infection. One way to address that is using the suppressive subtractive hybridization (SSH) method. Different studies have used SSH technique to identify genes differentially transcribed involved in defense mechanisms of shrimp exposed to changes on environmental conditions (He et al. 2004; de Lorgeril et al. 2005; Pan et al. 2005; de la Vega et al. 2007; Reyes et al. 2007; Zhao et al. 2007; García et al. 2009; Pongsomboon et al. 2009).

The aims of this study were to identify genes that are differentially transcribed in gills of *L. vannamei* exposed to different osmotic conditions and further to evaluate the influence of the osmotic conditions in the transcription responses of some of those selected genes from SSH and after challenging with the pathogenic virus WSSV.

Materials and methods

Osmotic stress, WSSV challenge and experimental design

Specimens of juvenile shrimp L. vannamei (weighting 11 ± 1.9 g) were collected at the Marine Shrimp Laboratory from the Universidade Federal de Santa Catarina, Florianopolis, Brazil, and kept in aquaria at the density of 5 m^{-2}, fed ad libitum, with constant aeration, 29°C and 35 ppt salinity. In order to minimize the influence of sex and the ecdise cycle on the gene transcription pattern, only females in the intermolt stage were used in the experiments, based on Robertson et al. (1987) and Vijayan and Diwan (1996).

Two experiments were conducted. In the first experiment, the animals were kept in two osmotic conditions: hyposmotic (salinity 10) and isosmotic (salinity 20). The decrease of salinity from 35 to 20 and 10 was carried out by the slow addition of filtered freshwater in two tanks of 200 L during 7 days. Two groups of organisms were kept respectively at both salinities for 16 days. About 20% of the water in the tank was renewed daily.

A second experiment was carried out with shrimps kept in the two osmotic conditions (salinities 10 ppt and 20 ppt) and challenged with the WSSV virus. Before starting the virus challenge, hemolymph samples from all shrimps were collected individually, immediately frozen in liquid nitrogen and stored at -80°C. WSSV detection in hemolymph was carried out by 2-step PCR according to Lo et al. (1996) in order to confirm the absence of WSSV infection before the starting of the experiment. All shrimps used in this study were tested for the presence of WSSV through Nested PCR test before the beginning of the experiments and were diagnosed as negative for the presence of the virus (data not shown).

The WSSV challenge was carried out using a viral inoculum prepared according to the methodology adapted from Prior et al. (2003). Briefly, frozen muscle tissue (10 g) of WSSV-infected shrimp, confirmed by PCR analysis, was homogenized in the presence of sterile Tris-NaCl (20 mM Tris, 0.4 M NaCl, pH 7.4) (1:10 w/v) and centrifuged at 2,000x*g* for 20 min at 4°C. The supernatant was recovered and centrifuged at 9,000x*g* for 10 min at 4°C, then was filtered (0.45 μM) in sterile conditions. The viral solution was aliquoted (200 μl) and kept in liquid nitrogen. The virus titer inoculum of infection was 1.04 x 10^7/10g muscle tissue (Müller 2009). The shrimps were inoculated through i.m. injection in the second abdominal segment

with 100µl of viral inoculum (treated) or isotonic solution to shrimp (control). After 48h, the gills from both groups were collected aseptically and preserved in RNA Later ™ solution (SIGMA®). A subset of individuals infected with WSSV was used to control the mortality and confirm the viral infection. We observed 100 % of mortality after 5 days p.i. viral.

Water quality parameters

Analysis of pH, ammonia and total alkalinity in the water tanks was carried out using a commercial kit for seawater analysis (Alfakit®) and an electronic probe was used to measure dissolved oxygen (YSI Incorporated® 550A).

Ionic concentration of water and hemolymph

Hemolymph from three animals collected in solution containing 10mM HEPES, 20 mM EDTA were pooled, centrifuged at 8,000x*g* for 10 min at 4°C and stored at -80°C until analysis.

Levels of Ca^{+2}, Mg^{2+} and K^{+}, both in the hemolymph and water tanks, were performed using atomic absorption spectrophotometer (Hitachi, Model Z 8230) (Vijayan and Diwan 1996).

Total RNA and messenger RNA purification

Five shrimps not infected with WSSV were killed from each group. Gill samples were excised, immersed in RNAlater™ solution (SIGMA ®), left at 4°C overnight and stored at -80°C. Total RNA was extracted from gills individually using TRIzol reagent (Invitrogen®) according to manufacturer's instructions.

Messenger RNA was isolated and purified using the kit MicroPolyA (Ambion®) from 2µg total RNA according to manufacturer's instructions. The concentration and purity of total RNA and mRNA was checked by spectrophotometer Nanodrop 2000 (Thermo Scientific) at 260nm and verified the ratio 260/280nm.

Construction of the suppression subtractive hybridization (SSH) library and sequencing

SSH was performed using the hyposmotic exposed shrimp as the tester and isosmotic exposed shrimp as the driver, in order to identify

genes that were up-regulated in the hyposmotic condition. Messenger RNA (1.25μg) was reverse-transcribed using the system Clontech PCR-Select cDNA Subtraction Kit (Clontech®) according to manufacturer's instructions. The differentially transcribed genes were amplified by nested PCR using primers specific for the adapters and the products were visualized by electrophoresis on 2% agarose gel. PCR products were purified using the Illustra GFX PCR DNA system and Gel Band Purification (GE Healthcare®) according to manufacturer's instructions. The purified cDNA was cloned into pGEM-T Easy kit Vector ® (Promega, Madison) according to manufacturer's specifications and inserted into DH5-α cells by heat shock. Positive bacteria colonies were grown overnight and the plasmid DNA purified. The products were sequenced in an automatic sequencer MegaBACE 1000 DNA Analysis System® (GE/Amersham Biosciences, Upsalla), using the sequencing reaction prepared from the Kit ET Dye Terminator DYEnamic® according manufacturer's guidelines. The sequencing reaction was performed from 5.0 pmol of primer M13-F or M13-R and about 800ng plasmid DNA. The sequences were analyzed for quality using the package Phred /Phrap /Consed (Ewing and Green 1998). The identification of the fragments was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1997) and subsequently merged using the CAP3 sequence assembler program (http://pbil.univ-lyon1.fr/cap3.php) to form consensus sequences.

Functional characterization of shrimp sequences was based on Gene Ontology (GO) annotation and was carried out by means of the universal platform Blast2GO (http://www.blast2go.org/) using default parameters (Conesa et al. 2005). Additional protein domain information was added to the annotation through Interpro (IPS) analysis (http://www.ebi.ac.uk/interpro/), also using the Blast2GO platform. Final annotation was based on top blast hits, GO terms and IPS results.

Gene transcription analysis by qPCR of WSSV challenged shrimp in different osmotic conditions

For each shrimp, total RNA from gill was extracted using TRIzol® reagent (Invitrogen®) according to the manufacturer's protocol, and 2μg of total RNA was reverse transcribed to cDNA using Omniscript Reverse Transcription Kit (Qiagen®).

The primers were designed using the Primer Quest program (Integrated DNA Technology, IDT), based on genes identified in the cDNA library (Table 1). Among the up-regulated genes list were selected the *TGF beta inducible nuclear protein 1*(*TINP1*) , *Trypsin-like serine proteinase* (*TRYP*) and *QM protein* (*QM*), and among the down-

regulated genes were selected the mitochondrial *ATP synthase F chain* (*ATPsy1*), *Malate dehydrogenase 2-2 NAD* (*MDH*), *Lectin-C* (*LEC-C*), *Cyclophilin-type peptidyl-prolyl* (*PPI*) and *ATP synthase subunit 9 mitochondrial precursor* (*ATPsy2*). In addition, specific primers were designed for the housekeeping genes *ribosomal 18S*, based on a sequence from *Penaeus aztecus* (Genbank M34362), and *ribosomal L8* based on a sequence from *L. vannamei* (Genbank DQ316258) (Table 1). Primer pairs were tested for *ribosomal L8* and *18S* as housekeeping genes for normalizing the data, but only the primers for *18S* were selected, because the hyposmotic or WSSV treatment affected the transcription of *ribosomal L8*. The primers used in the qPCR, showed in Table 1, were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 2000) avoiding hairpin and dimer formation using the software PrimerQuest (http://www.idtdna.com/scitools/applications/primerquest/) and FASTPCR (Kalendar et al. 2009).

For the amplification of PCR products, 0.3 mM for each primer pair was used to amplify the target and normalizer genes. The cycling conditions used were: 95°C for 15 min, 40 cycles of 10s at 95°C, 15s at 53°C, 20s at 72°C. A melting curve was performed to confirm amplification of specific products. Quantifast^{TM} SYBR Green (Qiagen®) was used as a fluorescence marker to detect specific PCR products. Data were collected as CT using the Rotor Gene 6000 software version 1.7 (Corbett Research®). Analysis was performed in duplicate for each sample.

Statistical analysis

Homogeneity of variances was tested (Cochran C; Hartley; Bartlett) accepting $p<0.05$, and the normal probability test was used to verify data normality. Gene expression calculations and statistical analysis were carried out using the REST software (Pfaffl et al. 2002).

