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BIOMARCADORES MOLECULARES NO CAMARÃO BRANCO, *Litopenaeus vannamei* (CRUSTACEA: DECAPODA), SUBMETIDO A ESTRESSE AMBIENTAL E INFECTADO PELO VÍRUS DA SÍNDROME DA MANCHA BRANCA (WHITE SPOT SYNDROME VIRUS, WSSV)

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Resumo

O vírus da síndrome da mancha branca (*White Spot Syndrome Virus* - WSSV) é o agente etiológico da doença viral que afeta a maior parte dos cultivos de camarão marinho no mundo. A ocorrência do WSSV em Santa Catarina, no início de 2005, revelou a fragilidade das medidas sanitárias aplicadas nas fazendas de criação de camarões marinhos e do sistema de vigilância sanitária animal. A saúde dos camarões e, conseqüentemente, a produtividade de uma fazenda de cultivo são fortemente influenciadas pelas condições físicas, químicas e biológicas que prevalecem ao longo do processo de cultivo. Compostos tóxicos, como pesticidas, alterações na salinidade temperatura da água de cultivo, são alguns dos fatores que podem promover estresse e induzir alterações bioquímicas, moleculares e fisiológicas nesses animais, podendo, ainda, favorecer a incidência de enfermidades virais. A permetrina é o inseticida piretróide mais utilizado em práticas de cultura de arroz em muitos países. Concentrações sub-letais de permetrina podem causar impacto nos cultivos, afetando sua produtividade. Neste estudo, camarões juvenis, *Litopenaeus vannamei* foram expostos permetrina (0,01, 0,1, 0,2, 0,3, 0,4 e 0,8 $\mu g.L^{-1}$) por 96h e em um segundo experimento (0,8 $\mu g.L^{-1}$) por 6, 12, 24, 48, 72 e 96h. Ensaios enzimáticos envolvendo a determinação da atividade da superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa S-Transferase (GST) e acetilcolinesterase (AChE), foram realizados em brânquias e hepatopâncreas de camarão exposto a permetrina. Níveis de transcrição dos genes que codificam estes biomarcadores enzimáticos foram investigados por PCR em tempo real (qPCR) em ambos os tecidos. Num segundo experimento, os camarões foram expostos a 0,8 $\mu g.L^{-1}$ de permetrina durante 7 dias antes de serem inoculados com o WSSV. O perfil de expressão de proteínas de estresse e de defesa, tais como ferritina, β-actina, proteína quinase Cξ, ubiquitina, HSP90 Hsp60, Hsp70, caspase-3 e proteína QM, foi avaliado no tecido branquial destes animais, através de qPCR e por Western blot. Mudanças nos níveis de expressão sugerem que a toxicidade de permetrina promove alterações na expressão de genes-alvo no camarão *L. vannamei*, modulando a transcrição e os níveis de proteínas, bem como a atividade enzimática de biomarcadores de estresse oxidativo. Além disso, a infecção e a carga viral poderiam ser associadas a um diferencial de transcrição de alguns dos genes que codificam essas proteínas. O padrão de respostas provocadas após a exposição à

permetrina, através da análise mútua de respostas transcricionais, traducionais e enzimáticas relacionadas a estes genes, apóiam o desenvolvimento de um sistema multi-biomarcador que poderia ser usado como ferramenta para o monitoramento de ambientes aquáticos e, consequentemente, de saúde dos camarões de cultivo. Outra etapa deste trabalho teve como objetivo avaliar o efeito do estresse térmico sobre a taxa de replicação do WSSV em camarões marinhos, mantidos em água quente (29±0,5°C), em comparação com camarões mantidos em água fria (18±0,5°C), além disso, pós-larvas e crustáceos selvagens capturados foram selecionados quanto à presença ou ausência do WSSV, após serem mantidos por 48h em estresse térmico. No caso de camarões silvestres, previamente negativos para o vírus, foram obtidos resultados positivos para o WSSV após estresse térmico de 48h. Os resultados apóiam a aplicação de um protocolo de estresse térmico como estratégia de seleção de reprodutores e para monitoramento dos ambientes de cultivo.

Palavras-chave: *Litopenaeus vannamei*, biomarcadores, WSSV, permetrina, temperatura

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LISTA DE SIGLAS

AChE - acetilcolinesterase
ANVISA - Agência Nacional de Vigilância Sanitária
CAT - catalase
cDNA - sequência nucleotídica complementar de DNA
DDT - Dithiothreitol
DNA - ácido desoxirribonucléico
EPAGRI - Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina
GPx - glutationa peroxidase
GST - glutationa *S*-transferase
HSP - proteína de choque térmico (Heat Shock Protein)
IHHNV - vírus da necrose hipodermal e hematopoiética infecciosa (*Infectious Hypodermal and Hematopoietic Necrosis Virus*)
L8 - gene ribosomal L8 (*housekeeping gene*)
OIE - Organização Internacional de Epizotias
PAGE - eletroforese em gel de poliacrilamida (*PolyAcrylamide Gel Electrophoresis*)
PMSF - *phenylmethylsulfonyl fluoride*
QM - proteína QM (*QM protein*)
qPCR - reação em cadeia da polimerase quantitativa ou em tempo real (*quantitative PCR; real time PCR*)
RNA - ácido ribonucléico
RNAm - RNA mensageiro
SDS - dodecil sulfato de sódio (*sodium dodecyl sulfate*)
SOD - superóxido dismutase
WSD - doença da mancha branca (*White Spot Disease*)
WSSV - vírus da síndrome da mancha branca (*White Spot Syndrome Virus*)
18S - gene ribosomal subunidade 18S (*housekeeping* gene)

Introdução Geral

A aqüicultura é uma atividade de grande potencial para a produção de alimentos, diminuição da pobreza e geração de bem estar para populações que vivem nas áreas costeiras, muitas das quais, consideradas as mais pobres do mundo. No entanto, uma série de problemas tem sido associada ao desenvolvimento da aqüicultura costeira, podendo ser destacadas a vulnerabilidade da atividade frente à má qualidade da água e a poluição aquática causada por dejetos domésticos, industriais, agrícolas e da própria aqüicultura, e o crescimento não sustentado, devido ao uso indevido dos recursos, problemas sociais e em alguns casos de doenças nos cultivos (SEIFFERT, 2003).

O litoral catarinense apresenta inúmeras áreas propícias à implantação de fazendas de camarões, destacando-se as regiões do complexo Lagunar Sul (Laguna, Jaguaruna, Imbituba e Imaruí), Grande Florianópolis (Paulo Lopes, Biguaçú, Tijucas) e Baía da Babitonga (São Francisco do Sul, Araquarí e Barra do Sul), áreas desprovidas de vegetação de mata atlântica, arenosas e não competitivas para pecuária (COSTA et al., 1999).

Segundo dados da EPAGRI, em 2001, a produção de *Litopenaeus vannamei* em Santa Catarina foi de 572,1 toneladas (SOUZA, 2001), relativas a 270 ha de tanques distribuídos em 23 fazendas. Em 2002 e 2003, a produção catarinense foi de 1.679 e 3.442 ton., respectivamente. Após atingir a marca de 4.189 ton. em 2004, relativas a 1500 ha de tanques distribuídos em 107 fazendas, a produção caiu para 2.750 ton. no ano seguinte, em decorrência do aparecimento da enfermidade da mancha branca.

Desde então, houve um decréscimo significativo na produção estadual. Entre 2006 e 2007, apenas cerca de 100 toneladas foram produzidas. A ocorrência do WSSV provocou a diminuição súbita das atividades, gerando grandes prejuízos para a carcinicultura catarinense. Na safra 2006/2007, apenas três fazendas da região Sul obtiverem sucesso na produção de camarões, uma delas, inclusive, vitimada anteriormente pela mancha branca, mostrou sinais de recuperação, com ótimos resultados técnicos. No entanto, a produção da região Sul ainda encontra-se bastante comprometida (MELLO; FARIAS, 2007). Em 2009 a produção estadual de camarões cultivados atingiu 172 toneladas, com 19 produtores em operação (SANTOS et al., 2010).

A ocorrência recente da enfermidade da mancha branca, em Santa Catarina (OIE, 2005), revelou a fragilidade das medidas sanitárias então aplicadas nas fazendas de criação de camarões marinhos e, ainda, do próprio sistema de vigilância animal, uma vez que as importações de espécies exóticas como o *L. vannamei* estão proibidas (MARQUES; MOSER; MULLER, 2006). As fronteiras brasileiras foram fechadas para a entrada de crustáceos em 1999, com o objetivo de impedir a entrada do vírus da mancha branca (*White Spot Syndrome Virus* – WSSV) no país. Entretanto, sintomas da doença começaram a ser detectados em novembro de 2004 em fazendas de camarão de Santa Catarina. Amostras coletadas na região e enviadas para diagnóstico, realizado na Universidade do Arizona (UAZ) onde se encontra um dos laboratórios de referência da Organização Mundial de Saúde Animal (OIE), foram diagnosticadas como sendo positivas para a síndrome da mancha branca em 11 de janeiro de 2005. A presença dos vírus também foi confirmada pela Universidade Federal de Santa Catarina (UFSC), pelo Laboratório de Biomarcadores de Contaminação Aquática e Imunoquímica em 15 de janeiro de 2005. As 15 fazendas contaminadas, nos municípios de Imaruí e Laguna, em uma área de 200 hectares (SEIFFERT, 2005), foram cloradas e o repovoamento foi suspenso até setembro de 2005, promovendo-se o vazio sanitário.

1.1. Vírus da Síndrome da Mancha Branca

Entre as enfermidades virais de camarões peneídeos, a síndrome da mancha branca, cujo agente etiológico é o *White Spot Síndrome Virus* - WSSV, tem causado grandes mortalidades nos cultivos de camarão, desde 1993 (LO et al., 1996). O WSSV destaca-se por seu alto poder de infecção, causando grandes perdas nos cultivos, em um ou mais estágios de vida desses crustáceos. De acordo com Wang, Zhan e Xing (2005), a síndrome da mancha branca é um dos principais fatores que dificultam o desenvolvimento do cultivo de camarões. Desde o surgimento do WSSV até os dias de hoje, têm sido enormes os esforços técnicos, científicos e econômicos que se vêm realizando em vários países para entender e enfrentar esta doença viral. Recentemente, um estudo conduzido por Müller e colaboradores (2010) com o vírus isolado de camarões infectados no Brasil confirmou que este isolado é diferente de WSSV isolado de outros países nas Américas, além de exibir diferenças entre o vírus encontrado em duas regiões do país, na Bahia e em Santa Catarina.

O WSSV é extremamente virulento e infecta tecidos de origem ecto e mesodérmica, como o epitélio cuticular, tecidos conectivos, e

tecidos hematopoiéticos (CHANG et al., 1996; SHI et al., 2005). A síndrome da mancha branca é caracterizada pela presença de inclusões brancas na cutícula e foi registrada em diferentes espécies de peneídeos (LIGHTNER, 1996). Camarões infectados apresentam também redução no consumo de alimento, letargia, anorexia, coloração avermelhada nos apêndices ou mesmo no corpo (DURAND et al., 1997; MORALES-COVARRUBIAS, 2004). A taxa de mortalidade é muito alta, podendo alcançar 100%, após 3 a 10 dias do aparecimento dos sinais clínicos (LIGHTNER, 1996; HE; QIN; XU, 2006) e, ao contrário da maioria dos vírus, possui afinidade indiscriminada por uma grande variedade de crustáceos (ZHANG, et al., 2006; ESCOBEDO-BONILLA, et al, 2008; MARQUES, et al, 2010).

1.2. Qualidade da água e fatores ambientais

Entretanto, a simples presença de um dado patógeno em uma amostra de camarão submetida ao diagnóstico laboratorial não implica, necessariamente, em doença (SNIESZKO, 1973). É o desequilíbrio na relação patógeno – ambiente – hospedeiro, provocado geralmente por algum tipo de "gatilho" que promoverá condições para que o patógeno se alastre e que a doença se instale.

A saúde dos camarões e, conseqüentemente, a produtividade de uma fazenda de cultivo são fortemente influenciadas pelas condições físicas, químicas e biológicas que prevalecem ao longo do processo de cultivo. Mais especificamente, qualquer elemento presente na água que afete a sobrevivência, a reprodução, o crescimento, a produção ou o manejo da população cultivada de camarões é uma variável importante na qualidade da água (BOYD, 1979). Inúmeros fatores ambientais, como temperatura e pH extremos, baixas concentrações de oxigênio dissolvido, mudanças abruptas na salinidade e presença de substâncias tóxicas endógenas ou xenobióticos podem agir como agentes estressores (NUNES; MARTINS, 2002; JIANG; YU; ZHOU, 2004).

1.2.1. Pesticidas

Pesticidas podem ser introduzidos em lagoas costeiras ou estuários através da aplicação direta, derrames acidentais, lixiviação, drenagem ou precipitação dos resíduos de plantações, além do uso direto na aqüicultura como controle de pragas (COMOGLIO, 2005; FAO, 2006).

Em Santa Catarina, algumas das áreas ocupadas com cultivos de camarão encontram-se próximas a estuários e lagoas costeiras e utilizam

a mesma água que recebe a drenagem de áreas de rizicultura (EMBRAPA, 2004).

Segundo Mello (2007), no início das mortalidades de camarões ocorridas no fim de 2004 em decorrência do início da incidência da mancha branca nas fazendas catarinenses, produtores e técnicos do setor acreditavam haver uma relação direta com os agroquímicos utilizados nas culturas de arroz. Estes compostos eram aplicados em áreas muito próximas aos plantéis, inclusive com aviões, que sobrevoavam as fazendas e poderiam estar contaminando as águas de captação destas. Contudo, em relação a crustáceos marinhos, existe uma escassez de dados que suportem a hipótese, segundo a qual mudanças ambientais induzem alterações do sistema imune, levando a uma maior susceptibilidade a agentes infecciosos (LE MOULLAC; HAFFNER, 2000; BACHÈRE, 2000).

A utilização de diversos produtos químicos, nas várias etapas do cultivo do arroz, dentre eles compostos da classe dos piretróides (FREITAS; LANNA; FERREIRA, 2005), desde 1993 vem gerando conflitos entre pescadores e rizicultores. Ainda hoje, pesquisas têm demonstrado a eliminação de uma série de pesticidas nas águas de drenagem de riziculturas (DESCHAMPS; TOLEDO; NOLDIN, 2003; MOLOZZI; PINHEIRO; SILVA, 2006).

Dentre os piretróides existentes, a permetrina é o inseticida mais persistente, e o mais utilizado para o controle de insetos nas culturas de arroz. Considerando-se que as práticas utilizadas na rizicultura envolvem o revolvimento do solo e aplicação de agroquímicos, a cultura do arroz apresenta-se como potencial degradador da qualidade da água (BETHUNE; AUSTIN; MAHER, 2001) podendo contribuir para uma situação de estresse e favorecer a incidência de infecções virais nos camarões cultivados em áreas adjacentes.

Porém, a maior parte dos estudos envolvendo piretróides em ambientes aquáticos é realizada em peixes. Em peixes da espécie *Channa punctatus* a exposição por 48 horas a uma dose de deltametrina (0,75 μg/l), causou indução de vários sistemas antioxidantes no fígado, rim e brânquias. A peroxidação lipídica foi induzida em todos os órgãos, principalmente nas brânquias, e a glutationa e ácido ascórbico foram significantemente aumentados (SAYEED et al., 2003). Reddy e Philip (1994) utilizaram uma concentração subletal de cipermetrina em machos de *Cyprinus carpio* e verificaram a inibição da atividade da enzima Acetilcolinesterase e da ATPase.

Em relação a crustáceos, Ferrero; Gutiérrez; Cervellini (2001) constataram que o caranguejo *Chasmagnathus granulata* é mais

tolerante ao inseticida malation do que a deltametrina, ao determinarem o valor de CL50 para deltametrina de 0,27 µg/l (0,25-0,45) em indivíduos machos desta espécie de crustáceo. Ensaios de toxicidade aguda com *Daphnia similis*, desenvolvidos por Silva (2005) permitiram estabelecer valores de permetrina para organismos aquáticos através de CL50; 24h de 0,0011 g/ml e a CL50;48h de 0,0003 g/ml. Para camarões peneideos, o único estudo desenvolvido visando estabelecer valores toxicidade da permetrina em camarões cultivados foi desenvolvido por Mello (2007), em juvenis de *Litopenaeus vannamei*, e resultou em valores de CL50;96h de 0,009 µg/l para o inseticida comercial Talcord.

Alguns trabalhos têm buscado determinar as concentrações de xenobióticos que podem interferir na qualidade de água e seu potencial efeito na biota existente. Porém, poucos estudos são focados nas alterações que inseticidas podem desencadear nos organismos aquáticos quando em concentrações subletais e longe da população alvo.

1.2.2. Temperatura

Dentre os fatores abióticos, a temperatura é um dos fatores mais importantes que afetam os animais aquáticos. Muitos organismos ectotérmicos se adaptam e sobrevivem em amplas faixas de temperatura. A falta de capacidade de produzir calor dos animais endotérmicos, faz com que seu metabolismo fique à mercê das mudanças de temperatura ditadas pelas estações e outros distúrbios sazonais e termais. Estas flutuações térmicas não são insignificantes. Muitos processos biológicos importantes, incluindo o desenvolvimento, o crescimento e a adaptação ao *habitat*, são dependentes da temperatura. Em resposta à variação térmica no ambiente, muitos organismos desenvolveram adaptações bioquímicas para a proteção contra mudanças de temperatura (SPEES et al, 2002)

A temperatura tem influência direta sobre o metabolismo, o consumo de oxigênio, a taxa de crescimento, o ciclo de muda e a taxa de sobrevivência (CHENG; CHEN, 2000).

A temperatura ótima para o crescimento e sobrevivência dos camarões varia de acordo com a fase do seu ciclo de vida e da espécie. Para *Litopenaeus vannamei*, por exemplo, faixas de temperatura ideal variam entre 27°C e 30°C (WYBAN et al, 1995), sendo a maior taxa de sobrevivência de juvenis desta espécie observada entre 20°C e 30°C (PONCE-PALAFOX et al., 1997).

A maioria dos estudos envolvendo a avaliação das condições de temperatura para camarões peneídeos tem por finalidade erradicar ou minimizar danos a saúde dos animais de cultivo e a mortalidades dos

plantéis. Zhu et al. (2009) observaram que o WSSV é capaz de promover infecção mesmo após ter sido exposto a 37°C por 24 horas ou a 50°C por 30 minutos. Momoyama et al. (1998) relataram a presença de WSSV viável por pelo menos 30 dias na água do mar a 30°C em condições de laboratório. No ambiente, esse vírus recebe proteção térmica natural, pois, a temperatura da água raramente é superior a 35ºC.

A replicação do WSSV é inibida, devido à proteção parcial por hipertermia, quando camarões são mantidos em água a 32°C, reduzindo, assim, a replicação viral em alguns viveiros. Rahman et al. (2007) descrevem que animais expostos por 6 horas a condições de hipertermia apresentaram redução da mortalidade e inibição da replicação do WSSV

Em um estudo recente conduzido por Costa et al. (2010) em fazendas catarinenses infectadas com o WSSV, a variação na temperatura da água dos viveiros entre 24,6 e 29,3ºC favoreceu a manifestação da enfermidade da mancha branca em camarões *Litopenaeus vannamei*. As temperaturas observadas nos períodos de mortalidade estão dentro do intervalo de oscilação diária de 26 a 30ºC, registrado em ocorrências de mortalidades em cultivos no México, em 2007. A elevação na temperatura da água também foi relacionada ao aumento da mortalidade de *L.vannamei* infectados experimentalmente com WSSV (SONNENHOLZNER; RODRÍGUEZ; CALDERÓN, 2002).

Os mecanismos sugeridos para explicar tais resultados abrangem alterações metabólicas dos camarões, a redução da replicação viral (DU et al, 2006), mecanismos de apoptose (GRANJA et al, 2003;GRANJA et al, 2006) e alterações na expressão de genes do WSSV (REYES et al, 2007).

1.3. Avaliação das respostas ao estresse através do uso de biomarcadores

Os biomarcadores são definidos por Walker, Hopkin e Sibly (1996) como alterações biológicas em nível molecular, celular ou fisiológico que expressam os efeitos tóxicos causados por contaminantes nos organismos expostos. Os biomarcadores podem indicar tanto a exposição dos organismos aos contaminantes (biomarcadores de exposição), como a magnitude da perturbação causada em resposta a poluentes (biomarcadores de efeito) (CAJARAVILLE et al., 2000). Contaminantes orgânicos podem causar, além de outras alterações, a

indução de enzimas que atuam na biotransformação ou detoxificação dos mesmos, e, em alguns casos, produzir metabólitos ativos. Durante o processo de biotransformação, outras alterações envolvem a indução de enzimas decorrente de interações destes contaminantes com receptores celulares e, ainda alterações no DNA (COMOGLIO, 2005; LYLE-FRITCH; ROMERO-BELTRAN; PÁEZ-OSUNA, 2006).

Estas respostas podem não causar nenhum dano evidente ao organismo, porém podem trazer conseqüências para as células ou para todo o organismo, afetando, por exemplo, seu crescimento e reprodução. Neste sentido, as alterações bioquímicas são a primeira resposta de ação biológica e representam a base molecular da toxicidade (WALKER; HOPKIN; SIBLY, 1996).

Segundo Abessa (2006), uma série de biomarcadores pode ser utilizada em estudos ecotoxicológicos, todos eles baseados em processos envolvidos na eliminação dos contaminantes do interior das células. Durante todo o processo, é possível avaliar os efeitos causados pelos contaminantes, através da mensuração da atividade e da expressão de enzimas envolvidas ou de subprodutos das reações de biotransformação, conjugação, oxidação lipídica e ativação de defesas atioxidantes, entre outros.

Dentre os sistemas enzimáticos envolvidos em processos de biotransformação de xenobióticos, destaca-se a enzima glutationa-*S*-transferase (GST) que atua na conjugação destes compostos, facilitando a sua excreção. As enzimas superoxide dismutase (SOD) e catalase (CAT) estão envolvidas em processos de estresse oxidativo. Por outro lado, a acetilcolinesterase (AChE) tem sua atividade inibida na presença de compostos organofosforados e carbamatos (CHIEN et al, 2003).

Outra abordagem inclui a avaliação das proteínas de choque térmico – *Heat Shock Proteins* (HSP) – ou proteínas de estresse, como um instrumento válido para o biomonitoramento de exposições a contaminantes, na tentativa de prevenir conseqüências biológicas que possam vir a afetar o organismo ou níveis de organização superiores. As HSP têm a função de manter a conformação natural das proteínas celulares, agindo como chaperonas. Além do choque térmico, uma variedade de outros agentes estressores pode induzir a síntese destas proteínas, inclusive salinidade (GONZALES; BRADLEY, 1995) e agentes tóxicos (LINDQUIST; CRAIG, 1988). As proteínas de estresse têm sido identificadas em todos os organismos estudados até o presente, sendo o mais conservado sistema genético conhecido (LINDQUIST; CRAIG, 1988; CIMINO et al., 2002). A exposição das células a agentes estressores resulta em um conjunto de alterações metabólicas rápidas,

referidas como respostas ao estresse. Tais alterações incluem a ativação e elevação da expressão de um pequeno conjunto de genes, resultando num aumento da síntese e acumulação de proteínas de estresse (NASCIMENTO et al., 1998).

Técnicas bioquímicas clássicas, associadas a técnicas de biologia molecular, vêm auxiliando na descoberta de ferramentas promissoras para o biomonitoramento ambiental. A partir da caracterização molecular e a construção de iniciadores específicos para a amplificação de genes envolvidos nas respostas de defesa bioquímica e celular é possível avaliar sua expressão frente a diferentes condições ambientais (TAVARES-SANCHEZ et al., 2004; GOMEZ-ANDURO et al., 2007; ZHANG et al., 2007). Estes biomarcadores moleculares podem ser utilizados no monitoramento das condições de cultivo dos camarões

Considerando que a ativação e/ou inibição dos processos moleculares e celulares de defesa ou de eliminação dos contaminantes podem ser avaliadas e mensuradas por uma série de biomarcadores, um programa de avaliação e de monitoramento do estado de saúde dos animais, aliado a ferramentas bioquímicas, poderia contribuir para a detecção precoce de problemas existentes nos cultivos (MARQUES; BARRACCO, 2000; BAINY, 2000). Desta forma, seria possível adotar ações para controlar, minimizar ou excluir os efeitos negativos sobre a produção aquícola e contribuir para a sanidade dos cultivos.

Dentro desse contexto, o presente trabalho teve como objetivo avaliar biomarcadores moleculares no camarão *Litopenaeus vannamei* submetido a estresse ambiental e infectado pelo vírus da síndrome da mancha branca.

Através da análise diferencial de transcritos e da atividade enzimática de biomarcadores envolvidos em processos de defesa antioxidante, foi possível avaliar as respostas do camarão *L. vannamei* frente à contaminação por um pesticida piretróide. Além disso, foi avaliada a expressão de algumas proteínas de defesa e sua relação com a susceptibilidade ao vírus da mancha branca, após o estresse causado pela exposição à permetrina e pela inoculação com o WSSV.

Outra abordagem foi realizada para avaliar o efeito da temperatura na replicação do WSSV em camarões de cultivo, *L.vannamei* inoculados com o vírus e submetidos a estresse térmico. O efeito da temperatura sobre a replicação viral também foi monitorado em crustáceos silvestres.

Os resultados aqui apresentados estão reunidos na forma de três artigos científicos.

CAPÍTULO 1

Antioxidant defense biomarkers in the white shrimp, *Litopenaeus vannamei,* exposed to permethrin

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Abstract

Permethrin is the most common pyrethroid insecticide used to grow rice crops in many countries. Sub-lethal concentrations of permethrin may negatively impact the productivity of shrimp farms located in the vicinity of rice fields due to the effects on physiological and biochemical responses in cultivated shrimp. In the present study, we investigated the activity of antioxidant defense enzymes, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as glutathione-*S*-transferase (GST) and acetylcholinesterase (AChE) in juvenile *Litopenaeus vannamei* following two different conditions of exposure to permethrin (0.01; 0.1; 0.2; 0.3; 0.4 µg.L¯1 for 96h and 0.8 µg.L¯1 for 6, 12, 24, 48, 72 and 96h). Enzymatic assays were conducted in gills and hepatopancreas of exposed shrimp. Transcription levels of the genes encoding these enzymatic biomarkers were addressed by real time PCR in both tissues. Changes were seen in CAT and GPx activities in gills, in contrast with the hepatopancreas. GST activity was increased at 24 and 48h of exposure in both tissues while SOD was increased only in hepatopancreas. Decreased AChE activity was seen between 6 and 12h in both target tissues. Pesticide stress induced GPx and SOD gene expression in gills between 48 and 96h while these genes were induced earlier in hepatopancreas. The pattern of transcriptional and enzymatic responses elicited after permethrin exposure supports a multi-biomarker approach as a useful tool for monitoring the aquatic environment.

Keywords: *Litopenaeus vannamei*, permethrin, gene expression, antioxidant enzymes, quantitative real-time PCR

1. Introdution

The coastal environment is continuously challenged by exogenous chemicals or xenobiotics released by urban communities, farms and industries (Van der Oos et al, 2003). Pesticides may be released into coastal lagoons or estuary by direct application, accidental spills, leaching, and precipitation of drainage or waste from crops, in addition to their direct use in aquaculture as pest control (Comoglio, 2005; FAO, 2006).

Decades of research and development by agrochemical industries resulted in a wide variety of pesticides. Consequently, the widespread use of pesticides represents a serious issue to animal health (FAO, 2006), once the generated waste can be leached and cause poisoning and environmental contamination. Natural water bodies around shrimp ponds may receive contaminated effluents with high loads of pesticides. Sub-lethal concentrations of pesticides, such as permethrin, can cause physiological and biochemical changes in exposed shrimp and, therefore, directly affect the productivity of farms.

Due to restrictions applied to the use of organophosphorous pesticides, pyrethroids have been developed in order to be effective insecticides with reduced human toxicity (He et al, 2007) and their usage is expected to increase further (Parvez and Raisuddin, 2006; Qin and Gan, 2006). Among the pyrethroids, permethrin is the most persistent. After 30 days of application, permethrin has been found at concentrations very close to the residual values determined after 48 hours of its dispersion (0 to 15.7 mg) (Lutnick et al, 1999).

Light sensitivity range is diverse among various types of pyrethroids. In water, deltamethrin, permethrin and cypermethrin present an average life ranging from 17 to 110 days. In aqueous solution, pyrethroids tend to be stable in acid and neutral pH, but become increasingly susceptible to hydrolysis at pH above neutral. Nonetheless, permethrin is considered stable even in basic medium and has an average life of 240 days (Laskowski, 2002).

Permethrin is the most common pyrethroid insecticide used to grow rice crops in many countries. In Santa Catarina State, southern Brazil, some shrimp farms are located close to estuaries and coastal lagoons and make use of the same water that receives drainage from rice growing areas. The soils cultivated with rice occupy about 7% of the total area of Santa Catarina, and include mainly coastal plains (90% of lowland areas) in the southern part of the state, extending from the

border of Rio Grande do Sul to Cabo de Santa Marta, as well as from Bay of Babitonga to the cities of Joinville and Itajai in the north (EMBRAPA, 2004). The use of various chemicals in diverse stages of rice cultivation, including compounds belonging to the class of pyrethroids (Freitas et al, 2005), has been generating conflicts between fishermen and rice farmers ever since. Even today, studies have still shown the elimination of pesticides in drainage water from rice growing areas (Deschamps et al, 2003; Molozzi et al, 2006).

The effects of pesticides in the environment, considering the contamination of water and the impact on non-target organisms, may not be seen immediately but have consequences for the cells or the entire organism, affecting, for example, growth and reproduction (Comoglio, 2005; Lyle-Fritch et al, 2006). In this sense, biochemical changes are seen as the first response of biological defense mechanisms and therefore, represent the molecular basis of toxicity (Walker et al, 1996). It is possible to evaluate the effects caused by contaminants by measuring the activity and expression of enzymes involved in the reactions of biotransformation, conjugation and activation of cellular defenses, as well as the transcription levels of their encoding genes.

Among the enzymatic systems involved in biotransformation processes, the enzymes superoxide dismutase (SOD) and catalase (CAT) and glutathione peroxide (GPx) are involved in the cellular defense against oxidative stress conditions, whereas glutathione S-transferase (GST) acts on the conjugation of the biotransformed compounds, facilitating their excretion. On the other hand, acetylcholinesterase (AChE) activity is inhibited in the presence of organophosphates and carbamates (Chien et al, 2003).

Biochemical analyses associated with classical molecular biology assays are useful and complementary approaches in order to investigate and validate new biomarkers for monitoring the quality of the aquatic environment as well as the health status of target organisms. These molecular biomarkers can be used for monitoring the conditions of cultured shrimp as well as other aquatic crustaceans (Tu et al, 2010).