Table 1. Primers used in the qPCR:

Gene description (sequence origin SSH)	Code	Primer	Sequence (5' – 3')	Product size
Lectin-C (contig 60)	LEC-C	F (forward)	AGGCCTGCATCGCCATTCTCAAC	171
		R (reverse)	AGCTCATCGGAGTCCTCGCACA	
QM protein (contig 27)	QM	F	GGCATCTCACGGACATCGGACTTC	139
		R	ACCCCAAGTCGCGTTTCTGTCGT	
TGF beta inducible nuclear protein 1 (contig 2 and d09)	TINP1	F	TAGACCGTGAGGGTGAAACGAGAGC	82
		R	TCCCATTTACCAGCCTTCTCCTTCC	
Peptidylprolyl isomerase B - cyclophilin B (a11)	PPI	F	GACGTATTGAAATCGGCCTGTTTGG	79
		R	TCAGGCTTGGTTGCCAATTCCTTG	
Malate dehydrogenase 2-2, NAD (contig 54)	MDH	F	ATGCCATTGGGACCCAGGACAAG	101
		R	ATGAACGGAGAGCAGGGTGTGGTG	
Mitochondrial ATP synthase F chain (contig 44)	ATPsy1	F	TCCCGCCAGGTTCTATGGCAAAC	125
		R	TTGTGCTGCCATCTCCACCAAGC	
ATP synthase subunit 9 mitochondrial precursor(contig 34)	ATPsy2	F	TGTCTTATGATGGCCTTCCTGTTGC	121
		R	GGAAGTGGTCCACAAACACCATGC	
Trypsin (contig 25)	TRYP	F	GCTGATGCAGGCTACTATGGCGTCT	97
		R	GCTCCTCTCAGCAGAAGTTCCCAGA	
Ribossomal 18S (housekeeping)	DECRT1	F	CCGAATGGTCGTGCATGGAATGAT	127
		R	GAATTTCACCTCTAGCGTCGCAGT	
Ribossomal L8 (housekeeping)	L8	F	TAGGCAATGTCATCCCCATT	167
		R	TCCTGAAGGGAGCTTTACACG	

Results

Shrimp survival following the osmotic stress

No mortality was observed in all groups. An increased frequency of soft-shelled shrimp was observed in the treatment of hyposmotic condition (data not showed).

Water quality and osmotic condition

The water quality parameters pH, oxygen, ammonia and total alkalinity (8.12 ± 0.06, 5.5 ± 1.0 mg l^{-1}, 0.47 ± 0.35 mg l^{-1}, and 100 mg l^{-1} $CaCO_3$, respectively) measured in the tank of the isosmotic group were within the acceptable range for this species according to Boyd (1990). In the hyposmotic tank, total alkalinity was 80 mg l^{-1} $CaCO_3$. Lower levels of Ca^{2+}, Mg^{2+} and K^+ were observed in the water from the tank kept at salinity 10 , when compared to the tank kept at salinity 20 (Fig. 1). The levels of these ions in the shrimp hemolymph remained quite similar regardless of salinity (Fig. 1).

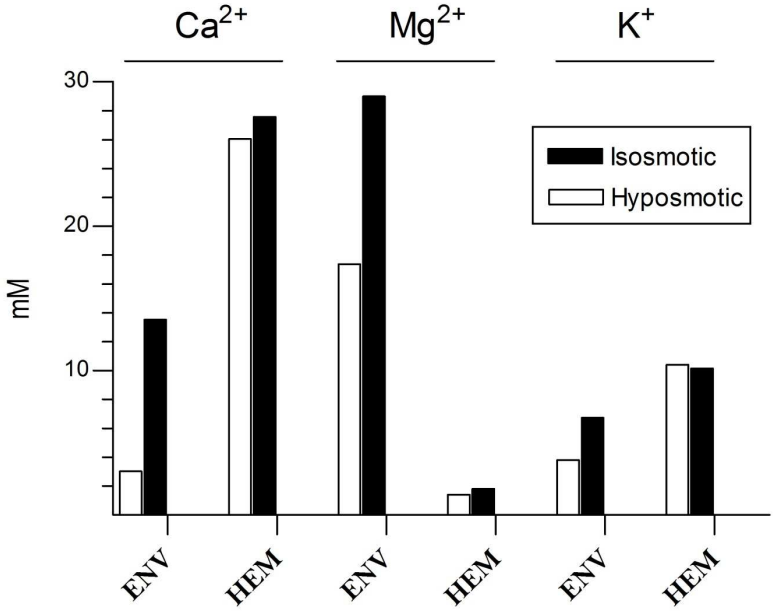

Figure 1. Ca^{2+}, Mg^{2+} and K^+ concentrations (mM) in the environmental medium (ENV) and hemolymph serum (HEM) of *L.vannamei* kept for 16-days at

salinities 20 (isosmotic) and 10 (hyposmotic). Bars represent measurements carried out in pools of three shrimps each.

Identification of genes that were differentially transcribed using SSH

Two cDNA libraries were generated in this study containing, respectively, the up-and down-regulated genes in gills of shrimp kept under hyposmotic conditions compared to organisms kept at salinity 20 ppt. Using the software CAP3, from 192 sequenced clones we identified 61 consensus sequences and 120 isolated sequences. The inserts had a size range between 95 bp and 1582 bp, with an average size of 653 bp. Blasting to NCBI database, 46 genes were identified, 19 up-regulated (Table 2) and 27 down-regulated genes (Table 3). The remaining sequences had no similarity with known genes.

Twenty-eight sequences were putatively annotated using GO terms obtained from the first 20 BLAST hits or/and from protein domains obtained from the InterPro database. Several basic biological processes were represented. Among the list of up-regulated genes are those belonging to immune defense (3), protein synthesis (3) and proteolysis (1), mitochondrial electron transfer (2), signal transduction (2), cell metabolism (2), metalloprotease (2), transcription factor (2) and others. The down-regulated genes are related with energy production (5), cuticle synthesis (4), protein synthesis (4), osmoregulatory process (1), mitochondrial electron transfer chain (2), protein folding (1), citric acid cycle (1), intracellular proton transport (1), immune defense (1), aminopeptidase activity (1), palmitoyl hydrolase activity (1) and others.

Table 2. Subtractive library of the up-regulated genes:

Genbank access code	Seq. Name	Seq. Description	Species	Seq. Length	E value	(%) Id	N	Putative feature/function
YP_001315043.1	Contig1	Cytochrome b*	*Litopenaeus vannamei*	480	8e-90	93	1	Mitochondrial Electron Transfer
AC101299.17	Contig 13	Ubiquinol-cytochrome c reductase binding protein**	*Mus musculus*	302	9e-09	91	1	Mitochondrial Electron Transfer
ACH70872.1	Contig2 d09	TGF beta inducible nuclear protein 1*	*Salmo salar*	260	7e-37	85	2	Signal transduction
NG_013337.1	Contig 23	Amyloid beta (A4) precursor protein-binding (APBB2) **	*Homo sapiens*	514	4e-10	86	1	Signal transduction
DAA34115.1	Contig4	Ribosomal protein rpl7a*	*Amblyomma variegatum*	314	2e-20	81	1	Protein synthesis
DQ534543.1	Several	Mitochondrion**	*Litopenaeus vannamei*	-	-	-	5	Protein synthesis
BC091659.1	e10	Tu translation elongation factor, mitochondrial (tufm)**	*Danio rerio*	221	1e-11	95	1	Protein synthesis
CAA60129.1	Contig25	Trypsin*	*Litopenaeus vannamei*	1025	4e-18	66	1	Proteolysis
ACV72062.1	Contig27	QM protein*	*Penaeus monodon*	744	4e-11	66	1	Cell growth, differentiation and apoptosis

continuation …

ACR34045.1	a09	Cell wall hydroxyproline-rich glycoprotein*	*Zea mays*	727	1e-07	39	1	Cellular cell wall organization, primary cell wall
ACU31809.1	Contig26	Alpha-2-macroglobulin isoform 3*	*Fenneropenaeus chinensis*	1008	6e-20	54	1	Immune defense
NM_001134701.1	a04	MHC class I RT.Aa alpha chain (Rt1.aa)**	*Ratus novergicus*	820	1e-11	89	1	Immune defense
AEH05998.1	Contig 11 a12	Mitochondrial C type lectin containing domain protein*	*Litopenaeus vannamei*	855	1e-38	97	5	Immune defense
EFP85449.1	e03	Zinc metalloproteinase nas-10*	*Ascaris suum*	297	6e-08	52	1	Proteolysis, metalloendopeptidase activity, zinc ion binding
ACR48141.1	Contig 22	SpAN-like protein**	*Rimicaris exoculata*	953	2e-12	50	1	Metalloprotease
NM_006366.2	Contig 15	CAP, adenylate cyclase-associate d protein (yeast) (CAP2)**	*Homo sapiens*	439	3e-09	88	2	Unknown, appears to be able to interact with adenylyl cyclase-associated protein and actin
NG_011881.1	e09	Ataxin 2-binding protein 1 (A2BP1)**	*Homo sapiens*	399	4e-08	85	1	mRNA processing, mRNA splicing

continuation …

AM402994.1	Contig 28	Transposase IS630**	*Listonella anguillarum*	1130	9e-10	84	5	Transcription factor
DQ630805.1	Contig 5	Putative accessory gland protein**	*Gryllus rubens*	286	4e-12	95	1	Unknown

Seq. Name: code of EST sequence; Seq. Description: EST sequence describe with similarity identified from blastx*/blastn**/NCBI; Seq. Length: EST length (base pair); E value: E value describe in the blast/NCBI; (%) Id: EST similarity; N: EST number. * *similarity identified from blastx*. ** *similarity identified from blastn.*

Table 3. Subtractive library of the down-regulated genes:

Genebank access code	Seq. Name	Seq. Description	Species	Seq. Length	E value	(%) Id	N	Putative feature/function
HM163157.1	Contig 55	V-H-ATPase subunit A*	*Litopenaeus vannamei*	2654	2e-72	94	1	Osmoregulatory process
ABX56859.1	Contig 34	ATP synthase subunit 9 mitochondrial precursor*	*Litopenaeus vannamei*	116	7e-10	100	1	Energy production
GQ848643.3	f02	ATP synthase subunit alpha precursor**	*Litopenaeus vannamei*	280	4e-114	92	1	Energy production
NP_001040526.1	d02	H+ transporting ATP synthase O subunit*	*Bombyx mori*	209	1e-32	53	1	Energy production
ACJ64319.1	Contig 44	Mitochondrial ATP synthase F chain*	*Culex tarsalis*	518	8e-44	74	1	Energy production
BC088540.1	Contig 30	Zinc binding alcohol dehydrogenase, domain containing 2 (zadh2)**	*Xenopus (Silurana) tropicalis*	289	6e-11	86	2	Energy production
NP_001133198.1	Contig 54	Malate dehydrogenase 2-2, NAD (mitochondrial)*	*Salmo salar*	338	1e-26	81	1	Citric acid cycle
ABD98763.1	g04	Vacuolar ATPase G subunit-like protein*	*Graphocephala atropunctata*	119	3e-26	65	1	Intracellular proton transport
AB264633.1	Contig 60	C-type lectin*	*Papilio xuthus*	1100	4e-42	75	1	Immune defense

continuation …

NP_001013474	Contig 53	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5*	*Danio rerio*	116	2e-08	49	1	Mitochondrial electron transfer chain
YP_001315034.1	Contig 46	Cytochrome c oxidase subunit II*	*Litopenaeus vannamei*	229	5e-57	92	1	Mitochondrial electron transfer chain
ABB91676.1	Contig 37 f10	Calcified cuticle protein CP14.1*	*Callinectes sapidus*	144	6e-25	60	2	Cuticle synthesis (calcification inhibitor in epi- and exocuticle)
ADI59749.1	Contig 58	Early cuticle protein 1*	*Callinectes sapidus*	1078	1e-05	69	1	Cuticle synthesis
ACR78694.1	b06	Peritrophin*	*Rimicaris exoculata*	107	6e-06	52	1	Cuticle synthesis
AAR82845.1	Contig 43	Actin D*	*Litopenaeus vannamei*	297	1e-58	99	1	Cytoskeletal
ABI52808.1	Contig 39	40S ribosomal protein S23*	*Argas monolakensis*	143	3e-48	94	1	Protein synthesis
AEB54637.1	Contig 47	40S ribosomal protein S24*	*Procambarus clarkii*	110	4e-10	79	1	Protein synthesis
HQ127458.1	h01	16S ribosomal RNA gene**	*Litopenaeus vannamei*	1132	5e-82	100	1	Protein synthesis
DQ534543.1	Several	Mitochondrion**	*Litopenaeus vannamei*	-	-	-	25	Protein synthesis
NM_001242544.1	a11	Peptidylprolyl isomerase B (cyclophilin B) *	*Apis mellifera*	937	1e-49	76	1	Protein folding

continuation …

BC040792.1	h10	Alanyl (membrane) aminopeptidase**	*Mus musculus*	262	1e-10	91	1	Aminopeptidase activity
BC052330.1	f08	Palmitoyl-protein thioesterase 2**	*Mus musculus*	175	5e-09	92	1	Palmitoyl-(protein) hydrolase activity
NM_001131463.1	Contig 38	Tripartit motif containing 37 (TRIM37)**	*Pongo abelii*	267	3e-32	92	2	Diverse cellular functions such as developmental patterning and oncogenesis, chelate zinc and might be involved in protein-protein and/or protein-nucleic acid interactions
AM941347.1	Contig 56	finTRIM family protein (ftr02 gene)**	*Danio rerio*	395	1e-09	91	1	Interacting selectively and non-covalently with zinc (Zn) ions
NM_015261.2	Contig 40	Non-SMCcondensin II complex , subunit D3 (NCAPD3)**	*Homo sapiens*	459	1e-07	93	1	Condensin complexes I and II play essential roles in mitotic chromosome assembly and segregation

continuation …

ZP_03450878.1	Contig 33	Endo/excinuclease domain protein**	*Burkholderia pseudomallei 576*	904	1e-09	57	1	Involved in many cellular processes, such as class I homing GIY-YIG family endonucleases, prokaryotic nucleotide excision repair proteins UvrC and Cho, type II restriction enzymes, the endonuclease/reverse transcriptase of eukaryotic retrotransposable elements, and a family of eukaryotic enzymes that repair stalled replication forks.
AAH11176.1	c06	Proline-rich protein BstNI subfamily 1**	*Mus musculus*	1582	4e-08	34	1	Unknown

Seq. Name: code of EST sequence; Seq. Description: EST sequence describe with similarity identified from blastx*/blastn**/NCBI; Seq. Length: EST length (base pair); E value: E value describe in the blast/NCBI; (%) Id: EST similarity; N: EST number. * *similarity identified from blastx.* ** *similarity identified from blastn.*

Effects of osmotic conditions in the gene transcription levels of *L. vannamei* infected or not-infected with WSSV

Eight genes were selected and their differential transcription levels were evaluated through qPCR in shrimp kept at hyposmotic and isosmotic conditions and both infected and not-infected with WSSV.

Despite the high individual variability observed in the samples within a given experimental group, shrimp kept under hyposmotic conditions showed significant induction in gene expression for all tested genes, excepting *TRYP*, comparing with the group kept in the isosmotic condition. This pattern was observed in both WSSV-infected and WSSV-non-infected shrimps (Fig. 2). The shrimp kept in the hyposmotic condition showed an increased transcription rate of *TINP1*, *QM*, *ATPsy1-2*, *PPI* and *MDH* from 200 to more than 1000-fold when compared to the organisms kept at salinity 20. LEC-C gene expression was more discretely up-regulated (105 fold).

When infected with WSSV, the *TINP1*, *QM*, *PPI*, *MDH*, *LPS*, *ATPsy1-2* and *LEC-C* transcripts were up-regulated significantly from 43 to 200 fold in hyposmotic over the isosmotic condition. Despite the lower levels of changes observed in the WSSV-infected shrimp after the osmotic challenge, comparing to the non-infected shrimp, the virus infection did not affect statistically the transcription of tested genes.

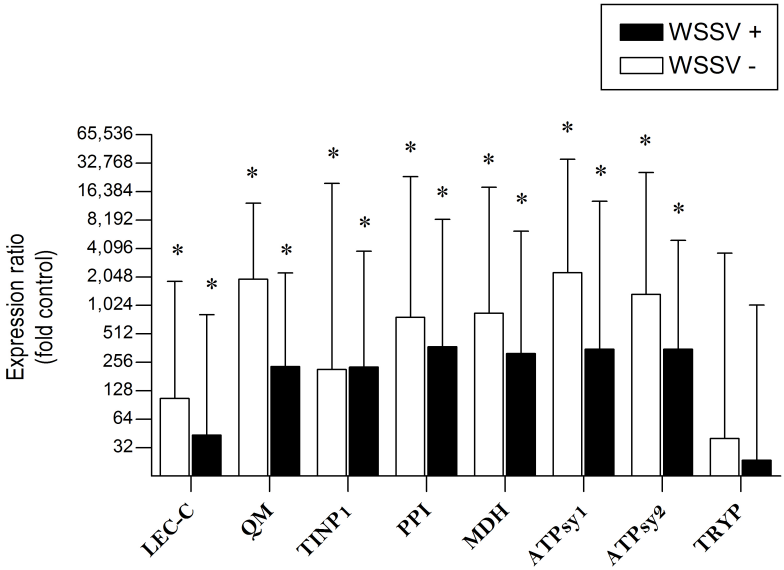

Figure 2. Real time qPCR expression analyses for eight different sets of genes in shrimp kept for 7 days at hiposmotic conditions with or without WSSV challenge (*LEC-C* = lectin type C; *QM* = QM protein; *TINP1* = TGF-beta inducible nuclear protein 1; *PPI* = cyclophilin; *MDH* = malate dehydrogenase; *ATPsy1* = mitochondrial ATP synthase F chain; *ATPsy2* = ATP synthase subunit 9 mitochondrial precursor; *TRYP* = trypsin); white bars: hyposmotic condition in relation to isosmotic condition without WSSV (WSSV -); black bars: hyposmotic condition with WSSV in relation to isosmotic condition with WSSV (WSSV +). *Significant differences in gene expression levels between hyposmotic and isosmotic conditions ($P < 0.05$). N=6 shrimp per treatment. Data are presented as Ratio = $E_{\text{target genes}}^{\Delta CP \text{ target (Mean Control – Mean Sample)}} / E_{18S \text{ gene}}^{\Delta CP \text{ 18S (Mean Control – Mean Sample)}}$.

Discussion

Water salinity and ionic levels

Most of shrimp farms worldwide are concentrated in coastal regions and often uptake water from the surrounding estuaries (FAO 2010). Estuarine waters are directly affected by the rainfall, which changes salinity, the ion levels and alkalinity of the water, compromising its quality for shrimp ponds (Costa et al. 2010; Ferreira et al. 2011).

The ability of crustaceans to osmoregulate is determined by the difference between hemolymph and medium osmolarity at a given salinity (Charmantier et al. 1989). Despite the observed decrease of about 45% in the levels of Mg^{2+}, Ca^{2+} and K^{+} in the tank maintained at salinity 10, compared to the tank kept at salinity 20, the hemolymph levels of these ions in the *L. vannamei* kept for 16 days at lower salinity did not change, suggesting an active osmo-regulatory process. These data are consistent with previous studies (Chen and Liao 1986; Ferraris et al. 1986; Lin et al. 2000; Tantulo and Fotedar 2007). The levels of Mg^{2+}, Ca^{2+} and K^{+} detected in the experimental conditions are similar to what have been observed in estuarine waters regularly used for shrimp ponds supply in south of Brazil (Ferreira et al. 2011).

Ca^{2+} concentration in the medium was within the recommended range (121 mg/L) for penaeid farms in hyposmotic condition (Wyk 1999; Nunes et al. 2005). However, Mg^{2+} (422 mg/L), K^{+} (149 mg/L) and alkalinity (80mg/L $CaCO_3$) were below the recommended values (1200 mg/L, 375 mg/L and 100 mg/L, respectively) (Boyd 1990; Nunes et al. 2005; Chávez 2009). K^{+} and Mg^{2+} are essential for crustacean growth, survival and osmoregulation (Mantel and Farmer 1983; Pequeux 1995; Saoud et al. 2003). The molting abnormalities have been observed in waters with less than 30 mg Ca^{2+}/L (Morrissey 1970; Fieber and Lutz 1982). Ca^{2+} in the hemolymph decreased slightly (from 27.6 to 26.0 mM) (Fig. 1).

Salinity effects on gene expression

Considering that the osmo-regulatory mechanism in penaeid is controlled by the gill, gut and hypodermis (Mantel and Farmer 1983; Muramoto 1988), we decided to investigate which genes could be up or down regulated in gill of shrimp submitted to a hyposmotic condition.

In this study, although the gills were collected from shrimp in the intermoult stage, we observed an increased frequency of soft-shelled shrimp in the hyposmotic tank. *Calcified cuticle protein*, *early cuticle protein 1*, *peritrophin* and *actin D* transcripts, involved in the exoskeleton mineralization, were identified as down-regulated genes (Table 3). Their expression seems to be affected under low salinity, possibly due to the lower levels of Ca^{2+} in the water, since calcium carbonate ($CaCO_3$) is the most commonly mineral found in the hard structures of invertebrates (Passano 1960).