The aim of the present study was to investigate biochemical and molecular responses related to the activity and gene transcription of antioxidant (SOD, CAT, GST and GPx) and a pesticide sensitive enzyme (AChE) in gills and hepatopancreas of the white shrimp, *Litopenaeus vannamei*, after exposure to permethrin under laboratory conditions.

2. Materials and methods

2.1. Animals

Juvenile intermolt shrimp (8.5 ± 2.0 cm in body length) were obtained from a local shrimp farm in the northern region of Santa Catarina State (Brazil). In the laboratory, shrimp were maintained in a 6000 L fiberglass tank, with water temperature at 23±1°C and salinity of 20 $g.L^{-1}$. Animals were held for a week to acclimate to the laboratory conditions prior to experimental use and were fed once daily with commercial shrimp feed. Feeding was stopped 24 h before treatment.

2.2. Exposure experiments

After acclimation, shrimp were randomly distributed into tanks (20L). Each tank contained six shrimp. Experimental exposure to different concentrations of permethrin was done in duplicate and one group of shrimp was reared in pesticide free water and used as a control group. Shrimp (n=6/group) were exposed to 0.01, 0.1, 0.2, 0.3, 0.4 and 0.8 $\mu g.L^{-1}$ of permethrin for 96h. Pesticide for the experiments was prepared by diluting proper volume of permethrin (SIGMA) stock solution in filtered sea water.

Based on a preliminary analysis of mobility and enzymatic activity of shrimp, the final concentration of 0.8 $\mu g.L^{-1}$ was chosen as sub-lethal concentration for the following experiment.

Shrimp (n=6/group) were exposed to 0.8 $\mu g.L^{-1}$ of permethrin for different periods of exposure. Among the groups of shrimp exposed to permethrin, additional groups were reared in permethrin-free water and used as a control group. Shrimp were collected after 6, 12, 24, 48, 72 and 96h of exposure. As in the experiment conducted previously, shrimp were monitored every 6 hours for survival, pattern of behavior, mobility and body coloration. Exposed shrimp had gills and hepatopancreas excised individually and tissue was immediately frozen in liquid nitrogen for further analyses.

2.3. Tissue preparation for enzymatic analysis

Gill tissue and hepatopancreas homogenization was performed in 0.02 M Tris-HCl, 0.25 M sucrose, 2 mM DTT, 0.1 mM PMSF (pH 8.0) cooled buffer, using the Glas-Col homogenizer (Terre Haute, USA). For the enzymatic determination, homogenized tissues were centrifuged at 9.000xg for 30 minutes, followed by a new centrifugation at 37.000xg for 90 min at 4°C. The supernatants were stored at -80°C for analysis.

2.3.1. Determination of enzyme activity.

Total SOD activity was assayed following the method of McCord and Fridovich (1969). CAT activity was measured according to Beutler (1975). GPx and GST activity was assayed according to Keen et al. (1976). AChE activity was measured according to Ellman et al. (1961). The soluble protein content was determined by the Lowry method modified by Peterson (1977) using bovine serum albumen as standard. All of these methods have been adapted for the micro-plate reader (Tecan, Germany).

All the measurements were made in duplicate. The results were subjected to analysis of variance (ANOVA) followed by Tukey test, performed with GraphPad Prism software.

2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from gill and hepatopancreas samples using the Trizol method (Invitrogen), according to the manufacturer's instructions. Extracted RNA samples were subjected to DNA-free (DNase) treatment to prevent genomic DNA contamination. The cDNA synthesis was performed using the reverse transcription system Omniscript RT Kit (QIAGEN), following the protocol recommended by the manufacturer.

2.4.1. Quantitative real-time PCR (qPCR) assay

The mRNA level of target genes in hepatopancreas and gills was measured by qPCR. The specific primers were designed by Primer Quest program (Integrated DNA Technology, IDT)(Table 1). Amplifications were carried out with Quantifast SYBR green Kit (QIAGEN) in a total volume of 20 µL containing 20 µM forward and reverse primer and 100 µg of cDNA. Real-time PCR was carried out with a Rotor-gene 6000 real-time PCR machine (QIAGEN). The cycling parameters used were as follows: an initial denaturation at 95°C for 5min, 35 cycles at 95°C for 10s, 56°C for 30s.

A melting curve analysis of the amplified products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. Further, samples were subjected to electrophoresis on agarose gel to confirm the primer specificity. cDNA of each shrimp from time point exposure was used for analysis in qPCR assays each were run in triplicates. 18S ribosomal subunit from decapods – DEC, and L8 transcript levels were used to normalize samples. The relative quantification of gene expression among the treated groups was analyzed by the ΔΔCt method (Livak and

Schmittgen, 2001) and by the REST software (QIAGEN). Thus, the PCR efficiencies of target genes used in the present study were detected and only efficiencies close to 100% (E = 2) were used. For each assay, a standard curve of target gene was obtained by using serially diluted cDNA pooled samples.

Table 1: Primers for quantitative real time PCR amplification of biomarkers genes.

Target gene	GenBank #	primer sequences	Products (bp)
Ribosomal L8 (housekeeping gene)	DQ316258	5′-TAGGCAATGTCATCCCCATT-3′ 5′-TCCTGAAGGGAGCTTTACACG-3′	167
Ribosomal 18S (housekeeping gene)	M34362	5'- CCGAATGGTCGTGCATGGAATGAT-3' 5'- GAATTTCACCTCTAGCGTCGCAGT-3'	127
Superoxide dimutase	AY486424	5'-TCATGCTTTGCCACCTCTC-3' 5'- CCGCTTCAACCAACTTCTTC-3'	143
Catalase	AY518322	5'- CAAGTGGCGATTACCCCTCAT-3' 5'- CCGCTTCAACCAACTTCTTC-3'	110
Glutathione Peroxidase	AY973252	5'-AGGGACTTCCACCAGATG-3' 5'- CAACAACTCCCCTTCGGTA-3'	117
Glutathione S-Transferase	AY573381	5'- AAGATAACGCAGAGCAAGG-3' 5'- TCGTAGGTGACGGTAAAGA-3'	146
Acetylcholinesterase	DQ533868	5'- GGCACTGAAATGGATCCAGGAA-3' 5'- CGTGACTTCACCAGGGTTACC-3'	121

3. Results

3.1. Shrimp mortality, behavior, mobility and body coloration

Mortality was observed in the groups exposed to 0.1, 0.3 and 0.8 $\mu g.L^{-1}$ of permethrin. Only one dead shrimp was recorded per concentration on the last day of exposure. Since one dead shrimp was also seen in the control group, recorded mortalities may have been caused by either limited water quality or non-availability of food, and not directly by the exposure to permethrin.

Typical signs of stressed shrimp, such as red coloration of body appendices, were observed only during the exposure to the more elevated doses of the xenobiotic, e.g., 0.3, 0.4 and 0.8 at 96h. No red coloration was observed in the control groups.

Changes in behavior patterns, such as restless swimming, was observed during permethrin exposure over time. Erratic swimming was observed in the early hours of exposure. On the other hand, the reddish color in shrimp body (uropods and pleopods) was observed after 48 to 72 hours of exposure, indicating a stress condition.

3.2. Enzymatic activities

The effects of permethrin concentration and the period of exposure were estimated by changes in enzymatic activities.

The enzyme determinations were performed in the gills of animals exposed for 96h to 0.001 to 0.8 $\mu g.L^{-1}$ of permethrin (Fig.1). In the supernatant fraction of homogenized gills, significant differences were observed in the enzymes CAT, GST and GPx in shrimp exposed to 0.8 $\mu g.L^{-1}$. On the other hand, exposed shrimp showed no alteration in the activity of AChE when compared to the control group, independently of permethrin concentration and period of exposure.

Permethrin effects over time in enzymatic activities of gills are showed in Figure 2. Permethrin promoted significant higher activities of SOD in shrimp gills, after 24, 48 and 96h of exposure. CAT activity was significantly higher in gills of animals exposed for a period of 6 to 24 and 96h, compared with the control shrimp. In addition, between 24 to 96 hours of pesticide exposure promoted significantly higher activity of GPx in gills of exposed shrimp when compared to the control.

The activity of AChE in gills was inhibited in shrimp collected after permethrin exposure for 6 and 12h. After a 24 and a 48 period of exposure, an increase in the activity of AChE was observed. GST activity increased in gills after 24 and 48h of exposure. Nonetheless, after a 96h exposure the activity of both enzymes returned to the levels seen in gills of control shrimp.

SOD determination in shrimp hepatopancreas exposed to permethrin was significant higher in all time of exposure. Higher CAT and GPx activities were seen after 48h under permethrin exposure, compared with control shrimp. Significant differences in GST activity were observed at 6, 24, 48 and 96h post-permethrin exposure (Fig. 3).

Decrease of AChE activity was also observed in shrimp hepatopancre as after 6h, 12h, as well as 96h of exposure to permethrin.

3.3. Gene transcription after permethrin stress

Quantitative real-time PCR was used to investigate the mRNA levels of antioxidant and non-antioxidant enzyme genes (*SOD*, *CAT*, *GPx*, *GST* and *AChE*) in gills and in hepatopancreas after exposure to permethrin over time.

Transcription of *SOD* in shrimp gills was slightly inhibited after 6 and 12h of exposure, whereas, significant induction was observed at 48h (2.78 fold) and 72 h (1.80 fold) after exposure to the pesticide. In addition, *CAT* expression was induced at 12, 24 and 96h of exposure (2.53, 2.29 and 2.51 fold, respectively). At 48h, expression levels were

increased remarkably (22.16 fold) in exposed shrimp when compared to the control group. *GPx* gene expression was significant induced in gills sampled after 48 (4.94 fold) and 72h (1.95 fold) of exposure in comparison of control groups (Fig.4)

Inhibition in *GST* transcript levels in gills was seen at 12, 48 and 96h post-permethrin exposure. In contrast, permethrin significantly induced *AChE* after 24 (4.5 fold) and 48h (8.02 fold) of exposure.

Figure 5 shows the transcription profile of the same biomarkers in hepatopancreas. In this tissue, *SOD* was induced in the groups exposed for 6, 24, 48 and 96 h (1.58, 1.87, 14.77 and 1.57 fold, respectively). Slight inhibition was seen in *CAT* after 6 and 12h of exposure while significant induction (2.88 fold) of this enzyme was observed after 24h of exposure. *GPx* transcription analysis showed induction in exposed shrimp after at 6h (1.71 fold) and after 24, 48 and 72h (2.10, 2.62, 2.04 fold, respectively).

Despite the changes seen in gills of exposed shrimp, no inhibition in *GST* transcripts was observed in hepatopancreas. Significant induction was seen after 6h and 48h of exposure. Permethrin did not promote any changes in *AChE* transcript levels in shrimp hepatopancreas.

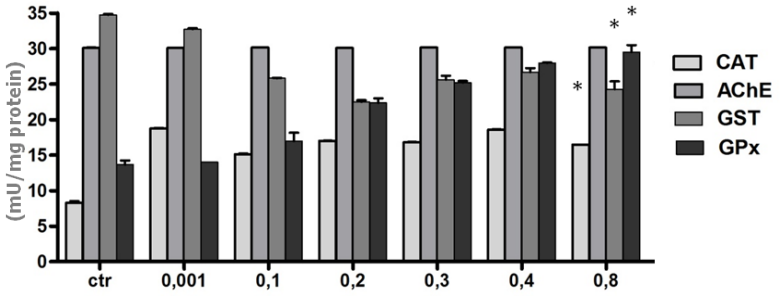

Figure 1: Enzymatic activity of Catalase (CAT), Acetylcholinesterase (AChE), Glutathione Peroxidase (GPx) and Glutathione S-Transferase (GST) in *Litopenaeus vannamei* exposed to different concentration of permethrin 0.01, 0.1, 0.2, 0.3, 0.4 and 0.8µg.L^{-1} for 96h. (*) p<0,001.

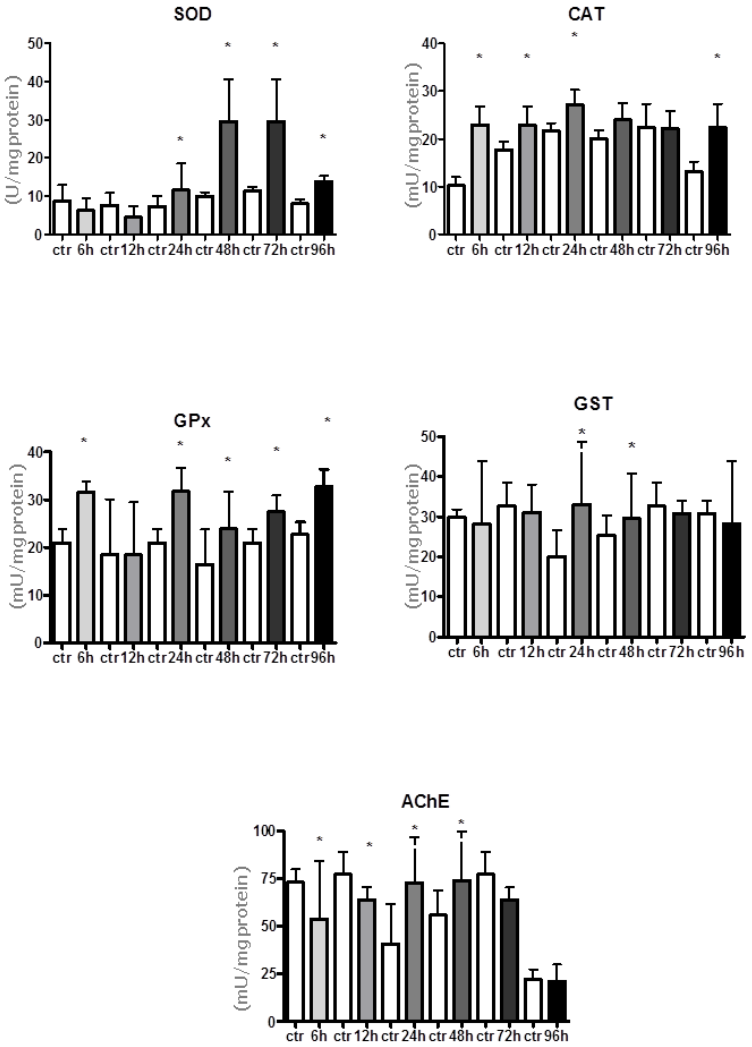

Figure 2: Enzymatic activity of SOD, CAT, GPx, GST and AChE in gill of *Litopenaeus vannamei* exposed to permethrin over time. Significant differences between treatments and control are indicated with superscripts (* $p<0,001$).

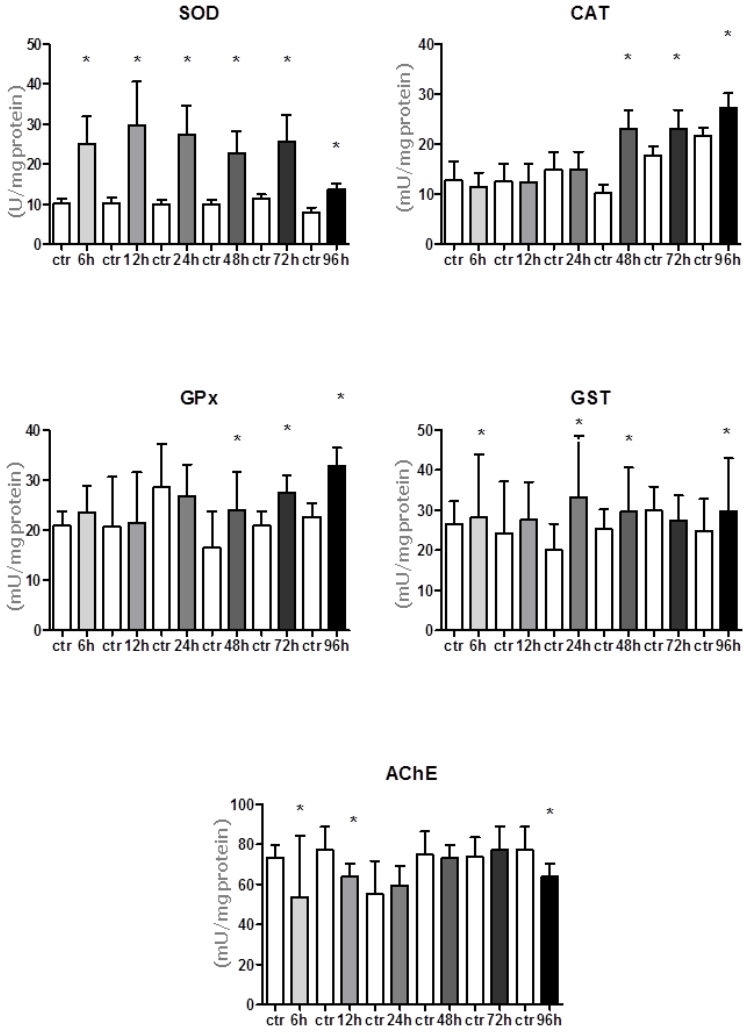

Figure 3: Enzymatic activity of SOD, CAT, GPx, GST and AChE in hepatopancreas of *Litopenaeus vannamei* exposed to permethrin over time. Significant differences between treatments and control are indicated with superscripts (* p<0,001).

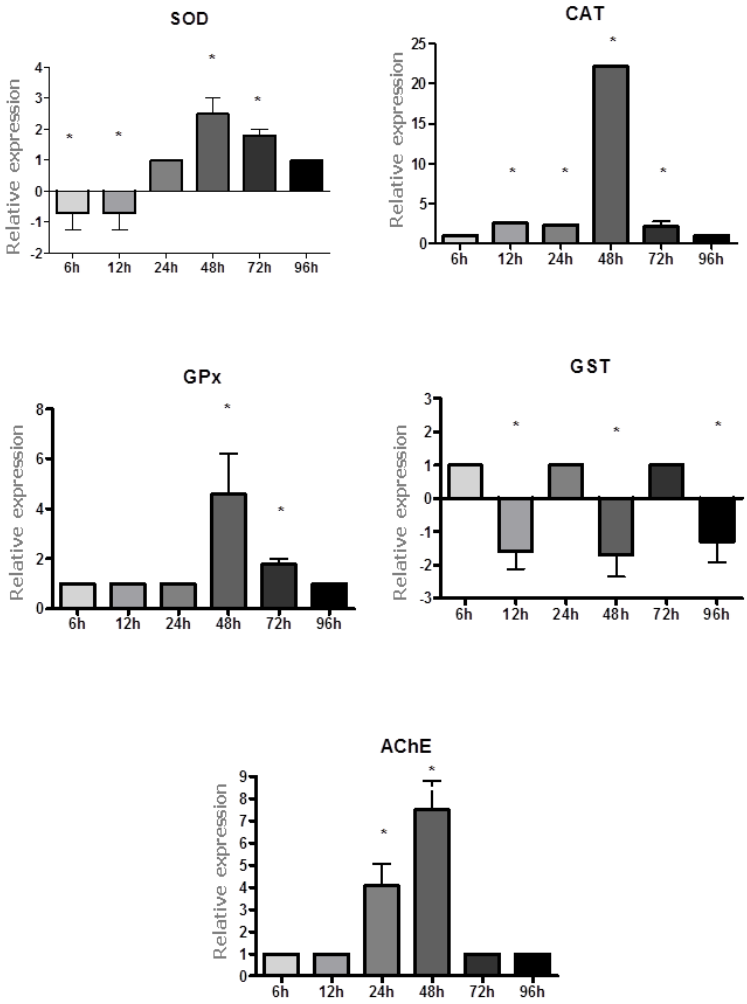

Figure 4: Transcription levels of *SOD*, *CAT*, *GPx*, *GST* and *AChE* genes after permethrin stress in gills of *L.vannamei*. Significant differences between treatment and control are indicated with superscripts (* $p<0,05$).

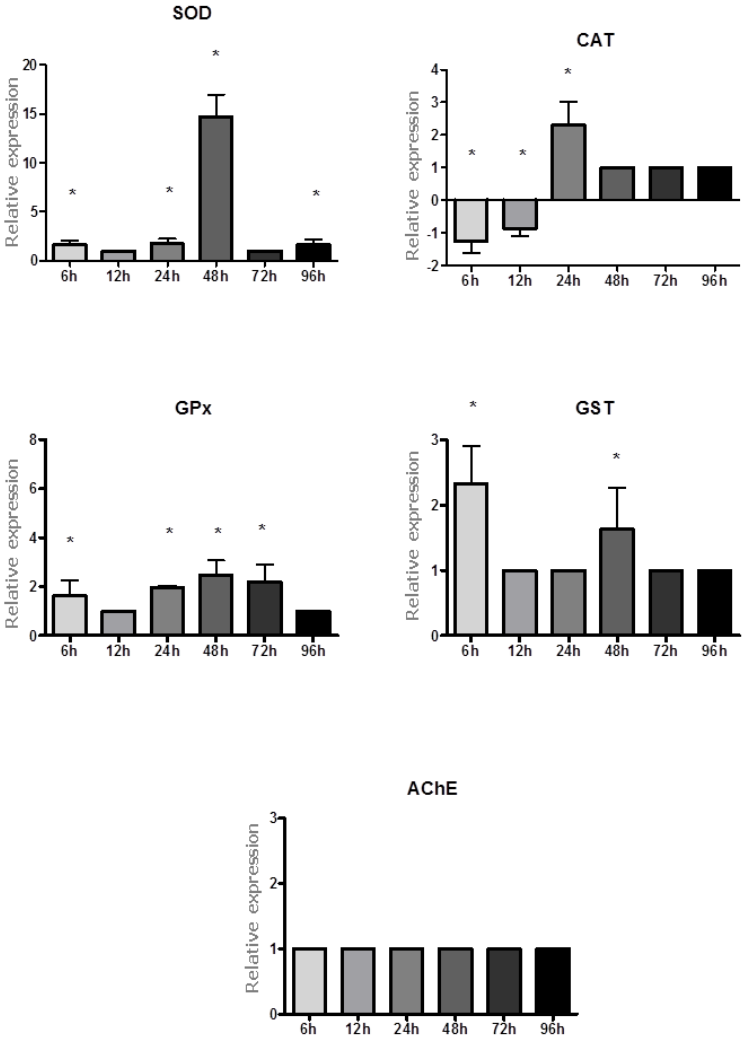

Figure 5: Transcription levels of *SOD*, *CAT*, *GPx*, *GST* and *AChE* genes after permethrin stress in hepatopancreas of *L.vannamei*. Significant differences between treatment and control are indicated with superscripts (* $p<0,05$).

4. Discussion

Antioxidant responses are potential indicators of oxidative stress in marine organisms and abundant literature supports the validity of biomarkers as indicators of organism responses to a contaminant or stressor (Adams, 1990; Cajaraville et al, 2000; Downs et al, 2001).

Changes in shrimp behavior patterns, such as restless and erratic swimming and reddish body color, were observed during permethrin exposure over time in the present study. Studies reported a syndrome consisting of aggressive sparring behavior, tremor, hyperthermia, and decreased motor activity in rats exposed to Type I pyrethroids (permethrin) (McDaniel and Moser, 2002), along with hypersensitivity, hyperexcitability, muscle cramps and seizures (ANVISA). However, despite the toxicological effects seen in fishes and insects, information concerning alterations in shrimp behavior patterns associated with pesticides contamination is scarce. Nonetheless, marine crustaceans have been considered as being susceptible to the toxic effects of pyrethroids (Fairchild et al., 1992).

The present study evaluated SOD, CAT, GPx, GST and AChE activities and transcription levels as biomarkers in shrimp *Litopenaeus vannamei* exposed to permethrin.

The expression of genes encoding these enzymes revealed different patterns of response. Overall, kinetic profiles were not associated with concentration or time of exposure. However, one can consider that each one of these biomarkers plays a relevant role, since antioxidant and conjugation enzymes are pivotal biochemical strategies to the organism defense response against contaminants. Furthermore, we found that the expression of genes encoding these enzymes differs between gills and hepatopancreas.

SOD, CAT, GPx and GST have been widely used as biomarkers to evaluate stress caused by xenobiotics in the aquatic environment, whereas real-time PCR is one of the most sensitive assay to address transcriptional levels of target genes. Our results confirmed that transcription of genes in juvenile shrimp can be rapidly and efficiently induced by short term exposure (hours) to permethrin. Moreover, differences in the activity of some enzymes (CAT, GPx and GST) were observed as early as 6h post-stress. Although in gills, the major effects were observed at 12h post-exposure, all five proposed biomarkers showed significant differences when compared to the control group.

Superoxide dismutase (SOD) is one of the main antioxidant defense enzymes generated in response to oxidative stress. It converts

the highly toxic superoxide anions into hydrogen peroxide (Fridovich, 1995). In the present study, the activity of SOD was significantly increased in hepatopancreas at all post-exposure intervals, while in gills the higher activity was observed after 24h. The expression profile of the *SOD* coding gene showed that the gene was initially down-regulated in gills and after 48h its expression was higher in the same tissue.

The kinetic profile of CAT showed an up-regulation in gills of shrimp exposed for 12h, and an increase in the enzymatic activity was detected at 24h after exposure time.

In response of permethrin stress GPx activity was increased in gills and hepatopancreas at the same time points. Wang et al (2009) reported the induction of antioxidant genes as a consequence of stress caused by pH in *L vannamei*, after 12h.

Secondary enzymes in antioxidant defense include those of glutathione metabolism. Glutathione-*S*-transferase (GST) catalyzes the conjugation of reduced glutathione (GSH) and cellular components damaged by reactive-oxygen species (ROS) attack, to nucleophilic xenobiotics, which leads to their detoxification (Clark, 1989). These enzymes exist in multiple forms and are known to be involved in insect resistance to organophosphorous insecticides (Terriere, 1984, Contreras-Vergara et al, 2007). The high specific activity of GST may be related to a highly effective detoxification mechanism necessary in gills since they are the first tissue to be exposed to permethrin. Tu et al. (2010) found elevated levels of GST in gills and hepatopancreas of shrimp sampled in intensive and rice-shrimp integrated systems.

Once the aim of this study was the evaluation of molecular biomarkers (gene transcriptional profile) and enzymatic alteration in response to permethrin exposure, the activity of AChE has been also studied. Inhibition of AChE has been extensively used as a biomarker of effect and exposure to insecticides, especially organophosphates and carbamates, in order to monitor the effects of these contaminants in living organisms (Bocquené et al, 1997). The cypermethrin and ë-cyhalothrin have been associated to the significant inhibition in the activity of AChE in freshwater fishes, *Channa punctatus* and *C. batarchus* (Kumar et al, 2009). Tu et al. (2009) assessed the potentiality to use cholinesterase activity in *Penaeus monodon* and observed significant decrease of the enzyme activity in muscle after 4 days of exposure of 0.07 $\mu g.L^{-1}$ deltamethrin. According to Montserrat et al (1997), inhibition of AChE varies with the species, the type of compound and exposure conditions.

To our knowledge this is the first study that assesses the effects of permethrin at the enzymatic and gene expression in *L.vannamei*. Evaluation of alterations in gene expression is advisable to monitor the potential adverse effect of permethrin in shrimp health and allow an early warning of the ecotoxicological impact caused by the xenobiotic.

In conclusion, antioxidant and non-antioxidant enzyme coding genes are modulated in response to permethrin stress. These transcription data can serve as sensitive multi-biomarkers for monitoring aquatic environments

From our results and previous studies (Ferrero et al, 2000; Galindo-Reyes et al, 2000; Sánchez-Fortún and Barahona, 2005), it is clear that pyrethroid pesticides, such as permethrin, display high acute aquatic toxicity to shrimp, promoting changes in enzymatic and transcription levels of target genes in response of permethrin exposure. These results indicate that environmental changes may compromise the health of shrimp and also the economic viability of farming. These findings can be useful to the future management and regulation of pyrethroid insecticide use near aquaculture areas.

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CAPÍTULO 2

Molecular defense responses in *Litopenaeus vannamei* exposed to permethrin and challenged with white spot syndrome virus (WSSV)

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Abstract

The effects of environmental contaminants, such as pesticides, on non-target organisms may impact and bring consequences at the molecular level even at sublethal concentration. There is a lack of data supporting the hypothesis according to which environmental stress modulates the immune system, leading to an increased susceptibility to infectious agents in marine organisms. The present study was focused on changes occurring in cellular defense gene and proteins in *Litopenaeus vannamei* exposed to permethrin (0.8 $\mu g.\mu L^{-1}$) for 1, 2 and 7 days and challenged by white spot syndrome virus (WSSV). Real-time quantitative PCR was used to evaluate the transcriptional levels of genes encoding ferritin, β-actin, protein kinase Cξ, ubiquitin, Hsp60, Hsp70, HSP90, caspase-3 and QM protein in gill tissue. Furthermore, expression of some of these proteins was addressed by Western blot analysis. Changes in transcript levels were supported by immunedetection analysis, suggesting that permethrin toxicity to shrimp promotes changes in the expression of target genes. Furthermore, WSSV infection could be associated to a differential transcription of some of the genes encoding these proteins.

Keywords: *Litopenaeus vannamei*, WSSV infection, permethrin, gene expression, Western blotting

1. Introdution

The occurrence of white spot disease (WSD) in Santa Catarina State, southern Brazil (OIE, 2005), revealed the fragility of sanitary measures applied to marine shrimp farming and, also the lack of a systematic health surveillance system for cultivated shrimp in the region (Marques et al, 2006). Brazilian frontiers were closed to the entry of crustaceans in 1999, aiming to prevent the occurrence of WSD in the country. However, symptoms of the disease began to be detected in November 2004 in shrimp farms in the south of Santa Catarina. WSD was diagnosed in January 2005, in samples collected in 15 farms in the municipalities of Laguna and Imaruí in an area of 200 hectares (Seiffert, 2005).

Among the viral diseases of penaeid shrimp, WSD, whose etiologic agent is white spot syndrome virus (WSSV), has deeply impacted shrimp cultivation worldwide, causing massive mortalities of shrimp since 1993 (Lo et al., 1996). According to Wang et al. (2005), WSD is a major limiting factor to the development of shrimp farming. Since the appearance of WSSV, massive technical efforts, associated to the development of research and studies addressing the etiological agent, the investigation of potential hosts, the molecular basis of the infection as well as the development and standardization of diagnosis tools have been performed. Recently, a study conducted by Müller et al. (2010) with WSSV geographical isolates has shown that that WSSV isolates from Brazil are different from WSSV isolates from other countries in the Americas. This study has also pointed out differences between WSSV isolates found in two regions in Brazil, Bahia and Santa Catarina.