The V-type H^+-ATPase B-subunit, responsible for the active Na^+-exporting pump, expressed preferentially in the gills (Weihrauch et al. 2001), was found to be down-regulated in *L. vannamei* kept at hyposmotic conditions, possibly associated with the need to keep the Na^+ levels in the hemolymph. This result is in accordance with the presence of five energy producer genes also identified as down-regulated. Lower ATP production would limit the activity of Na^+-transporting ATPase. Likewise, we could hypothesize that lower transcription rate of energy producer genes could affect growth, molt cycle and surviving under osmotic stress.

Under osmotic stress, penaeids could elicit a decreased immune response triggering WSSV infection (Wang and Chen 2006; Joseph and Philip 2007). Liu et al. (2006) observed an increase in the WSSV load in *Fennerepenaeus chinensis* when submitted to a fast decrease in the salinity, from 22 to 14 ppt in 1h. The host defense in crustaceans depends on innate immune mechanisms. Among the cytokines known to be involved in the immunity of vertebrates and invertebrates, the transforming growth factor-b (TGF-beta) members are involved in innate immunity (Lieber and Luckhart 2004; Nicholas and Hodgkin 2004). TGF-beta has various pleiotropic functions in development, extracellular matrix synthesis, wound repair and inflammation. The *TGF-beta inducible nuclear protein 1* was identified in the SSH up-regulated library and its transcription rate quantified by qPCR was significantly up-regulated in the gills of shrimp in hyposmotic condition with or without WSSV. The presence of a nuclear protein modulated by TGF-beta suggests the existence of a TGF-beta signaling in *L.vannamei*. He et al. (2004) and Prapavorarat et al. (2010) identified in haemocytes from penaeid shrimp injected with microorganisms that there was an up-regulation of TGF-beta. The involvement of shrimp TGF-beta inducible protein 1 in the osmotic stress response suggest that this gene could play a role in environmental stress response, beyond their possible functions in innate immunity and development.

The *QM protein* gene was identified in the up-regulated library. *QM protein* was described as a candidate tumor-suppressor gene in the Wilms' tumor cell line (Dowdy et al. 1991). These proteins are highly conserved and have a role in cell growth, differentiation and apoptosis (Marty et al. 1993; Green et al. 2000; Lillico et al. 2002). qPCR data showed that *QM protein* was significantly up-regulated in gill of *L. vannamei* under hyposmotic condition, either with and without WSSV challenge. Furthermore, the strongest up-regulation in the shrimp that were not challenged with WSSV (1900–fold), in respect to the WSSV challenged ones (230–fold), despite not be statistically different, might denote that WSSV infection could influence the shrimp´s capability to induce *QM protein* gene under hyposmotic stress. Whether or not this capability to induce *QM protein* is directly involved in the shrimp´s adaptation to hyposmotic stress conditions needs further investigation. As revealed by RNAi assays, *QM protein* is involved in the invertebrate innate immunity responses, such phenol oxidase activity (PO), and a key enzyme in the proPO activation system. Likewise, Müller (2009) found that the *QM protein* is among the up-regulated genes in *L.vannamei* after WSSV infection. Induction of *QM protein* by WSSV infection have been also showed in other tissues, such hemolymph, hepatopancreas, gill, heart, intestine and muscle, and other shrimp species, such the *Penaeus japonicus* (Xu et al. 2008) and other invertebrate species, such in the mollusk abalone *Haliotis discus* gills in response to bacterial and viral challenge (Oh et al., 2010). Considering that *QM protein* is up-regulated under hyposmotic stress in gill of adult shrimp, we could suggest that this gene is involved in other more complexes metabolic changes that have not been described so far.

C-type lectins (CTLs) play key roles in pathogen recognition, innate immunity, cell-cell interactions and comprise the largest lectin family in animals (Drickamer and Taylor 1993). They are Ca^{2+}-dependent proteins and function outside of cells as secreted or membrane-bound proteins. Sugar-binding activity of CTLs attributes to a carbohydrate-recognition domain (CRD). Invertebrate CTLs are involved in immune responses including proPO activation (Yu and Kanost 2000), hemocyte nodule formation (Koizumi et al. 1999), opsonization and microbial clearance (Jomori and Natori 1992; Yu and Kanost 2003). *CTL* was identified as one of the down-regulated genes in gill of shrimp kept for 16 days at hyposmotic conditions. Contrariwise, we observed an up-regulation of *CTL* in shrimp kept 7 days in hyposmotic condition, but in the presence of WSSV this increased transcription was less intense (Fig. 3). There seems to be an interaction

between host, environment and viruses, since when the virus is present, under low salinity, there is an apparent reduction in the ability to enhance *CTL* transcription. Other authors have also observed higher transcription rates of *CTL* in hepatopancreas and hemocytes of shrimp infected with WSSV (Pan et al. 2005; Zhao et al. 2007, 2009; García et al. 2009) and IHHNV virus (Costa et al. 2011), but none of them have tested this transcription under hyposmotic conditions.

Adaptation to salinity changes in crustaceans is regulated through transport mechanisms including Na^+/K^+-ATPase, $V(H^+)$- ATPase, $HCO3^-$-ATPase, K^+ and Cl^- channels, Na^+ channels, $Cl^-/HCO3^-$ exchangers, $Na^+/K^+/2Cl^-$ co-transporter, Ca^{2+}-pumps, Na^+/Ca^{2+} exchangers and carbonic anhydrase (Mantel and Farmer 1983; Henry 1984; Böttcher et al. 1990; Péqueux 1995; Henry and Watts 2001; Freire et al. 2008). Most of these proteins are ATP-dependent transporters. Paradoxically, four genes of the mitochondrial ATP synthase complex (*ATP synthase subunit 9 mitochondrial precursor*, *ATP synthase subunit alpha precursor*, *H^+transporting ATP synthase O subunit*, *Mitochondrial ATP synthase F chain*) and one gene of the citric acid cycle (*malate dehydrogenase)* were identified in the list of the down-regulated genes, suggesting a low ATP production status in the shrimp kept at hyposmotic condition. However, using qPCR we observed that three of these genes were up-regulated in the gill of shrimp kept at hyposmotic condition with and without WSSV challenge (Fig.2). The difference in these metabolic responses might be related with the period of exposure that the shrimp were submitted to osmotic stress. The SSH library was constructed in samples of shrimp exposed for 16 days to hyposmotic conditions and the qPCR experiment was carried out in samples of shrimp exposed for 7 days. We could hypothesize that during the time-course of energy production, transcription genes changes along with the time of adaptation to hyposmotic conditions. Towle et al. (2011) observed that ATPsy and MDH were up-regulated in gills of green crabs *Carcinus maenas* 24h and 48h after salinity decrease. After 7 days of the decreased salinity, the *ATPsy* transcription reached a plateau and after 15 days there was no difference. These results seem to agree with those found in our study, since at 7 days of exposure of shrimp to the hyposmotic condition there was an increased transcription of ATPsy and after 16 days, the transcripts seem to be repressed by the treatment. In the same way, the mitochondrial *malate dehydrogenase* showed a somewhat earlier up-regulate at 7 days, but after 16 days was down-regulated.

Conclusion

Based on the obtained data we could conclude that the hyposmotic conditions of water elicits change in the gene transcriptional profile of shrimp, particularly on those associated with energy production and cellular defense. WSSV infection does not seem to significantly influence the transcription of the genes evaluated in this study. However, more studies needs to be carried out to clarify whether there is an interaction between the adaptation to low salinity and higher susceptibility to WSSV infection.

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5. CAPÍTULO 2

Expression and activity of glutathione-S transferases and catalase in the shrimp *Litopenaeus vannamei* inoculated with a toxic *Microcystis aeruginosa* strain

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Abstract

Microcystin (MC) produced during cyanobacteria blooms is notably toxic to human and wildlife. Conjugation with reduced glutathione (GSH) by glutathione *S*-transferase (GST) and the antioxidant enzymes defenses (e.g. catalase, CAT) are important biochemical defense mechanisms against MCs toxicity. We investigated the enzymatic activity of CAT and GST and the gene expression levels of *CAT* and eight *GST* isoforms in the hepatopancreas of the globally farmed shrimp *Litopenaeus vannamei* 48-hrs after injection with a sublethal dose of 100 μg kg^{-1} of a toxic *Microcystis aeruginosa* extract. MCs caused up-regulation for *GST* ω, μ and MAPEG isoform, by 12, 2.8 and 1.8 fold, respectively, and increases in the total GST enzyme activity and CAT enzyme activity. The study points to the importance of further characterization for the *L. vannamei* GST isoforms and GST/CAT post-translational regulation processes to better understand the key mechanisms involved in the shrimp´s defense against MC exposure.

Keywords: Microcystin; glutathione *S*-transferase; catalase; *Litopenaeus vannamei*; *Microcystis aeruginosa*; pollution; biomarker; environmental toxicology; algal toxins.

1. Introduction

Toxic cyanobacterial blooms are observed associated to nutrient imbalances in eutrophic waters. Since the 1970s, there was a suspicion that shrimp mortality by toxin bioaccumulation could be related to the presence of marine cyanobacteria (e.g. *Microcystis*, *Nodularia*, *Lyngbya* and *Oscillatoria*) (Lightner, 1975). Cyanotoxin exposure occurs via ingestion of living cells or direct toxin uptake from the water (Cazenave et al., 2005) affecting the health of aquatic organisms (Codd, 1995). The understanding of biochemical mechanisms involved in the defense and toxicity to common toxins, such the microcystins (MCs) produced by the worldwide spread *Microcystis aeruginosa*, is crucial to understand chronic and acute fate to aquatic organisms in the environment and aquaculture.

MCs are cyclic heptapeptides, and its cellular uptake requires organic anion transporter polypeptides (OATP) activity to entry the cells (Fischer et al. 2005). Into the cells, the mechanism of MCs toxicity involves the inhibition of protein phosphatases, which leads to the hyperphosphorylation of cellular proteins and increased reactive oxygen species (ROS) formation (Zegura et al., 2011). Recently, more and more evidences have shown that oxidative stress, produced by ROS, such superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) may play a significant role in the pathogenesis of MCs toxicity (Guzman and Solter, 1999; Bouaicha and Maatouk, 2004; Sun et al., 2008; Puerto et al., 2009; Jiang et al., 2011).