WSSV is extremely virulent and infects tissues of ecto-and mesodermal origin, such as the cuticular epithelium, connective tissue, and hematopoietic tissues (Chang et al. 1996; Shi et al., 2005). WSD can be characterized by the presence of white inclusions in the cuticle and was recorded in different penaeid species (Lightner, 1996). The mortality rate is very high and can reach 100% after 3 to 10 days of the onset of clinical signs (Lightner, 1996; He et al, 2006) and unlike most viruses, has a great affinity for indiscriminate number of families of crustaceans (Zhang et al., 2006).

The emergence and rapid spread of diseases in cultured organisms, including crustaceans, are directly related to environmental degradation. Farmed shrimp, subjected to high stocking densities, nurseries, degraded soils and reduced water quality; experience a high

level of stress that can predispose the organism to a wide range of diseases (Roch, 1999, Hernández, 2000). Other environmental factors, such as temperature and pH, low dissolved oxygen concentrations, abrupt changes in salinity and the presence of toxic endogenous compounds or xenobiotics may act as stressors (Nunes and Martins, 2002; Jiang et al, 2004).

In Santa Catarina, Brazil, when mortality in cultivated shrimp occurred in late 2004, at first, producers and technicians believed the events were directly related with the use of agrochemicals in crops of rice located in areas surrounding shrimp farms.

Nonetheless, as far as marine crustacean are concerned, there is a lack of data supporting the hypothesis according to which environmental changes induce alterations in the immune system, leading to increased susceptibility to infectious agents (Le Moullac and Haffner, 2000; Bachère, 2000). On the other hand, several studies have aimed to identify and monitor the presence of pathogens in aquatic animals, especially those with economic relevance. At present, analysis by hybridization and PCR methods offer greater sensitivity of detection of some penaeid viruses, such as WSSV (Peinado-Guevara, Lopez-Meyer, 2005, Powell et al., 2006, Souza, 2008) and have been employed in routine laboratory tests for purposes of diagnosis, certification processes, selecting of spawning and also in quarantine surveys (Lamela, et al, 2004; Moser et al, 2011). Recent studies have been conducted in order to identify genes involved in the processes of virus infection (Gomez-Anduro, et al. 2006; Clavero-Salas et al., 2007; Muller, 2010). From the molecular characterization of these genes and construction of specific primers for their amplification it is possible to investigate their pattern of expression in the face of different environmental conditions and stressors (Tavares-Sanchez et al. 2004; Zhang et al., 2007).

It is possible to evaluate the effects caused by contaminants, as well as the effects originated by the process of viral infection, by measuring the activity and expression of enzymes and proteins involved in cellular defense mechanisms. Biochemical changes are the first response of the biological defense action and represent the molecular basis of toxicity (Walker et al, 1996). The investigation and characterization of these responses, combined and applied to the diagnosis and identification of genes involved in molecular defense responses and related to desired zootechnical parameters, represent an important strategy to improve not only shrimp cultivation, but the whole aquaculture activity (Pan et al., 2005).

Therefore, molecular biomarkers can be used as tools for monitoring the impact of cultivation conditions in shrimp health and sanitary status, as well as in other aquatic crustaceans (Tu et al., 2010). Thus, the present study was focused on evaluating changes in the expression of cellular defense genes and immunecontent of proteins in gills of juvenile *Litopenaeus vannamei* exposed to permethrin and infected with WSSV.

2. Materials and methods

2.1. Animals

Juvenile intermolt shrimp (8,5 ± 2cm in body length) were obtained from a local shrimp farm in Northern of Santa Catarina State (Brazil), maintained in a 6000 L fiberglass tank, with water temperature at 23±1°C and salinity of 20 $g.L^{-1}$. Shrimp were held for a week to acclimate to laboratory conditions prior to experimental use. During this period, shrimp were fed once daily with commercial shrimp feed. Feeding was stopped 24 h before treatment.

2.2. Selection of animals

Shrimp were analyzed previously in order to detect genomic sequences of Infectious Hematopoietic Necrosis Virus Hypodermal (IHHNV) and White Spot Syndrome Virus (WSSV). Analyses were performed prior to the experiments using hemolymph samples. The hemolymph (100 µL) of each animal were collected separately and applied directly on FTA card (WHATMAN). DNA extraction was performed according to the manufacturer's protocol (FTA Technnology) and samples were then submitted to nested PCR using primers described by Moser (2005) and Lo et al. (1996), respectively. Only animals negative for WSSV and IHHNV were used in the experiments.

2.3. Experimental design

Pesticide for the experiments was prepared from a permethrin (SIGMA) stock solution in filtered sea water. After acclimation, selected shrimp were exposed to permethrin (0.8 $\mu g.L^{-1}$) and sampled after 1, 2 and 7 days of exposure. At the end of this procedure, 10 shrimps were placed in permethrin-free water and kept for another 7 days, to be further analyzed as a depurated group.

After 7 days of permethrin exposure, surviving shrimp were challenged with WSSV by injection. Inoculum was prepared as follow: WSSV-infected *L. vannamei* tissues were diluted 1:5 (w/v) with sterile

PBS buffer (pH 7.8) and homogenized. The homogenate was clarified by centrifugation, filtered (0.45 µm filter) and used for injecting experimental animals. The concentration of WSSV inoculum was assessed by real-time PCR. Shrimp were injected intramuscularly into the third dorsal segment with 50µL of WSSV inoculum (8.6×10^2 copies/µL) using a sterile 1 mL syringe fitted with a 21G needle. The control group was injected with the same volume (50µL) of PBS buffer.

After challenge, shrimp were monitored for survival, pattern of behavior, mobility and body coloration. Shrimp were collected after 7 days of permethrin exposure and in 1 and 2 days after WSSV challenge. The gills were excised and immediately frozen in liquid nitrogen for further analysis. Pleopods were excised and used to determine viral load in WSSV-injected shrimp.

2.4. DNA extraction and WSSV qPCR analysis

DNA extraction was performed according to Maciel (2002), in WSSV-infected shrimp pleopods. Viral load of positive samples was performed with Quantitec SYBR green Kit (QIAGEN), as follow: initial denaturation step at 95°C for 5 min and then 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 10 s. The set of primers used in the reaction were designed based in the sequence amplified by the nested PCR reaction described by Lo et al, 1996: WS2F:5'-TGCCTTGCCGGAAATTAGTGTGTG-3' and WS2R:5'ACAACATCCAACAATGGTCCCGTG-3).

2.5. RNA extraction and cDNA synthesis

Total RNA was isolated from gill samples using the Trizol method (Invitrogen) according to the manufacturer's instructions. Extracted RNA samples were subjected to DNA-free (DNase) treatment to prevent genomic DNA contamination. The cDNA synthesis was performed using the reverse transcription system Omniscript RT Kit (Qiagen), following the protocol recommended by the manufacturer.

2.5.1. Quantitative real-time PCR assay

Real-time quantitative (qPCR) was used to evaluate the expression levels of target genes in gills of shrimp subjected to permethrin stress and WSSV-infected. Genes were selected partially based on a previous study conducted by Muller, 2009. The specific primers designed accordingly are listed in Table 1. Amplifications were carried out with Quantifast SYBR green Kit (QIAGEN) in a total volume of 20 µL containing 10 µM forward and reverse primer and 100

μg of cDNA. Real-time PCR was carried out with a Rotor-gene 6000 real-time PCR machine (QIAGEN). The cycling parameters used were as follows: an initial denaturation at 95°C for 5min, 35 cycles at 95°C for 10s, 53 to 56°C for 30s. Melting curve analysis and electrophoresis gel of amplification products was performed to confirm the primer specificity. The cDNA of shrimp was used for analysis in qPCR, run in triplicates. 18S ribosomal from decapods (DEC) and L8 transcript levels were used to normalize samples. The relative quantification of gene expression among the treatment groups was analyzed by the ΔΔCt method (Livak and Schmittgen, 2001) and by REST software (QIAGEN). For each assay, standard curves of target gene were obtained by using serially diluted pooled cDNA.

2.6. Tissue preparation

Gill homogenization was performed in Tris-HCl 0.02 M, 0.25 M sucrose, 2 mM DTT, 0.1 mM PMSF (pH 8.0) buffer, cooled, using the Glas-Col homogenizer (Terre Haute, USA). For the protein detections homogenized tissues were centrifuged at 20.000xg for 20 minutes at 4°C. The supernatants were stored at -80°C for analysis. The total soluble protein content was determined by the Lowry method modified by Peterson (1977), using bovine serum albumen as standard.

2.6.1. Immunedetection of proteins (Western Blotting)

Gills homogenates at protein concentration range from 30 to 60μg.μL^{-1}, were subjected to poliacrylamide gel electrophoresis (PAGE) under denaturating conditions (0.1% SDS), according to the method of Laemmli (1970). Following electrophoresis, proteins were transferred to nitrocellulose membrane (Hybond, ECL). The membranes were incubated for 2 h with anti-β-actin (1:5000), anti-PKCξ (1:3000), anti-caspase-3 (1:250), anti-Hsp60 (1:3000), anti–Hsp70 (1:3000) and anti–Hsp90 (1:3000), followed by incubation with AP-conjugated, anti-mouse or anti-rabbit (1:2000) secondary antibodies (GE Healthcare or Calbiochemistry).

The immune complex was detected by chemiluminescence with Luminol reagent and densitometry of immunoreactive bands was performed with Gel Quant software (AMPL). All measurements were made in duplicate. The results were subjected to analysis of variance (ANOVA) followed by Tukey test, performed by GraphPad software.

Table 1: Primers used in quantitative real time PCR - qPCR

Target gene	Primer sequences
Ribossomal L8 (housekeeping)	5′-TAGGCAATGTCATCCCCATT-3′ 5′-TCCTGAAGGGAGCTTTACACG-3′
Ribossomal 18S (housekeeping)	5'-CCGAATGGTCGTGCATGGAATGAT-3' 5'-GAATTTCACCTCTAGCGTCGCAGT-3'
Ferritin	5'-CAAGTCCGCCAGAACTAC-3' 5'-TGGCAAATCCAGGTAGAG-3'
Ubiquitin	5'-AGAAGGCCAAGATCCAGGATAAGGAG-3' 5'-AGTCAGACAGAGTGCGACCATCTT-3'
β-Actin	5'-GCCCATCTACGAGGGATA-3' 5-GGTGGTCGTGAAGGTGTAA-3'
Protein Kinase C	5'-GTGTCCTGTGTAAGGTTCTC-3' 5'-CCATAGTGGTTGGTCTTGAG-3'
Caspase	5′-CAAAGTCACCAGGGAAGTAG-3' 5'-GAGTGTCGAGTGGATGTAAG-3'
QM protein	5'-GGCATCTCACGGACATCGGACTTC-3' 5'-ACCCCAAGTCGCGTTTCTGTCGT-3'
Hsp60	5'-CAGTGGTGAAGGTTGGGAGGT-3' 5'-TGTGGCATGGAGTGTGGATA-3'
Hsp70	5'-TTCATGATATTGTGCTGGTGGGCG-3' 5'-ACCGCTTCATCCGGGTTAATGCTT-3'
Hsp90	5'-GCATGAAGGAGAACCAGAAGCACA-3' 5'-TGAACGCAGTATTCGTCGATGGGT-3'

3. Results

3.1. Effects of permethrin exposure: mortality, general behavior pattern, body coloration and viral load

Dead shrimp were found in sub-chronic stress by permethrin exposure (7days - 0.8 $\mu g.L^{-1}$). Mortality started at the fourth day and continued until the end of experimental period of exposure. Also, typical signs of stressed shrimp, such as red coloration of body appendices and erratic swimming, were observed during the exposure to the contaminant stressor.

Shrimp injected with WSSV started to show clinical signs, such as lethargy, from 30 to 48 hour post infection. Mortalities were seen in WSSV challenged group after 2 days from the beginning of the experiment. Experiment was ended three days after WSSV challenge, since all shrimp were dead between the second and the third day post virus injection.

In shrimp infected with WSSV after permethrin exposure, besides the restless swimming observed in earlier hours post-infection, changes

in body color were observed. Shrimps showed a reddish color in body appendices, which turned to dark-red at the second day pos-infection.

All dead shrimp, sampled after virus injection, were WSSV-positive. Moreover, significant differences ($p<0.05$) were seen in the viral load of WSSV-positive shrimp, according to the time interval following WSSV infection, e.g., one and two days. The average number of viral copies after one day of WSSV injection was 5.8 x10 copies/µl DNA, whereas after two days, the average number of viral copies in infected shrimp was 2.3 $x10^2$. No viral copies were detected in control shrimp.

3.2. Effects of permethrin exposure on the transcription levels of target genes in non-infected and WSSV-infected *L. vannamei*

Nine genes were selected and their transcription levels were evaluated by qPCR in gills of shrimp reared under permethrin exposure, followed by WSSV challenge (Fig 1).

After one and two days of exposure to permethrin, relative gene transcription profile in shrimp showed a significant induction in the transcription of the gene enconding *PKCξ* (5.4 and 6.6 fold, respectively). In contrast, transcription of *β-actin* gene was apparently slight inhibited after one day (1.8 fold) and two days (1.4 fold). *Ferritin* transcript levels were significant induced (5.5 fold) two days after permethrin stress. In addition, *Hsp60* gene was 5.0 fold, whereas *Hsp90* was 1.97 fold higher than control, on the second day of exposure.

Relative expression of *Hsp70* was seen slight inhibited after one day of exposure to permethrin. The apparent stress induced by permethrin exposure induced *QM protein* (2.7 fold) and *Caspase-3* (2.4 fold) at the end of the first day of exposure. At two days of exposure a significant induction in the transcription of the gene encoding *caspase-3* (7.9 fold) was observed. Surprisingly, no changes were seen in the *Ubiquitin* gene transcripts.

On the other hand, WSSV-infection after permethrin sub-chronic exposure induced the relative expression of the gene coding for *Ubiquitin*. One day after challenge with WSSV, *Ubiquitin* transcript levels were 4.5 and 9.9 fold higher when compared to samples from shrimp that were only exposed to permethrin (after one and two days, respectively). *β-actin* (5.0 fold) and *Ferritin* (7.2 fold) transcripts were also induced after WSSV challenge, but no significant changes were seen in these same transcripts after two days of WSSV infection.

No changes were seen in neither *PKCξ* nor *Hsp60*, whereas, two days after WSSV-injection, shrimp gills displayed an induction of

Hsp70 (1.3 fold) and an inhibition of *Hsp90* (2.0 fold) when compared with control shrimp.

Relative expression of *QM protein* gene was induced (2.3 fold) in WSSV-infected shrimp after two days, and a strong induction was observed in C*aspase-3* gene (14.9 and 7.9 fold, after one and two days, respectively).

In shrimp stressed by permethrin exposure for 7 days, the transcript levels of *ubiquitin* (2.0 fold), *β-actin* (2.0 fold), *PKCξ* (1.6 fold), as well as the chaperones *Hsp60* (1.8 fold), *Hsp70* (1.6 fold) and *Hsp90* (1.6 fold) were significant induced when compared to the control group (Fig 2). The main induced genes were *Ferritin* (8.0 fold) and *caspase-3*, that displayed the highest relative expression when compared to shrimp in control group (24 fold higher than control).

After depuration (recovery time) of shrimp exposed to permethrin for seven days, the relative transcriptional analysis revealed significantly inhibition in *Ferritin*, *PKCξ* and *Hsp70* (1.2, 2.5 and 3.9 fold, respectively). However, the levels of the other addressed genes were not significantly different when compared to control shrimp.

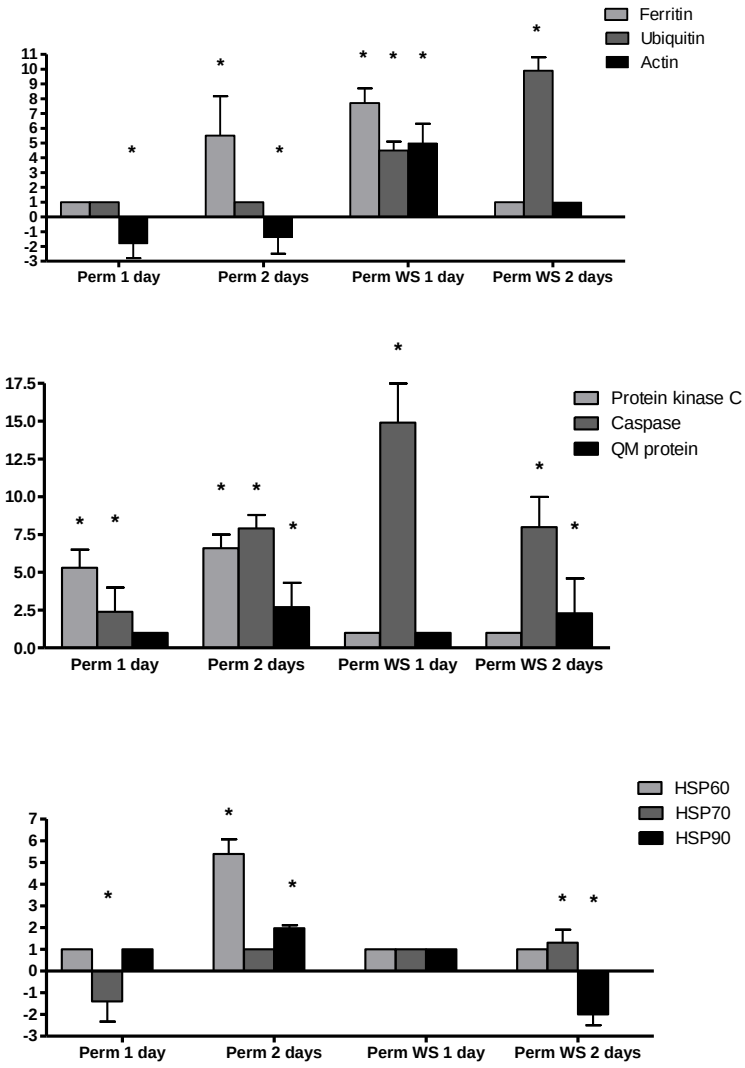

Figure 1: Effects of permethrin exposure in the transcription levels in gill of *L.vannamei* infected or not-infected with WSSV. Significant differences between treatment and control are indicated with superscripts (* $p<0,05$).

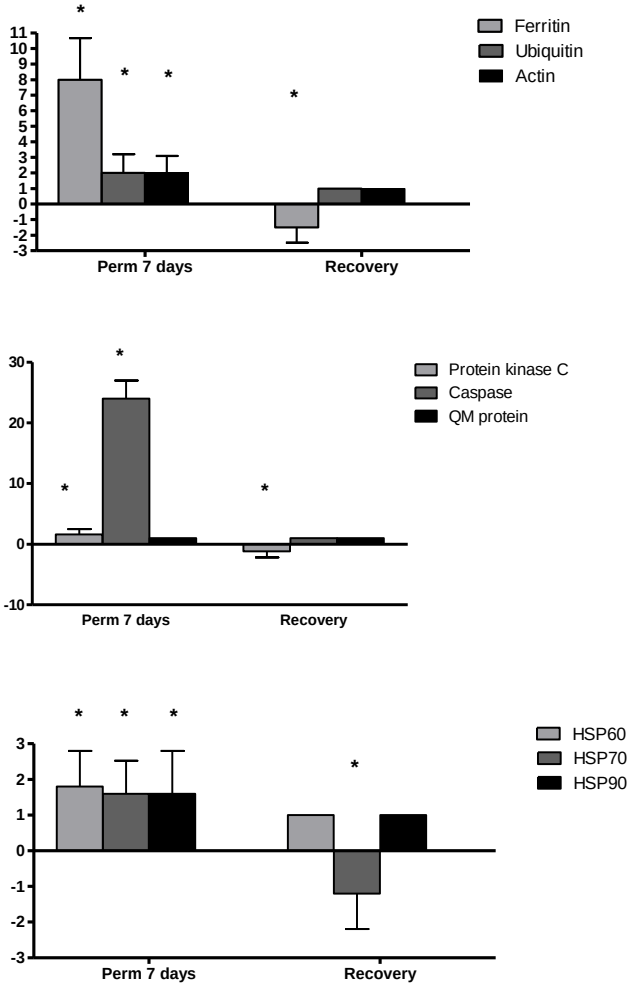

Figure 2: Effects of permethrin in the transcription levels of target genes in gills of *Litopenaeus vannamei* after sub-chronic exposure (7 days) and after recovery (7-day depuration in permethrin free-sea water). Significant differences between treatment and control are indicated with superscripts (* $p<0,05$).

3.3. Immunedetection

Expression levels of some proteins were investigated by the detection of specific immunecomplexes in gills of shrimp exposed for one and two days to permethrin and after challenge with WSSV.

In gill homogenates immunereactive bands were detected by cross-reactivity with specific antibodies, as seen in Figure 3, corresponding to the presence of β-actin (45 kDa), PKCξ (80 kDa), Hsp60, Hsp70, Hsp90 (60, 70 and 90 kDa, respectively) and caspase-3 (35 and 28 kDa).

Exposure to permethrin and challenge with WSSV were able to promote significant changes in Hsp60 and Hsp70 expression levels in treated shrimp (Table 2). Hsp70 expression was evidently increased after one and two days of exposure, as well as after two days upon WSSV challenge. On the contrary, Hsp60 was altered only in WSSV positive shrimp. Changes in Hsp90 levels were observed after exposure to permethrin in both groups, one-day and two-day exposed shrimp.

The expression of PKCξ, whose levels were significantly higher in shrimp exposed to permethrin, has not changed in WSSV-challenged shrimp. In contrast, significant levels of Caspase-3 were detected only in WSSV-positive shrimp. No immunoreactive bands were seen in Western blots with samples of gills from shrimp exposed to permethrin.

No changes were seen in β-actin levels in exposed shrimp. On the other hand, an increase in levels of this protein was seen in shrimp sampled after 2 days of WSSV-injection.

In permethrin stressed shrimp, sampled after 7 days of exposure (sub-chronic exposure), the levels of β-actin and PKCξ were evidently increased. Immunoreactive bands of Hsp60, Hsp70 and Hsp90 also revealed significant induction, when compared to the levels in the control group. However, no significant changes were detected in protein levels in gills of shrimp submitted to a 7-day recovery period (depuration) following permethrin sub-chronic exposure.

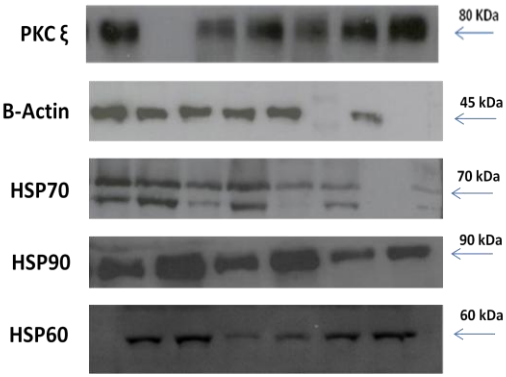

Figure 3: Immunereactive bands, corresponding to the presence of PKCξ, β-actin Hsp60, Hsp70 and Hsp90 proteins in gills of *Litopenaeus vannamei*.

Protein	Permethrin		Perm + WS		Permethrin	
	1 day	2 days	1 day	2 days	7 days	Rec
β-Actin	-	-	↑	↑	↑	-
Protein Kinase C	↑	↑	-	-	↑	-
Caspase	-	-	↑	↑	↑	-
HSP60	-	-	↑	↑	↑	-
HSP70	↑	↑	-	↑	↑	-
HSP90	↑	↑	-	-	↑	-

↑: the significant increase; ↓: the significant decrease; –: no significant changes, $p<0.05$.

Table 2: Expression levels of stress and defense proteins in gills of *Litopenaeus vannamei* exposed to permethrin and WSSV challenge and after sub-chronic exposure to permethrin (7 days) and after recovery (7-day depuration in permethrin free-sea water)

4. Discussion

The health of aquatic species is dependent on interactions between the environment, pathogens and the host itself. Diverse aspects related to each one of them can contribute to impact the interaction among pathogen and host. Farmed animals can coexist with potential pathogens with little or no impact on production (Bachére, 2003), while some pathogens dramatically impact production, like the WSSV. Hyperthermal, hypoxic or hyposmotic conditions, for instance, have been identified as triggers for the transition from chronic to acute viral infections in shrimp populations (de la Vega et al., 2004; Liu et al., 2006). This transition appears to be the result of a reduction in the shrimp's immunological and defense capacity (Hall et al., 1998; Le Moullac and Haffner, 2000). However, other potential environmental stress conditions, such as exposure to pesticide contamination, have been scarcely addressed the linkage between xenobiotic stressors and their impact on the health status of cultivated aquatic species.

Therefore, the present study aimed to focus on the changes in the transcriptional level of target cellular defense genes, as well on the expression of corresponding proteins in gills of *L.vannamei* exposed to permethrin and infected with WSSV. Based on previous studies of our group about the differential expression of genes in WSSV-naturally infected *L.vannamei* (Muller, 2009) and *L.vannamei* subjected to osmotic stress (Soares, 2011), we selected nine genes encoding proteins involved in responses to proteotoxicity, cellular signaling, cellular defense, cellular detoxification, protein modification, protein turnover and apoptosis.

Along the sub-chronic stress experiment of exposure to permethrin, mortality started at the fourth day and continued until the end of the period of exposure, at the seventh day. Mortality started after 2 days of challenging with WSSV. We hypothesized that permethrin effects on host defense responses as well as on viral load could be accelerating shrimp death. Infected shrimp have also reduced food consumption and showed lethargy and either general reddish color in the body or in the appendices. Similar signs have been considered as indicative of stressful conditions to shrimp (Durand et al. 1997; Morales-Covarrubias, 2004).

Ferritin is a storage protein that plays a key role in iron metabolism. The importance of ferritin is a consequence of its functional roles in a variety of biological processes, for instance, detoxification and

inflammation (Torti and Torti, 2002). As a result, ferritin is thought to play a key role in host defense responses against viral infection (Zhang et al, 2006), since it has been shown to enhance immunity, physiological responses and survival in WSSV challenged shrimp (Ruan et al, 2010).

We found ferritin transcript levels to be induced after stress caused by permethrin exposure. Besides, in WSSV-infected shrimp, ferritin transcripts also experienced induction. Thus, it is possible that ferritin is protecting shrimp cells since keeping tightly bound metals avoids free radical reaction (Grove and Wilkinson, 2005). Free radicals can be potentially generated in experimental conditions through toxic effects of permethrin or during its biotransformation, as well as in the defense immune responses against the virus. Pan et al. (2005) found that WSSV-resistant shrimp (*Penaeus japonicus*) showed an up-regulated expression of ferritin compared to normal shrimp. Zhang et al. (2006) used PCR to show that the expression of ferritin was up-regulated after shrimp (*Fenneropenaeus chinensis*) were challenged with either WSSV or heavy metal ions.

Muller (2009) identified in a WSSV-infected *P.monodon* EST library, the sequence of a receptor for activated protein kinase C, which also was up-regulated in her studies with WSSV-naturally infected *L.vannamei*. This gene is highly conserved and is involved in several processes, such as cell signaling, cell development, cell adhesion, growth and movement, as well as immune response. The protein product of this gene interacts with several viral proteins, such as WSSV protein VP9 (Tonganunt et al., 2009). In our study, protein kinase C expression was up-regulated after permethrin exposure, indicating a possible interaction between viral infection and permethrin.

Increased ubiquitin expression was seen in shrimp gills after sub-chronic permethrin exposure. Two days after WSSV-infection, expression of this protein was up-regulated (9.9 fold). Muller (2009) also reported a 14-fold increase in ubiquitin expression in WSSV-positive shrimp, naturally infected and collected in shrimp farms. Ubiquitin induction was also observed in other studies with shrimp (Wang et al., 2006, He et al., 2005).

Ubiquitin is a protein that takes part in the ubiquitin-proteasome pathway. This pathway is one of the main forms of protein degradation in the cell, taking part in several processes such as cellular progression, organelles biogenesis, transcriptional regulation, antigen processing, and apoptosis (Shen et al., 2009, Chen et al., 2008). It has been suggested that inactivation or overloading of the ubiquitin system leads to the induction of heat shock proteins. In addition, any denaturation of

proteins caused by stressful conditions, such as the presence of proteotoxic agents, is a common induction signal to induce a heat-shock response (Finlay et al, 1984, Robert et al., 2010). As seen in the present study, both defense systems were up-regulated in response to stress caused by permethrin and viral infection.

A number of studies have addressed the sensitivity of Hsp70 synthesis in response to environmental assault and showed that Hsp70 was induced by different mechanisms of toxicity and suggested the use of Hsp70 in mechanistic studies for the detection of toxic effects of certain pollutants such as pesticides (Kohler et al., 1992, Ceyhun et al, 2010) in response to cell stress (Ohashi, et al, 2000). According to the results, we observed that permethrin sub-chronic stress promoted changes in HSP70 and HSP90 expression. An increase in Hsp60 was seen after challenge with WSSV. Zhenyu et al. (2004) studied the distribution and induction of Hsp 70 in *Fenneropenaeus indicus* in response to various stressors. HSP70 genes in marine invertebrates have also been experimentally shown to decrease the mortality rate of certain organisms. The expression levels of Hsp70 and Hsp90 were increased as a part of an immune response against *Vibrio harveyi* in *P. monodon* (Rungrassamee et al. 2010). Xu et al. (2009a) propose that HSP70 is one of the binding partners of VP28 during virus infection and its expression was enhanced by WSSV infection at the early stage of the process.

HSP90 expression become markedly stimulated in stressful conditions, indicating that one of its main functions is to protect cells from damage (Csermely et al., 1998). In addition, this protein is associated with steroid receptors and protein kinases by forming specific complexes which bind to the responsive elements (RE) of gene promoter (Csermely et al., 1998; Gao et al., 2008). Zangh et al. (2009) suggested there were at least two Hsp90s in *P. trituberculatus*, which played different roles in physiological and stressful conditions.

Permethrin stress strongly up-regulated the expression of both QM protein and caspase-3. The elevated expression of caspase-3 was associated with the effects of permethrin contamination. Xu et al (2009b) reported that trichlorfon, an organophosphorus pesticide, increased intracellular reactive oxygen species and caused cytochrome c release, leading to caspase-3 activation. This is one of the most well defined pathways for caspase activation by translocation of the respiratory chain protein, cytochrome c, from mitochondria to the cytosol. Induction of cellular death via apoptosis, due to bacterial exposure, was seen in *P monodon* hemocytes (Sung et al, 2003).

Moreover, increase in caspase-3 transcripts were seen after permethrin exposure and WSSV challenge, followed by shrimp mortality. These finding supports a putative link between caspase-induced apoptosis and death. Oxidative stress appears to be the central key in the regulation of apoptotic pathways triggered by environmental stressors (Francoa et al., 2009).

Besides the findings related to QM protein in naturally-infected *L. vannamei* reported by Muller (2009), Soares (2011) showed a significant up-regulation of QM protein, influenced by hyposmotic stress either with or without WSSV challenge. Induction of this protein by WSSV infection has 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). QM proteins are highly conserved and play a role in cell growth, differentiation and apoptosis (Green et al. 2000; Lillico et al. 2002).