Antioxidants are important cellular defenses against MC toxicity, by inactivating ROS and repairing oxidized biomolecules (Halliwell and Gutteridge, 2007). Thus, it could be expected that exposure to MC and ROS generation, would modulate the antioxidant enzyme activities by regulating the mRNA levels through activation of signaling pathways (Xiong et al., 2010). However, most of the studies concerning oxidative stress induced by MCs have focused on the response of activities of antioxidant enzymes alone, and less is known about the effects of MCs on antioxidant enzyme genes at transcriptional level, especially in aquatic invertebrates.

The cellular defense system against ROS comprises enzymatic antioxidant defenses such superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). CAT is an oxidoreductase enzyme that catalyzes the conversion of two molecules of hydrogen peroxide into two molecules of water and one of oxygen. This enzyme is an ubiquitous hemoprotein formed by four identical subunits of

approximately 50–60 kDa and present in prokaryotes and eukaryotes (Kashiwagi et al., 1997; Klotz et al., 1997). The investigation of CAT responses against to MC in aquatic organisms is of special interest since changes in the catalytic activity and gene expression have been reported as an effect caused by MC exposure in vertebrates (Xiong et al., 2010). It has been hypothesized that changes in CAT activity could be due to the excess of superoxide anion radical caused by MCs, and may occurs along with a decreased expression of CAT mRNA (Xiong et al., 2010).

MC conjugation with glutathione (GSH) is catalyzed by glutathione S-transferases (GSTs) in different aquatic organisms such as plants (*Ceratophyllum demersum*), mollusk (*Dreissena polymorpha*), crustacean (*Daphnia magna*) and fish (*Danio rerio*) (Pflugmacher et al., 1998). This conjugation is generally considered the primarily route for MC detoxification in aquatic organisms by forming more polar compounds, and facilitating excretion (Pflugmacher et al., 1998; Beattie et al., 2003). GSTs comprise a family of multifunctional cytosolic, microsomal or mitochondrial enzymes that catalyze the nucleophilic attack of GSH to compounds that have an electrophilic carbon, nitrogen or sulfur atom (HAYES et al., 2005; HUBER et al., 2008). These enzymatic isoforms have been classically grouped into classes, such as α, π, μ and θ GSTs, and show organ-specific distribution (Sato et al., 1984; Hayes and Mantle, 1986; Hayes et al., 1987). Invertebrate subfamilies of GSTs, that are absent in mammals, are also described in the literature (Armstrong, 2000; Sheehan et al., 2001).

The aim of this study was to evaluate the responses of *Litopenaeus vannamei* GST isoforms and catalase after the injection with a sub-lethal level of microcystin [D-Leu1] MC-LR. The analysis of the transcriptional levels and enzymatic activity of these proteins may help in the understanding the mechanisms of metabolism and elimination of the toxin in marine crustaceans.

2. Material and Methods

2.1. Preparation of Microcystis aeruginosa extracts

Cells of *Microcystis aeruginosa* from strain RST 9501 were cultured in BG11 (8.82 mM of $NaNO_3$) medium (Rippka et al., 1979) at 25 ± 1 °C and employed as toxin source. Characterization of microcystins produced by the strain RST 9501 was carried out by Matthiensen et al. (2000). These authors found that methanolic extracts

of strain RST 9501 presented one major peak and three minor peaks with UV spectra characteristic of microcystins, with a λ_{max} of 238-239 nm. Interestingly, the retention time of the major peak (15.22 min) was different to that of microcystin-LR. Further analysis confirmed that the most abundant microcystin produced by strain RST 9501 is a [D-Leu^1]-microcystin-LR, a variant with a similar potency in terms of phosphatases inhibition respect the common [D-Ala^1]-microcystin-LR. The extract was prepared using lyophilized cells of *M. aeruginosa* and the protocol of Coyle and Lawton (1996). Briefly, the cells were dissolved in absolute methanol (Sigma), sonicated 3 times and centrifuged (10,000 *x* g) at 4 °C, during 10 min. Extracts were evaporated at 40 °C and then re-dissolved in ultra-pure water. Finally, samples were centrifuged and the supernatant was collected and stored at - 20° C. Microcystins content was determined using a commercial enzyme-linked immunoassay (ELISA) with polyclonal antibodies (EnviroLogix Inc., Portland, ME, USA). Different concentrations of microcystin were prepared after appropriate dilutions with saline phosphate buffer (Ca^{2+} and Mg^{2+} free) (PBS).

2.2. Experimental design

Females *L. vannamei* weighing 11.0 g ± 1.9 g were collected in the intermolt stage (Robertson, et al., 1987; Vijayan and Diwan, 1996) maintained in aquaria at a density of 5 m^{-2} with constant aeration, a controlled temperature of 29 °C and salinity of 35. We choose to use females in the intermoult stage to minimize the interference of the possible effects of sex and the ecdise cycle in the metabolic responses of the shrimp. Two bioassays were conducted to identify sub-lethal levels of microcystin (MC) since no LD50 information has been previously established for this species at this life stage. In the first bioassay, an intra-muscular injection was conducted at a dose of 50μg kg^{-1} biomass of shrimp, which was previously shown to cause mortality in post larvae shrimp of the native species *Farfantepenaeus paulensis* (Yunes, 2009). However, this dose was not sufficient to promote change in behavior or mortality in the shrimp evaluated. A second bioassay was performed with an intra-muscular inoculation of 200μg kg^{-1} biomass of shrimp and caused changes in behavior (erratic swimming) and a white color in muscle tissue. There was 100% mortality of the inoculated shrimp 96-hrs after inoculation. These results led us to choose an intermediate dose of 100μg kg^{-1} biomass of shrimp. Similar levels have been detected in tissues of fish exposed to a contaminated site (Romo et al., 2011). The intra-muscular injection into the shrimp was performed with an insulin

syringe containing MC or isosmotic solution (control group) in the lateral region of the animal's body between the second and third abdominal segment and the collection of shrimp hepatopancreas was performed 48-hrs after inoculation.

2.3. Identification of L. vannamei sequences and gene expression analysis

Glutathione *S*-transferase nucleotide sequences from the copepod *Tigriopus japonicos*, characterized previously by Lee et al. (2008) were employed as a starting point to collect homologous *L. vannamei* sequences in the EST (*Expressed Sequence Tag*) genetic sequence database at the National Center for Biotechnical Information (NCBI) using BLAST tool. The sequences collected were cleaned using VecScreen and contigs were constructed using CAP3 (Huang and Madan, 1999) to obtain single sequences. The preliminary annotation for those new *L. vannamei* GST sequences was made by blasting predicted protein from those contigs against the *genbank* protein database. Eight new identified *L. vannamei* sequences were grouped in the GST classes Ω*GST* (omega), *μGST* (mu), *δGST* (delta, three isoforms) *microssomal GSTs* (MAPEG, two isoforms) and *σGST* (sigma) based on identities and E values obtained by comparison to sequences previously annotated in the genebank (Table 1). The *L. vannamei CAT, ribosomal L8, ribosomal 18S* and *ATP synthase* (ATPsy) sequences used in this study were previously annotated (GenBank ID: AY518322, DQ316258, M34362 and EU194608, respectively). Primer pairs were tested for ribosomal *L8, 18S and ATPsy* as housekeeping genes for normalizing the data, but only the primers for *18S* and *ATPsy* were selected, because the MC-treatment affect the transcription of *ribosomal L8*. The primers used in the Quantitative Polimerase Chain Reaction (RT-qPCR), showed in Table 2, were designed using Primer3 (Rozen and Skaletsky, 2000) avoiding hairpin and dimer formation using the software *PrimerQuest* and *FASTPCR* (Kalendar et al., 2009).

Table 1.

List containing eight new *Litopenaeus vannamei* GST sequences classified in different isoforms based on the aminoacid similarities and E-values obtained after blasting to NCBI databank

***L. vannamei GSTs* obtained from ESTs**	**Predicted aa sequence**	**Protein blast E-value**	**Coverage**	**Similarity aa (%)**		**NCBI acession #**
				Identities	**Positives**	
Lv_mGST1	complete (144 aa)	2e-29	97%	48%	61%	*Heliothis virescens* microsomal glutathione transferase (ADH16761.1)
Lv_mGST2	complete (139 aa)	8e-36	97%	56%	67%	*Strongylocentrotus purpuratus* similar to microsomal glutathione S-transferase 3 (XP_793267.1)
Lv_δGST1	complete (203 aa)	1e-74	96%	65%	82%	*Eriocheir sinensis* delta glutathione S-transferase (ACT78699.1)
Lv_δGST2	incomplete (212 aa)	1e-55	99%	49%	71%	*Eriocheir sinensis* delta glutathione S-transferase (ACT78699.1)
Lv_δGST3	complete (219 aa)	7e-47	98%	47%	65%	*Eriocheir sinensis* delta glutathione S-transferase (ACT78699.1)
Lv_ ΩGST	incomplete (203 aa)	4e-39	85%	50%	66%	*Nasonia vitripennis* glutathione S-transferase O1 (NP_001165912.1)
Lv_μGST	complete (203 aa)	2e-83	99%	67%	82%	*Danio rerio* glutathione S-transferase M (CAX14388.1)
Lv_σGST	complete (211 aa)	3e-36	99%	42%	59%	*Gallus gallus* hematopoietic prostaglandin D synthase (NP_990342.1)

Legend of GST classes: mGST1 and mGST2, microssomal GSTs (MAPEG, two isoforms); δGST (delta, three isoforms); ΩGST (omega), μGST (mu) and σGST (sigma)

Table 2
Primers used in the Quantitative Polimerase Chain Reaction (RT-qPCR):