In conclusion, permethrin exposure triggered changes in the transcription pattern of target genes encoding proteins involved in responses to proteotoxicity, cellular signaling, cellular defense, cellular detoxification, protein turnover and apoptosis. The transcription of some of these genes was also impacted by WSSV infection. These results found a counterpart in the expression of some of the corresponding proteins in both stressful situations. Based on these results, potential molecular biomarker candidates can be selected and validated in further studies to monitor the health status of cultivated shrimp and to select shrimp less susceptible to environmental stressors, which may lead to minimize the negative impact of contamination and viral disease in the future.

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CAPÍTULO 3

Water temperature influences viral load and detection of white spot syndrome virus (WSSV) in *Litopenaeus vannamei* and wild crustaceans

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Abstract

The Pacific white shrimp *Litopenaeus vannamei* is particularly affected by White Spot Syndrome Virus (WSSV) as this virus can cause high mortality in infected populations. Presently, there are no known treatments for shrimp affected by WSSV and management tools for preventing this disease are limited to the exclusion of the virus from cultured shrimp populations. Previous studies have shown that warm-water culture conditions inhibit the replication rate of WSSV, as well as two other important shrimp viruses in *L. vannamei*. The purpose of this study was to evaluate the effect of thermal stress on the replication rate of WSSV in shrimp held in warm water (29±0.5°C), compared to the replication rate of WSSV in shrimp held in cool water (18±0.5°C), looking for improve virus detection in epidemiological programs. Furthermore, post larvae and captured wild crustaceans were screened for the WSSV after being held in warm water for 2 days (48h). The results indicate that water temperature had a profound effect on the replication rate of WSSV in *L. vannamei* and a protocol for WSSV screening after thermal stress is proposed. Our results support the findings of previous studies and further point out to the potential application of environmental temperature as a management strategy to selecting WSSV-free spawning shrimp within the shrimp farming industry in Mexico and possibly in other producing countries.

Key words: *Litopenaeus vannamei*, host crustaceans, WSSV, temperature, infectivity assay

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1. Introduction

Shrimp diseases have caused significant losses of production and jobs, reduced earning, export restrictions, failure and closing of business and decreased confidence of consumers (Bondad-Reantaso et al., 2005). White Spot Syndrome Virus (WSSV) is now one of the most devastating and virulent viral agents threatening the penaeid shrimp culture industry. This virus has been causing high mortality and huge economic losses in shrimp aquaculture worldwide. Shrimp cumulative mortality can reach 100% within 3 to 10 days under farming conditions (Chou et al., 1995; Lightner, 1996). WSSV has been detected in a wide range of wild crustaceans including penaeid and non-penaeid shrimp, as well as crabs and lobsters (Escobedo-Bonilla et al., 2008; Small and Pagenkopp, 2011).

The first WSSV epidemic was reported in shrimp farms of South East Asia in 1992 (Chou et al., 1995). The virus then spread to shrimp farms in countries in Asia, North, Central and South America and Middle East (Lightner, 1996; Rosenberry, 2002; Flegel, 2006). The most recent outbreak in a WSSV-free area was in Brazil (Seiffert, 2005). Until now there are neither treatments, nor vaccines, for WSSV eradication, and prevention or control through reliable diagnostic procedures is the first defense barrier against this pathogen (Roch, 1999; Bachère, 2000). Continuous and strict monitoring of the various components of shrimp farming is required to reduce the spread of WSSV within a region and to avoid the introduction of the pathogen into a new area. Moreover, such monitoring measure can also contribute to improve the design of sanitary and management strategies to minimize the negative impact of the disease on shrimp production. However, either persistent, very low level infections or virus latency in shrimp and other crustaceans can occur, sometimes at levels that are not detectable, even by the most sensible PCR procedure (Walker and Winton, 2010).

The amplification of viral loads and onset of disease can be induced by environmental or physiological stress or ambient temperatures (Lotz et al., 2005; Sanchez-Martinez et al., 2007). Optimum temperature for growth and survival of shrimp varies according to the life stage and the species. For *Litopenaeus vannamei*, for example, optimum temperature ranges from 27°C to 30°C (Wyban et al, 1995). Highest survival of juvenile *L.vannamei* is obtained between 20°C and 30°C (Ponce-Palafox et al., 1997). Vidal et al. (2001) discovered that warm-water (32°C) conditions provided a workable way

to control mortalities of *L. vannamei* from WSSV. Mortality of WSSV infected shrimp or crayfish was reduced or even totally stopped at a higher (32-33°C) or lower (<15°C) water temperature in comparison to the optimum temperature range (Guan et al., 2003; Jiravanichpaisal et al., 2004; Du et al., 2006). At optimum temperature (26-27 °C), differences in virulence between WSSV strains have been reported (Wang et al., 1999, Rahman et al., 2006). Guan et al. (2003) reported that viral concentration was lower at 15°C than at 23–28°C. The suggested mechanisms to explain this findings include reduced replication (Du et al., 2006), apoptosis (Granja et al., 2003; Granja et al., 2006) and altered gene expression of WSSV (Reyes et al., 2007). WSSV replication was also inhibited at 4°C and at 32°C in primary culture of hematopoietic tissue of crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2006).

Previews experiments conducted by Dr. Magallón and colleagues (not published) and field observations on temperature fluctuations and WSSV outbreaks in Mexico in the past five years showed that WSSV infection in northern areas of the country occurs when water temperatures display daily oscillations in the range of 26-30°C. One can speculate that perhaps this range of temperature falls within the optimum to viral proliferation and therefore, viral spread among shrimp farming areas in Mexico. On the other hand, at temperatures above 20°C WSSV could not be detected which could be keeping the virus in wild populations.

In 2010, almost all of Sonora State's shrimp farms had WSSV infection during the farm production cycle between May and November. During the cold period in the Gulf of California, from December 2010 to March 2011, all shrimp farmers agreed to suspend their operations and maintain empty all ponds and reservoirs, with no exception, in order to improve their sanitary status. During this dry sanitary period in farms, shrimp were collected in some channels outside the farms which are at an average temperature of 20°C. These shrimp were analyzed for the presence of WSSV but the results have been negative in all cases. However WSSV-positive wild organisms were detected in six areas close to shrimp farms. These WSSV reported cases clearly indicated a risk related to the permanence of this viral agent in host crustaceans found in surrounding areas. The virus may move outside the pond system by apparent healthy carriers and infect other organisms in the environment from where it could be introduced back to shrimp ponds during filling process in the next farm cycle.

Based on the foregoing, it is necessary to test the hypothesis that organisms maintained at temperatures below 20°C may contain the virus but its presence is undetectable, making diagnosis difficult. In an effort to contain the spread of this infectious agent in farms and hatcheries, the aim of present study was to evaluate the effects of temperature on WSSV replication. We propose the application of selective temperatures during the cold period before undertaking any WSSV diagnosis, called hyperthermic stress in wintertime, as a management strategy oriented to the selection of spawning shrimp and to a continuous and strict monitoring of the various wild organisms found outside shrimp farming.

2. Materials and Methods

2.1. Sample collection

Adults of marine shrimp *Litopenaeus vannamei* were collected from areas close to shrimp farms of Sonora (Mexico) during the sanitary dry period and maintained in a 3000 L tank, with water temperature at 18±1°C and salinity of 34 g L^{-1} at the facilities of the laboratory of Centro de Investigaciones Biológicas del Noroeste (CIBNOR), campus Hermosillo. Shrimp were tested for WSSV, Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Taura Syndrome Virus (TSV) and Yellow Head Virus (YHV). Only negative shrimp were used for the experiments.

Shrimp in post larvae stage were collected from a shrimp hatchery laboratory in Sonora and maintained in 60 L containers. Other marine crustaceans (crab, blue crab, red crab and shrimp) were collected from various shrimp ponds, breakwater and canals. Figure 1 shows Sonora State and the location of animal sampling. All the animals were brought to the laboratory of CIBNOR Hermosillo, and reared in 60 L plastic containers. The period of sampling was from December 2010 to March 2011.

2.2. Inoculum preparation and injection procedure

Approximately 15 g of WSSV-infected *L. vannamei* tissue were diluted 1:5 (w/v) with sterile PBS buffer (pH 7.8) and homogenized. The homogenate was clarified with two centrifugation cycles, 10000 ×g for 15 min and 15000 ×g for 20 min. The homogenate was filtered in a 0.45 µm filter and used for injecting experimental animals. The concentration of WSSV stock was assessed by real-time PCR. Shrimp were injected intramuscularly into the third dorsal segment with 50µL of

either PBS buffer or WSSV inoculum (6.2 x 10^3 copies/µL) using a sterile 1 mL syringe fitted with a 21G needle.

2.3. Experimental design

2.3.1 Effect of water temperature on viral replication

A total of 64 *Litopenaeus vannamei* adult specimens (average weight of 24.2 ± 1.4 g) were housed in 60 L plastic container with natural seawater (salinity of 34 g L^{-1}) and equipped with aeration and water heaters. Shrimp were kept at two water temperatures: warm water (29±0.5°C) and cool water (18±0.5°C). For the hyperthermic treatment, water temperature was raised from 18 to 29°C in 6 hours (at a rate of 2°C/h), and shrimp were kept at this elevated water temperature for 24 h. At the end of this period, shrimp were divided into four groups (six animals per tank, in triplicate): saline-injected shrimp held in warm water (29±0.5°C), saline-injected shrimp held in cool water (18±0.5°C), WSSV-injected shrimp held in warm water (29±0.5°C), and WSSV-injected shrimp held in cool water (18±0.5°C). After 72h, surviving shrimps from cool water were reared in warm water for another 72h. Water temperature was raised at a rate of 2°C/h. As control, a group was maintained in cool water. A commercial shrimp diet was provided daily. Hemolymph samples were collected at 0, 24, 48 and 72 hours post-inoculation (hpi) and post-water temperature increase to determine the viral replication by real time PCR. Shrimp were observed for clinical signs including anorexia and lethargy and mortality was recorded every 24h till the end of the experiment.

2.3.2. WSSV detection on post larvae and wild crustaceans held in warm water

A total of 207 crustacean specimens (Table 1) and approximately 600 post larvae were kept in 60 L plastic containers with artificial seawater (salinity of 34 g L^{-1}), equipped with aeration and water heaters, and initial temperatures was set at 18±0.5°C. Water temperature was raised from 18 to 29°C (at a rate of 2°C/h). Samples were collected at time 0 and 48h post-water temperature increase. In case of shrimp an additional sample was taken after 72 hours at thermal stress.

2.4. DNA extraction and WSSV qPCR analysis

DNA was extracted from hemocytes samples using a silica matrix (GeneClean Spin Glass Milk - MP Biomedicals, Inc) with slight

modifications of the manufacturer's protocol. Hemolymph (300 µL) was withdrawn from the ventral sinus, with a 1 mL syringe and a 21G needle containing anticoagulant solution (EDTA 20 mM, KCl 10 mM, NaCl 450 mM, HEPES 10 mM), proposed by Vargas-Albores et al. (1993) and transferred into a 1.5-mL centrifuge tube. Hemocytes were separated from the plasma by centrifugation at 9000 xg for 5 min and the plasma supernatant was discarded. In case of other crustacean, hemocytes, gills or whole body were used. Lyses buffer was added to the tissues. The solution was homogenized in fastprep equipment and the upper phase was recovered and added to a GeneClean silica matrix, vortexed and centrifuged at 6000 xg for 5 min. Two wash steps were performed before the nucleic acids were recovered in biology molecular water. The concentration of total nucleic acids was calculated by measuring the optical density (OD) at a wavelength of 260 nm using a Nanodrop spectrophotometer reader. Real time PCR (qPCR) using iQ SYBR Green Supermix (BIORAD) for WSSV detection was performed according to a protocol developed at CIBNOR. The amplification was performed in a Rotor-Gene thermocycler (Corbett Research), including vp28 primers (VP28F 5' – CTGCTGTGATTGCTGTATTT and VP28R 5'- CAGTGCCAGAGTAGGTGAC), with an initial denaturation step at 95°C for 5 min and then 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 10 s (acquiring), followed by a melt test from 72 to 95°C. The quantification of positive samples was performed with WSSV IQReal kit, as follow: initial denaturation step at 95°C for 5 min and then 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 10 s.

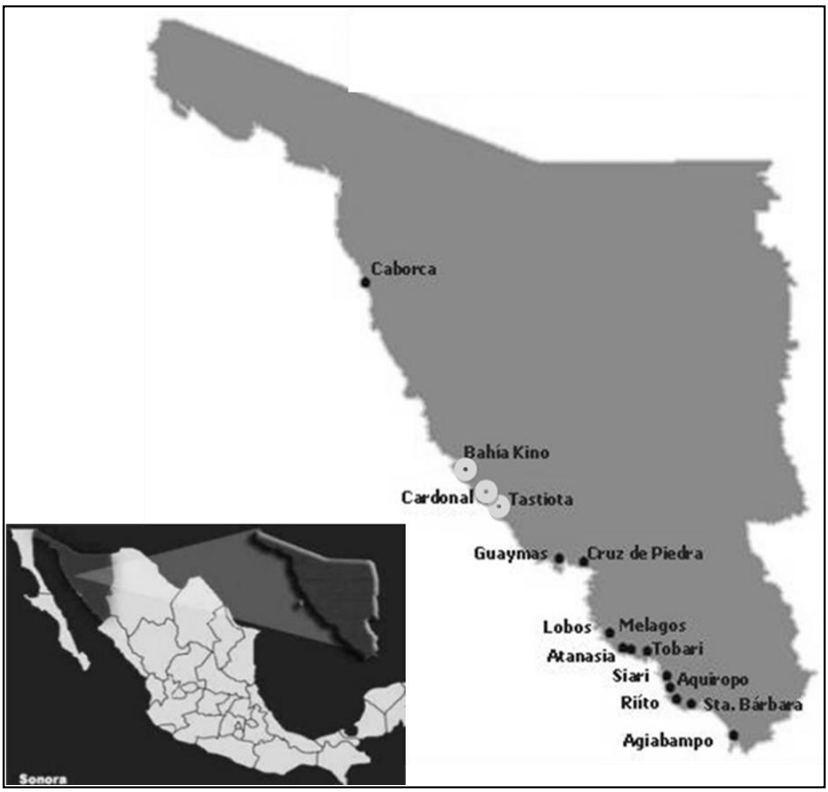

Figure 1: Sonora State in Mexico country and the location of animal sampling Bahía Kino, Cardonal and Tatiota.

3. Results

3.1. WSSV challenge and temperature effects on WSSV load

Shrimp inoculated with WSSV started to show clinical signs as lethargy from 24-36 hpi. Mortalities started from 36 hpi in WSSV-injected shrimp held in warm water and 48 hpi in WSSV- injected shrimp held in cold water. The cumulative percent mortality for *L. vannamei* is shown in Figure 2. WSSV caused 16%, 55% and 100% mortality of shrimp held in warm water, at 24, 48 and 72 hpi, respectively. WSSV-injected shrimp held in cold water showed 0, 11% and 22% of mortality in the same time period. All dead shrimp were WSSV-positive. Significant differences ($p<0.05$) were observed in the viral load of saline-injected shrimp and WSSV-injected shrimp held in both water conditions. Moreover, significant differences ($p<0.05$) were also seen in the viral load between WSSV-injected shrimp held in warm water and WSSV-injected shrimp held in cold water.

At 24 hpi, the average number of viral copies in injected shrimp held in warm water was 6.92×10^3 copies/µL DNA while no viral copies were detected in WSSV-injected shrimp kept in cold-water. At 48hpi, WSSV-injected group had an average number of viral copies of 7.75×10^5 in shrimp held in warm water versus 6.67×10^2 (copies/µL DNA) in shrimp held in cold water. At 72 hpi, all shrimp in hyperthermic condition died while WSSV-injected shrimp in cold-water had 2.52×10^3 copies/µL DNA.

After 72 hpi, among the 20 shrimp that survived WSSV challenge, 10 shrimp were brought to 29±0.5°C and 10 were held at 18±0.5°C. WSSV caused 16%, 33% and 100% mortality of shrimp held at 18°C water, at 96, 120 and 144 hpi, respectively. Among survived shrimp held in water temperature raised to 29°C, the cumulative mortality was 0, 16% and 100%, at 72, 96 and 120 hpi, respectively. After 72h both shrimp groups were blooded and tested for WSSV. The results showed that the group held in cold water had an average of 1.29×10^4 copies/µL DNA versus 1.41×10^5 copies/µL DNA displayed in the group held in warm water. Between 96-120h all shrimp maintained in hyperthermic condition were dead while WSSV-injected shrimp kept in cold-water shrimp had 8.59×10^4 copies/µL DNA.

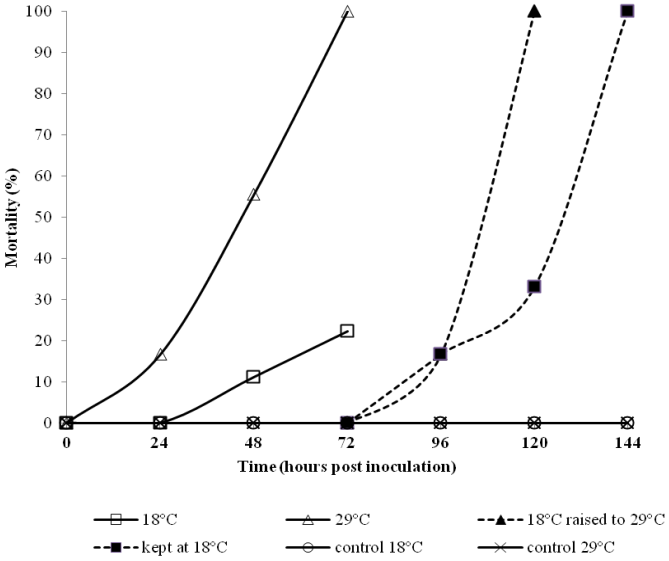

Figure 2: Cumulative mortality of *Litopenaeus vannamei* intramuscularly inoculated with WSSV and held either in warm water (29±0.5°C), cool water (18±0.5°C), survives at 18±0.5°C brought to 29±0.5°C and controls.

3.2. Hyperthermic stress to WSSV detection on wild crustaceans

A total of 207 marine organisms sampled in shrimp pond areas were tested for WSSV by qPCR. In 3 cases, WSSV negative wild shrimp at time 0 were positive in the second and third qPCR evaluation, after 48 and 72h at 29°C, respectively. These shrimp (*Farfantepenaeus californiensis*, *Litopenaeus vannamei*, *Litopenaeus stylirostris*) were sampled in distinct areas in December 2010, four months after the WSSV outbreak. Quantification analysis showed an average number of viral copies of 2.51 $x10^6$ in these shrimp after 72h held at 29°C. No WSSV positive crab, blue crab or red crab samples were detected at time 0 or after 48h at 29°C.

Table 1: Wild crustacean collected from shrimp ponds, breakwater and canals and held in warm water (29±0.5°C).

Organisms	Species	Life stage	Number
shrimp	Pacific blue shrimp (Litopenaeus stylirostris)	juveniles	11
	Pacific white shrimp (Litopenaeus vannamei)	adults, juveniles and larvas	36
	Brown shrimp (Farfantepenaeus californiensis)	juveniles	17
	(Macrobrachium tenellum)	adults, juveniles	24
blue crabs	Callinectes sapidus	adults and juveniles	84
crabs	Cancer sp.	juveniles	12
	red crab (langostillas)	adults and juveniles	20
	Hermit crab (Clibanarius vittatus)	adults	3

3.3. Hyperthermic stress to WSSV detection in post larvae

Approximately 600 post larvae were placed in 4 plastic containers (140 post larvae per container). WSSV positive samples were detected at time 0 in two post larvae samples, named group A and C. Quantitative analysis showed an average number of viral copies of 5.40 $x10^3$ at time 0. After thermal stress for 48h, an additional group, named group B, was found to be WSSV-positive (1.72 $x10^5$ copies/µL). WSSV positive post larvae at time 0 had an average of 8.83 $x10^6$ copies/µL after 48h and 3.50 $x10^7$ copies/µL DNA after 72h in water at 29°C. At 72h, most of post larvae had died. Table 2 shows the groups in which WSSV was detected as well those in which the virus was not detected at 0, 48 and 72h.

Table 2: WSSV detection in four groups of post larvae after 48 and 72h at thermal stress. ND - not detected; + detected (+ and ++ refers to comparative viral load among groups).

Group	T0	T48h	T72h
A	+	++	++
B	ND	+	+
C	+	++	++
D	ND	ND	ND

4. Discussion

WSSV infection depends on inherent variables of the pathogen like virulence of genetic strain and virus concentration (Zwart et al., 2010), however, the severity of infection due to environmental factors, e.g. temperature, could increase susceptibility of the host as well the viral replication process. Temperature may have positive or negative effects on disease, mortality and infection status of WSSV inoculated shrimp (Rahman et al., 2007).

Despite the use of protocols for transport disinfection, post-harvest, breeding and post larvae sanitary status verification, the presence of white spot virus was detected in 106 cases in Sonora´s shrimp ponds. During the viral outbreak in 2010, WSSV-positive wild organisms were detected in six shrimp pond areas. On the other hand, negative results for the presence of this virus could be due to low infectious levels of the virus that may be under the detection limits of the PCR methods used. This situation has been observed in other studies (Esparza-Leal et al, 2009).

In the State of Sonora, México, shrimp farms undergoing change in temperature throughout the growing season, display temperature ranges 26-30°C in spring and autumn. During summer, sea water temperatures are over 30°C and in winter below 20°C. In this cold season shrimp farms do not operate and wild crustaceans in the marine environment may be WSSV infected asymptomatic host organisms. Higher temperatures and sea water inputs generate a bloom of plankton in the marine environment, which creates suitable conditions for the permanence and transition of WSSV in the marine environment to asymptomatic host organisms (Sánchez-Paz, 2010; Small and Pagenkopp, 2011). At the present study we evaluate the effect of thermal stress on the replication rate of WSSV in shrimp held in warm water (29±0.5°C), compared to the replication rate of WSSV in shrimp held in cool water (18±0.5°C) by qPCR. A 6.2 $x10^3$ copies/µL inoculum was used to promote the WSSV infection in health shrimp. We also used the effect of elevated temperature in larvae and wild crustacean to increase the viral replication until becoming PCR detectable.

In WSSV-injected shrimp held at 29±0.5°C, we observed that shrimp showed clinical signals and mortality rates accelerated while at 18±0.5°C no clinical signs were observed and mortality occurred only after 36 hpi. Viral quantification showed that replication rates were lower at 18±0.5°C. Differences on WSSV load between infected animals held at 18±0.5°C compared with those kept at 29±0.5°C showed

that there are ranges of temperature that can keep shrimp as asymptomatic carriers and the virus at low rate of replication (15 to 22°C), or a non-detectable load (above 31°C). On the other hand, temperatures between 22 and 30°C allow the replication of WSSV at a high rate in infected shrimp maintained at these temperatures (Jiravanichpaisal et al., 2006; Ruiz-Velazco et al. 2010).

In addition, WSSV positive and negative post larvae were kept in warm water. Natural infected post larvae showed increased replication rate and high mortality when held at 29±0.5°C. The same was observed in adult shrimp artificially infected. Most surprising results were seen in negative post larvae at18±0.5°C that showed to be WSSV-positive before temperature exposure at 29 ±0.5°C. In this case, a normal diagnosis procedure will result in a false-negative status, once low WSSV viral load was responsible for a negative PCR result.

The question of whether the virus was in a "latent state" or in a very low replication rate was not determined in this work. However, our results support this hypothesis since we showed that the maintenance of WSSV-negative wild organisms and post larvae, at 18°C followed by a 2 day-period at 29°C resulted in WSSV-positive PCR results. These findings may indicate that organisms are infected but infection does not manifest because low temperature allows no proper replication of the WSSV. Thus, results showed that the virus remained in the environment, in wildlife, for at least four months after the outbreak, once positive wild shrimp samples were detected in December, 2010. Quang et al. (2009) assert that although the detection rates of WSSV genome were generally gradually declined in seawater environments of both diseased shrimp ponds and surrounding canals in Vietnam, WSSV was still detected with rates of more than 10% in the diseased ponds and lower in surrounding canals even 20 months after the WSSV outbreak.

To date, studies have shown temperature effects on WSSV replication as a possible explanation for the virus infection pattern. These findings suggest that WSSV may enter the target cells and replicate at high temperatures, while low temperatures may decrease infection, probably because the virus only attaches to the cell surface without replication (Jiravanichpaisal et al., 2006). However, in a chronic stage of infection, the increment of water temperature caused a rapid progression of disease and mortality in WSSV-infected shrimp which display high mortality rates (Rahman et al, 2007; Wongmaneepratep et al, 2010).

Our results suggest that increasing temperature to 29°C for 48h before collecting samples to PCR analysis might be applied to the

selection of spawning shrimp. Additionally, we propose the use of a thermal stress protocol for monitoring host organisms in order to reduce false negative PCR due to either low viral load or viral latency. We believe this proposed strategy may contribute to prevent or restrict WSSV spread in shrimp farming industry in Mexico and therefore reduce the probability of infection. This strategy may be also potentially applied in other shrimp producing countries that present similar temperature fluctuation pattern.

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CONSIDERAÇÕES FINAIS

Desde seu surgimento, em 1993, o vírus da mancha branca tem causado grandes perdas econômicas em países da Ásia e da América. A grave ameaça das enfermidades virais sobre a carcinicultura, em particular o WSSV, foi responsável pelo grande crescimento em estudos relacionados às respostas virais em camarões. Entretanto, até hoje, não foi desenvolvido um tratamento eficaz para esta doença, e pouco se sabe sobre o mecanismo de infecção do vírus.

O estresse resultante da baixa qualidade ambiental e do cultivo intensivo é um dos gatilhos para a transição de uma doença de um estado crônico para infecções agudas e parece ser resultado de uma redução na capacidade imunológica dos camarões, além de alteração de ordem bioquímica e molecular. Em áreas com muitos cultivos intensivos, os efluentes das fazendas são liberados na água e contaminantes químicos e biológicos recirculam entre as fazendas (KAUTSKY et al., 2000). A persistência de mortalidades altas em cultivos sucessivos possibilitou a permanência do WSSV no ambiente de cultivo e no seu entorno, provocando a contaminação das principais espécies de macro-crustáceos silvestres, potenciais vetores do vírus. Esta situação foi observada nos cultivos afetados pelo primeiro surto de WSSV em Santa Catarina, em 2005. De acordo com Seiffert (2005), a doença apenas se manifestou em cultivos com problemas de qualidade de água e de solo, como por exemplo, altos teores de sulfeto e teores de material orgânico acima de 10%.

Cinco anos após o seu surgimento em Santa Catarina, a enfermidade da mancha branca continua causando sérios prejuízos ao setor produtivo, provocando o fechamento da maioria das fazendas. Informações obtidas em diferentes estudos são indicativas de que a severidade da enfermidade está relacionada ao sistema de cultivo empregado e as características ambientais e climáticas encontradas no estado, os quais dificultam o seu controle.

Estudos envolvendo respostas bioquímicas, que reflitam o estresse causado nos organismos por fatores ambientais ou pela contaminação aquática ainda são pouco explorados, particularmente em camarões marinhos. Esta afirmação demonstra a relevância do presente estudo, na investigação sistemática das respostas do camarão peneídeo, *Litopenaeus vannamei*, frente ao estresse, causado por diferentes fatores ambientais (químicos e físicos).

O desenvolvimento deste estudo visou utilizar biomarcadores como ferramentas para avaliar a influência de parâmetros ambientais sobre a susceptibilidade a doenças, combinando a questão ambiental com a sanidade dos cultivos. Tais informações podem direcionar as medidas a serem adotadas nos cultivos, priorizando a qualidade ambiental e as técnicas de manejo, como ferramentas de caráter preventivo na manutenção da sanidade dos plantéis. Estudos moleculares que identifiquem biomarcadores de resistência ou envolvidos em mecanismos de defesa contra contaminação ambiental e/ou agentes virais podem melhorar as estratégias de enfrentamento desses problemas.

O presente trabalho consistiu de três linhas principais. A primeira parte avaliou biomarcadores em *Litopenaeus vannamei* exposto ao pesticida permetrina. Os resultados obtidos indicam que os biomarcadores de estresse oxidativo apresentam um potencial promissor como ferramenta para o monitoramento da qualidade da água dos cultivos. Este é um dos primeiros trabalhos a respeito da modulação na expressão gênica e na atividade enzimática deste biomarcadores, no camarão *L. vannamei*, em resposta a permetrina.

Na continuação, a susceptibilidade destes camarões ao vírus da mancha branca foi avaliada, através de alterações na expressão de genes e proteínas envolvidas em processos de defesa celular. Um grupo de animais foi infectado pelo WSSV em laboratório, através de inoculação. Em condições naturais, as respostas ao vírus podem ser diferentes daquelas encontradas em camarões infectados experimentalmente, uma vez que as condições ambientais influenciam a resposta imune dos animais. Estudos envolvendo o genoma do camarão, associados à proteômica, permitem a identificação de genes candidatos, responsáveis pela produção e desempenho traços de uma dada espécie. Alguns dos genes avaliados neste estudo, descritos no capítulo 2, podem ser utilizado para seleção de linhagens, contribuindo para a gestão dos estoques, além de possibilitar uma melhor compreensão da relação entre o agente viral e mecanismos de resistência ou defesa.

Além da qualidade da água, Costa et al (2010) ressalta que variações na temperatura da água dos viveiros têm sido associadas a manifestações da enfermidade da mancha branca no intervalo entre 25 a 28ºC. Em Santa Catarina a maioria dos surtos ocorreu dentro deste intervalo de temperatura, o qual foi registrado em todos os meses de cultivo. Observações sobre variações de temperatura e surtos WSSV no México, mostraram que a enfermidade ocorre quando a temperatura da água exibe oscilações diárias na faixa de 26-30°C. Pode-se especular

que estas sejam faixas de temperatura ideais para a proliferação viral e contribuam para a infecção nos cultivos, respeitando as diferenças entre as cepas virais e particularidades do clima destes dois países.

Além disso, baseado nos experimentos do capítulo 3, onde a replicação viral ocorreu numa taxa menor em camarões mantidos a 18°C do que a 29°C, é possível inferir que temperaturas abaixo de 20°C contribuam para que o vírus se mantenha em populações selvagens, em taxas que não podem ser detectadas. Desta forma, é recomendada a utilização de um protocolo de estresse térmico para o monitoramento de organismos hospedeiros selvagens, a fim de reduzir os falsos negativos devido à PCR ou carga viral baixa ou latência viral. Além disso, o protocolo de estresse térmico poderia ser aplicado na seleção de reprodutores, antes da desova, garantindo que apenas animais realmente sadios sejam utilizados na reprodução.

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