Gene	Description	Primer	Sequences (5'→3')	Product size (bp)
mGST1	microssomal	F (forward)	TGGACAACCCGGTGTTCGTGAGT	97
		R (reverse)	GTGATCCGGTAATAGCCGGTGATGG	
mGST2	microssomal	F	CGTGCCCACCAGAACACACTTGAG	97
		R	CACCACCAACGGCACACACAATG	
δGST1	delta (theta-like)	F	CCGCTTCGGCGAATATGTGTATCC	83
		R	CTTCGTGCAGCTTCTCCAGCTTCG	
δGST2	delta (theta-like)	F	AGTTGCTGACTTCAGCCTGGTGGC	102
		R	TTGCACCTCTTCAGCCACTGCAC	
δGST3	delta (theta-like)	F	AATTCGTGGCCATTAACCCGCAG	112
		R	TCCTTGCCGTGCTTGGCTATCAG	
Ω*GST*	omega	F	ACTTGGACGAGGCCTACCCTGAACC	145
		R	AGCGCCTCTTGATCACCCTTTGC	
μGST	mu	F	TGCCATCATCCGTCACATTGCTC	109
		R	GCCATTGCGGAAGTCAACAGCC	
σGST	sigma	F	TGACGCCTTGGCTGACACCATC	95
		R	TGAACTGCTTGCGCTTCTCCTC	

CAT	catalase	F	CCCGTACAAGGAACTACCAG	230
		R	GCTGACGTTCTGCCTCATTC	
L8	ribossomal L8 (housekeeping)	F	TAGGCAATGTCATCCCCATT	166
		R	TCCTGAAGGGAGCTTTACACG	
ATPsy	ATP synthase (housekeeping)	F	TGTCTTATGATGGCCTTCCTGTTG	121
		R	GGAAGTGGTCCACAAACACCATGC	
18S	ribossomal 18S (housekeeping)	F	CCGAATGGTCGTGCATGGAATGAT	127
		R	GAATTTCACCTCTAGCGTCGCAGT	

For each shrimp sample, total RNA was extracted from the hepatopancreas using TRIzol® reagent (Invitrogen®) according to the manufacturer's protocol, and 2μg of total RNA was reverse transcribed to cDNA using QuantiTect Reverse Transcription System (Qiagen). For the amplification of PCR products, 0.3mM for each primer pair of interest and normalizer gene were used. The following conditions were used for cycling: 95°C for 15 min, 40 cycles of 10 seconds at 95°C, 15 seconds at 53°C, 20 seconds at 72°C. A melting curve was performed to confirm amplification of specific products. Quantifast™ SYBR Green (Qiagen®) was used as a fluorescence marker to detect specific PCR products. Data were collected as CT using the Rotor Gene 6000 software version 1.7 (Corbett Research®). Analysis was performed in duplicate for each sample. Gene expression calculations were carried out according to the software REST (Pfaffl et al., 2002) using *18S* and *ATPsy* as housekeeping genes.

2.4. Enzyme analysis

Hepatopancreas were homogenized using a Tissue-Tearor (Biospec Prod. INC.) in four volumes of 20 mM Tris buffer pH 7.6, containing 1 mM EDTA, 0.5 M sucrose, 1 mM dithiothreitol, 0.15 M KCl, and 0.1 M PMSF. The homogenate was centrifuged at 9,000 *x g*, for 30 min at 4 °C, and the supernatant was stored at −85 °C. Glutathione S-transferase (GST, EC 2.5.1.18) activity was analyzed according to Habig and Jakoby (1981) using 2 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 2 mM reduced glutathione (GSH) in 0.1 M potassium phosphate buffer, pH 7.0. The absorbance was monitored for 2 min at 340 nm at 30 °C for a better detection in the microplate reader. One unit (U) of GST activity is the amount of enzyme which catalyzes the conjugation of 1 μmole of substrate per minute. Catalase (CAT, EC 1.11.1.6) activity was measured by the H_2O_2 decomposition in 0.1 M Tris buffer (pH 8.0) containing 0.5 mM EDTA and 10 mM H_2O_2 (Beutler, 1975) and expressed as unit (U) where one unit is defined as the amount of enzyme that decompose 1 μmole of H_2O_2 per minute. The enzymatic assay was performed for 1 min at 240 nm at 30 °C using 1-mL quartz cuvettes an Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech). Calculations for CAT and GST activity were carried out using the extinction molar coefficients of 9.6 $mM^{-1}.cm^{-1}$ and 0.04 $mM^{-1}.cm^{-1}$, respectively. Total protein concentration in the samples was determined according to Peterson (1977), adapted to microplate (TECAN Sunrise®), using bovine serum albumin as standard (Fraction

V, Sigma Chemical Co.), and the protein content was then used in the calculations to normalize the enzymatic activities. Results for CAT and GST enzymatic activities were expressed as U per mg of protein (U/mg protein) and mU per mg of protein (mU/mg protein), respectively.

2.5 Statistical analysis

Homogeneity of variances was tested (Cochran C; Hartley; Bartlett) using $p<0.05$, and the normal probability test was used to evaluate the data normality. The data that did not fit these features were logarithmically transformed. Differences among groups were evaluated using one-way analysis of variance (ANOVA; $p<0.05$), followed by the Tukey Post-test. Statistica software (version 8.0 for Windows, www.statsoft.com) was used to perform the analysis of enzymatic activities and the software REST (Pfaffl et al., 2002) was used in the qPCR analysis. The mathematical model used is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group and control group. Subsequently the expression ratio results of the investigated transcripts were tested for significance by a Pair-Wise Fixed Reallocation Randomisation Test and plotted using standard error (SE) estimation via a complex Taylor algorithm (Pflaff et al., 2002).

3. Results and Discussion

Different authors have associated the mortality of marine and freshwater crustaceans with the exposure to cyanobacteria blooms which produces cyanotoxins (Hauser et al., 1992; Hallegraeff, 1993; Lam and Wei, 1996; Smith, 1996; Ferrão-Filho et al., 2000) in particular MC (Lampert, 1981; Fulton and Paerl 1987; Reinikainen et al., 1995; Vasconcelos et al., 2001; Chen and Xie, 2005; Zimba et al., 2006).

The main mechanism associated with MCs toxicity in mammals is the inhibition of protein phosphatases, which leads to the hyperphosphorylation of cellular proteins, which is considered to be associated with their tumor-promoting activity (Honkanen et al., 1990; MacKintosh et al., 1990). Besides that, MCs induce reactive oxygen species production and, consequently, cause oxidative stress (Guzman and Solter, 1999; Bouaicha and Maatouk, 2004; Jiang et al., 2011). Despite their ecological importance, the molecular mechanism of MCs

toxicity in aquatic organisms is still not fully understood (Zegura et al., 2011).

The presence of high MC DLeu1-LR levels in the *M. aeruginosa* cyanobacterial extract used here was confirmed by HPLC (Fig. 1). The *M. aeruginosa* strain 9501 was isolated previously of water samples from the Patos Lagoon Estuary (RS, Brazil) and toxicity has been substantially tested and proved in different groups of organisms, such as mice, the brine shrimp *Artemia salina*, the tanaidacean *Kalliapseuds shubbartti* and the post larvae of the pink shrimp *Farfantepenaeus paulensis* (Yunes, 2009). The unique toxic peptide variant MC DLeu1-LR (Fig. 2) has been shown to correspond to 98 % of the total composition of the Brazilian *Microcystis* strains, thus representing the main compound in the environmental blooms. In recent years, several experiments were carried out with estuarine organisms, such as the polychaeta *Laeonereis acuta*, the crab *Chasmagnatus granulatus* (now *Neohelice granuta*) and also with the freshwater fish *Ciprinus carpio*, exposed to toxic *M. aeruginosa* RST 9501 cell extracts (Pinho et al., 2003; Leão et al., 2008; Amado et al., 2011). In all those tests glutathione *S*-transferase enzyme (GST) activity was evaluated, and seemed to be consistently increased in the crab by MC exposure. Some results from the literature that were extracted and adapted from Amado and Monserrat (2010) are shown in Table 3.

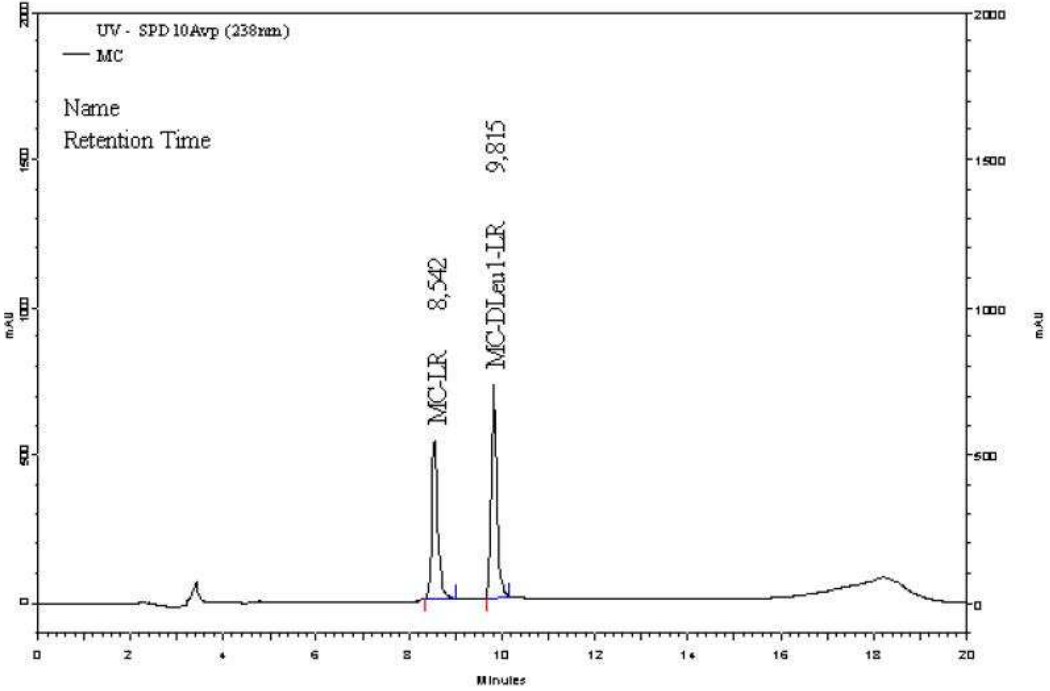

Figure 1. Chromatogram showing the results of liquid chromatography (LC) analysis from a sample containing microcystin (MC-DLeu1-LR), the most predominant MC form present in Brazilian cyanobacterial blooms. The MC-LR peak is included as a standard reference in the analysis.

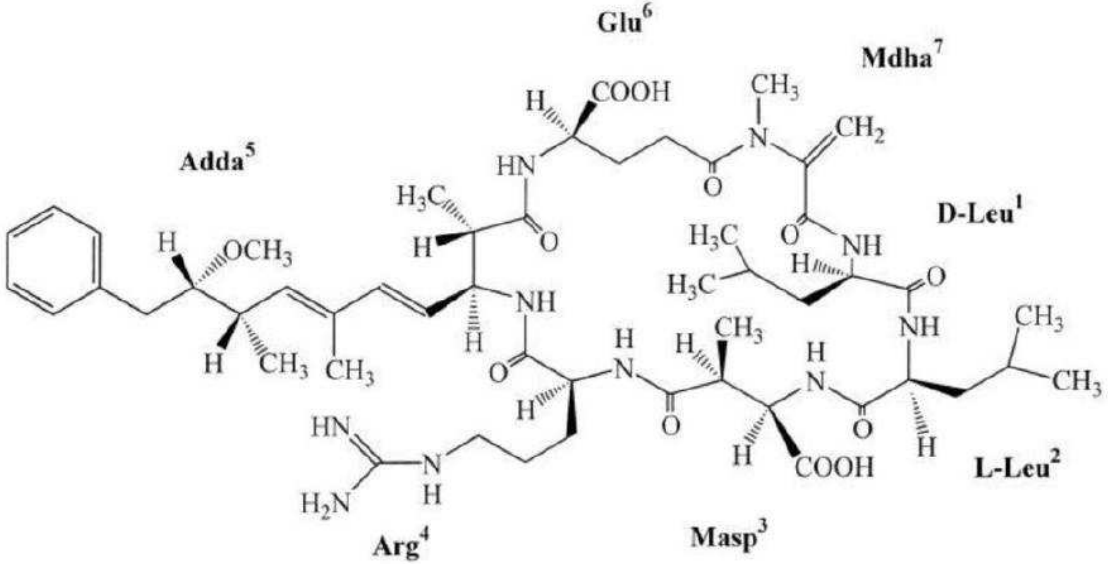

Figure 2. Structure of MC-1-D-Leu-LR. The main variant of *Microcystis aeruginosa* strain RST9501. (From Matthiensen *et al* 2000).

Table 3
Some evidences of alteration in GST activities of aquatic animals exposed to extracts from *Microcystis aeruginosa* strain RST 9501.

Species	Organ	Exposure	GST activity	Reference
Laeonereis acuta	Whole animal	Immersion in *M. aeruginosa* extract (~2 μg MC/mL) during 48h	No alteration on GST activity	Leão et al. (2008)
Neohelice granulata (former Chasmagnathus granulatus)	Gills (anterior and posterior)	Injected with *M. aeruginosa* extract (39.2 μg MIC/L) during 48 h	Higher GST in posterior gills	Vinagre et al. (2003)
Neohelice granulata	Hepatopancreas	Injected with *M. aeruginosa* extract (17.6 μg MC/L) during 72 h and one week of exposure	Higher GST activities	Pinho et al. (2003)
Neohelice granulata	Hepatopancreas	Forced ingestion (~1 and 5 μg MC/kg) during 168 h	Augmented GST activity	Pinho et al. (2005)
Neohelice granulata	Hepatopancreas	Gavage with *M. aeruginosa* extracts (34, 172, 860 μg MC/kg) during 6, 12 and 72 h	Higher GST activity in crabs exposed to 860 μg MC/kg for 12 h	Dewes et al. (2006)
Cyprinus carpio	Liver	Gavage of an aqueous extract from the toxic cyanobacteria *M. aeruginosa* in a final MC concentration of 50 ug/kg, 48 h	Decrease in GST activity and GSH concentration	Amado et al. (2011)

In the present study, we reported significant changes in both GST and CAT enzyme activities, as well as in the transcription levels of CAT and some *GST* isoforms. We observed that GST activity in the hepatopancreas of *L. vannamei* were increased in the MC-treated shrimps compared with the control group by 2.0-fold ($p<0.005$) (Fig. 3A). The increase in GST activity by the toxin is corroborated by other studies in aquatic organisms (Beattie et al., 2003; Pflugmacher, 2004; Pflugmacher et al., 2005). There is strong evidence of the involvement of GSH and GST in the elimination of microcystins in *L. vannamei*, but the mechanism has not been identified yet (Martins and Vasconcelos, 2009). GSH is the most abundant non-proteic thiol and is found at the milimolar range in most cells. Several studies have shown change in cytosolic GSH concentration due to MCs exposure and a biphasic response in terms of GSH levels has been also reported in rat hepatocytes during exposure to a *Microcystis* extract at 125 $\mu g.ml^{-1}$ of lyophilized cells (Ding et al., 2000). The initial increase of intracellular GSH is probably because of its conjugation with microcystin by GSTs (Pflugmacher et al. 1998), triggering the synthesis of new GSH. The subsequent GSH depletion was thought to be related to cell membrane damage and conjugated GSH efflux (Ding et al. 2000). In contrast, Runnegar et al. (1987) and Li et al. (2003) described only a decrease in GSH concentration, 10 and 15 minutes after rat and fish hepatocytes exposure to the purified MC-LR toxin, respectively.

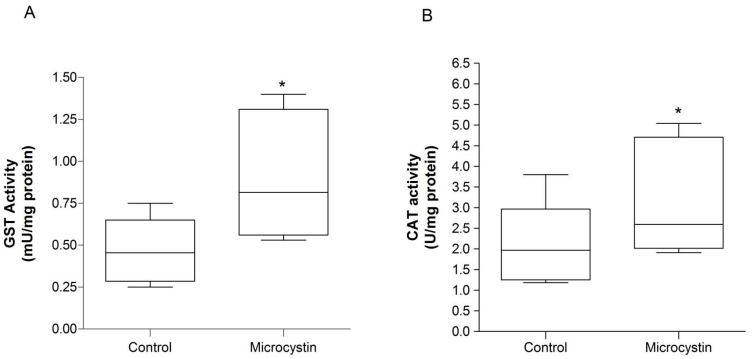

Figure 3. Total GST (A) and CAT (B) activity in the hepatopancreas of the shrimp *L. vannamei* collect 48h-post inoculation with 100 $\mu g\ kg^{-1}$ of a *M. aeruginosa* lysate (n=10). The results are expressed as mean values ± S.D. *Refers to significantly differences ($P<0.05$) among groups.

In recent years, more and more studies on marine GSTs focus on the biomarker traits of the enzyme (Blanchette et al., 2007). In crustaceans, the hepatopancreas is the key metabolic centre for the production of reactive oxygen species, it plays a major role in their immune defenses (Söderhall and Cerenius, 1998), and it is involved in both the synthesis of digestive enzymes and the detoxification of xenobiotics (Gibson and Barker, 1979; Vogt, 1994; Zhou et al., 2009). Contreras-Vergara et al. (2004) isolated from *L.vannamei* hepatopancreas a μ (mu)-class glutathione S-transferase (GST) and detected transcripts in hepatopancreas, hemocytes, gills, and muscle, but not in pleopods. μGST transcripts were expressed in all tissues examined from *L.vannamei*, but were most abundant in the hepatopancreas (Zhou et al., 2009). In the present study, we observed the increase in the transcription of three *L. vannamei* GST isoforms, Ω*GST*, *μGST* and *GST2-MAPEG*, which were induced 12.0-fold, 2.8-fold and 1.8-fold, respectively, when compared to the control group ($p<0.002$, $p<0.02$ and $p<0.05$, respectively, Fig. 4). Other five GST isoforms (*δGST* - 3 isoforms, *σGST* and *GST1-MAPEG*) were not changed by MC treatment. Based on these results we could hypothesize that elevated GST activity observed in the shrimp treated with MCs was mostly related with the transcription of *ωGST* followed by *μGST* and *GST2-MAPEG* isoforms.

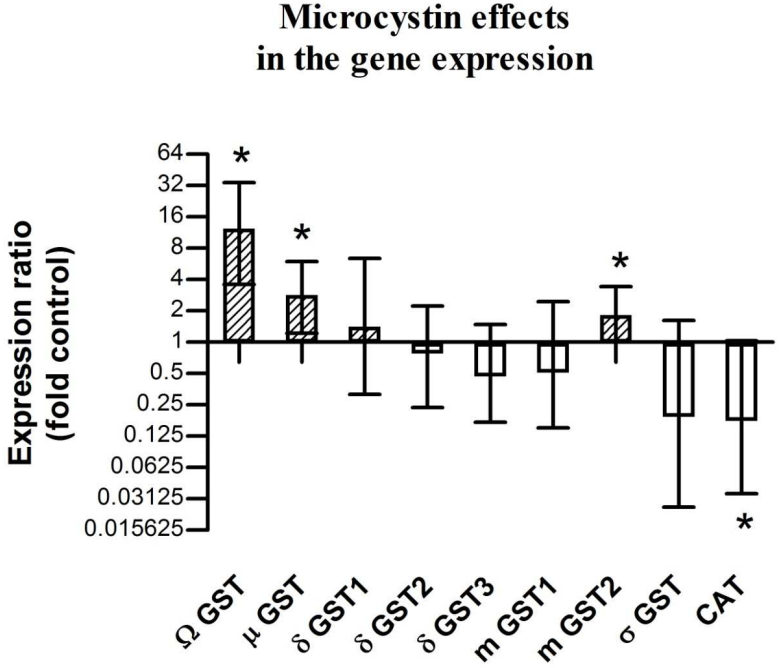

Figure 4. Relative expression of gene transcripts from hepatopancreas of *L. vannamei* 48h-post inoculation with 100 μg kg^{-1} of a *M. aeruginosa* lysate. The data are expressed as expression ratio for each gene among MC-treated and control shrimp (N = 8 individual shrimp for each group). *Refers to significant differences (REST 2009 software Qiagen®; $p<0.05$) among up- or down-regulated genes. The target gene expression was normalized by the expression levels of the non-regulated housekeeping genes 18S ribosomal and ATP synthase (M34362 and EU194608, respectively). Legend: Glutathione-S-tranferase isoforms (GST classes: Ω*GST* (omega), *μGST* (mu), *δGST* (delta, three isoforms) *microssomal GSTs* (MAPEG, two isoforms) and *σGST* (sigma) and catalase (CAT).

MCs toxicity involves the production of reactive oxygen species (ROS) (Guzman and Solter, 1999; Bouaicha and Maatouk, 2004; Jiang et al., 2011). These ROS activate several antioxidant enzymes such as superoxide dismutase (SOD), that dismutate two molecules of superoxide radical into hydrogen peroxide (H_2O_2) which in turns is

decomposed by catalase (CAT). CAT is ubiquitous and present in prokaryotes and eukaryotes (Kashiwagi et al., 1997; Klotz et al., 1997). A cDNA containing the complete coding sequence for catalase from the shrimp *L. vannamei* was sequenced and the mRNA was detected by RT-PCR in hepatopancreas and gills (Tavares-Sánchez et al., 2004). The *L. vannamei* injected with MCs showed a 1.5-fold increase in the CAT activity ($p<0.05$; Fig. 3B). Similar response in CAT activity and other antioxidant enzymes has been observed in crab hepatopancreas after 48h of exposure to MCs (Pinho et al., 2005). However, CAT activity in crabs exposed to the highest dose of *M. aeruginosa* aqueous extract was significantly reduced after 7-day exposure (Pinho et al., 2005). In a recent study, Sabatini et al. (2011) observed an increase in CAT activity in the digestive gland of the freshwater clam *Diplodon chilensis patagonicus* after feeding for 5 and 6 weeks with a toxic strain of *Microcystis aeruginosa* (NPJB1). Despite the elevated CAT activity after 48h of MCs-treatment, *CAT* mRNA transcription in *L vannamei*, was significantly decreased (0.26 fold) in treated group ($p<0.03$) (Fig. 4). We could hypothesize that at this time-point the elevation in the CAT activity could be associated with the decreased signaling pathway to *CAT* mRNA transcription. However, this hypothesis remains to be better investigated in marine invertebrates, such *L. vannamei*. It is reasonable to consider that other antioxidant parameters could be enhanced in this experiment since no mortality was observed in the treated shrimp.

4. Conclusion

Based on these results we could suggest that the glutathione conjugation can be an important MC´s detoxification mechanism in shrimp, similarly to what has been observed in mammals and other invertebrates. The elevated CAT activity suggests that reactive oxygen species are being produced during these processes. Better characterization of isoforms of *L. vannamei GST*, especially ω, μ and *MAPEG*, needs to be addressed in *L.vannamei* in order to understand the biochemical mechanisms involved in the toxin elimination from marine shrimp.

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6. CONCLUSÕES

Com base nos dados obtidos, foi possível concluir que as condições hiposmóticas da água provocam mudanças no perfil transcricional de genes do camarão eurhialino osmorregulador *Litopenaeus vannamei*, particularmente sobre àqueles associados com a produção de energia e de defesa celular. A infecção com WSSV não parece influenciar significativamente a transcrição dos genes avaliados neste estudo. No entanto, mais estudos precisam ser realizados para esclarecer se existe uma interação entre a adaptação à baixa salinidade e uma maior susceptibilidade à infecção viral.

Podemos sugerir que a conjugação de glutationa pode ser um mecanismo importante de desintoxicação de microcistinas em camarão, à semelhança do que tem sido observado em mamíferos e outros invertebrados. O aumento da atividade da catalase sugere que espécies reativas de oxigênio estão sendo produzidos durante este processo. A partir dos resultados obtidos, seria interessante melhor caracterizar as isoformas de GST de *L. vannamei*, especialmente ω, μ e MAPEG, no intuito de compreender os mecanismos bioquímicos envolvidos na eliminação de toxinas do camarão marinho.

7. CONSIDERAÇÕES FINAIS

O cultivo de camarões peneídeos é uma importante indústria nas Américas, e baseia-se quase que exclusivamente no cultivo da espécie de camarão marinho *L.vannamei* (LIGHTNER, 2011). Apesar do surgimento de enfermidades virais importantes na década de 90, como o WSSV, a carcinicultura em águas marinhas e salobras continua a expandir e, atualmente, está equiparada à pesca de captura com a produção em torno de 3 milhões de toneladas métricas (FAO, 2010). O aumento na produção de camarão de cultivo ocorreu apesar do relativamente pobre conhecimento básico das espécies cultivadas e apesar de enormes perdas devido a doenças, estimada em cerca de EUA $ 1 bilhão por ano desde o início de 1990 (FLEGEL et al 2008; TANTICHAROEN et al 2008). A grave ameaça das doenças virais sobre a carcinicultura foi a responsável pelo grande crescimento em estudos relacionados às respostas virais em camarões (FLEGEL; SRITUNYALUCKSANA, 2011). As enfermidades virais são mais problemáticas que as patologias de outras origens microbianas porque os invertebrados não possuem sistema imunológico adaptativo de defesa contra patógenos. Entretanto no ambiente natural existem mecanismos de defesa eficientes para combater os agentes infecciosos. As estratégias utilizadas para enfrentar esses obstáculos são o uso de animais livres de patógenos (da sigla em inglês SPF, ou *specific pathogen-free*) ou resistentes a patógenos (SPR, ou *specific pathogen-resistant)* (LIGHTNER, 2011). Estudos moleculares que identifiquem biomarcadores de resistência ou envolvidos em mecanismos de defesa contra agentes virais podem melhorar as estratégias de enfrentamento desses problemas.

Avaliamos o efeito do estresse osmótico sobre camarões *L.vannamei* desafiados ou não com WSSV em dois intervalos de exposição. No primeiro experimento os animais foram expostos durante 16 dias a uma condição hiposmótica sem o agente infeccioso. Identificamos genes envolvidos em diferentes processos biológicos, destacando moléculas associadas à produção de energia e defesa celular. Em um segundo experimento, o intervalo de exposição ao estresse osmótico foi reduzido para sete dias, e os camarões foram coletados 48h após o desafio com WSSV. A partir dos genes identificados na SSH, selecionamos oito genes para verificar sua expressão em animais em estresse osmótico e contendo o agente infeccioso. WSSV pareceu não

influenciar a expressão dos transcritos analisados, mas a condição osmótica da água foi capaz de aumentar a expressão de sete transcritos avaliados. A condição hiposmótica da água para essa espécie de camarão alterou a expressão dos transcritos em ambos os intervalos de exposição, porém o tempo de exposição pareceu interferir nos níveis de transcrição. Isso porque os transcritos de *ATPsy1-2, Lectina tipo-C, Malato desidrogenase* e *Ciclofilina* identificados na biblioteca de ESTs reprimidos através da SSH, foram induzidos na análise de PCR quantitativo no intervalo de exposição menor ao estresse osmótico. Esses resultados podem estar relacionados com sequências falso-positivas identificadas na SSH ou refletirem o período de adaptação à nova condição osmótica.

A ocorrência de florações de cianobactérias tem aumentado significativamente em muitas regiões do mundo no último século devido à eutrofização da água. Estas florações são perigosas para os seres humanos, animais e plantas devido à produção de hepatotoxinas. Há evidências de que certas toxinas de cianobactérias são genotóxicas e cancerígenicas, no entanto, os mecanismos de seu potencial carcinogênico não são bem compreendidos (ZEGURA et al., 2011). No intuito de esclarecer o efeito da microcistina sobre camarões *L.vannamei* estudamos a expressão e atividade enzimática de isoenzimas de glutationa-S-transferase e da catalase. Observamos que houve indução de três isoformas de *GSTs* e da atividade enzimática total da enzima 48h após a inoculação intra-muscular de 100µg por kg de biomassa de camarão da toxina. Esses resultados sugerem que a conjugação da glutationa com a microcistina é um importante mecanismo de detoxificação no camarão. Observou-se também o aumento da atividade da catalase o que sugere a produção de espécies reativas de oxigênio durante essa toxicidade. A diminuição de transcritos de *CAT* pode ser reflexo de um esgotamento da resposta antioxidante ou ainda o resultado de regulações pós-transcricionais sobre a expressão do gene. Entretanto, mais estudos dever ser feitos para compreender melhor o ciclo de biotransformação dessa cianotoxina.

De qualquer forma, esse mecanismo de detoxificação parece ser um importante processo na sobrevivência do camarão em condições de cultivo onde é comum a presença dessas cianotoxinas.

8. PERSPECTIVAS

A partir do conhecimento aprofundado das interações ambiente-hospedeiro-patógeno, melhorias podem ser sugeridas para manter a homeostase na saúde dos organismos aquáticos. Isso poderia auxiliar a minimizar os impactos econômicos provenientes das enfermidades infecciosas, como acontece mundialmente com o WSSV. Alguns estudos têm sugerido biomarcadores de estresse osmótico, entretanto, até o momento, nenhum viável para uso no setor produtivo. Através da análise de expressão gênica e protéica, da clonagem e expressão de proteínas identificadas em estudos que mantenham os animais em diferentes condições de estresse osmótico, sendo coletados diferentes tecidos e ao longo do tempo, poderiam auxiliar na identificação de biomarcadores para uso na seleção de organismos aquáticos mais resistentes às adversidades do meio. A análise de SSH realizada nesse estudo contribue fornecendo informações importantes que poderão auxiliar na identificação de genes relacionados com esse tipo de estresse.

Além disso, pouco se sabe do impacto das microcistinas produzidas por cianobactérias e liberadas no meio sobre organismos aquáticos. A clonagem e a expressão de isoenzimas de GSTs, além de estudos de cristalografia dessas proteínas, poderiam ser estudos alvo para a compreensão desse tipo de biotransformação. A compreensão das vias metabólicas envolvidas no processo de eliminação dessas toxinas pelos organismos poderia auxiliar a entender e justificar os impactos promovidos em ambientes eutrofizados, realidade cada vez mais presente em regiões estuarinas e costeiras, consequência da exploração humana. Além disso, o conhecimento sobre o estresse cianotóxico promovido sobre o metabolismo de invertebrados poderia servir de modelo para o entendimento desses processos em mamíferos.

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