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PATRÍCIA RAQUEL NOGUEIRA VIEIRA

ESTUDO *IN VIVO* E *IN VITRO* DA INFECÇÃO VIRAL POR IMNV E DESENVOLVIMENTO DE UM SISTEMA DE ENSAIO EM HEMÓCITOS DE CAMARÃO MARINHO *Litopenaeus vannamei* VISANDO A PROSPECÇÃO DE AGENTES ANTIVIRAIS

FORTALEZA

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Doutora em Biotecnologia. Área de concentração: Multidisciplinar.

Orientador: Prof. Dr. Gandhi Rádis Baptista

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Aprovada em: 28/06/2017.

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A Deus pela graça,

Dedico.

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A Deus por permitir que eu chegasse até aqui, me dando graça.

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RESUMO

O cultivo de camarão é uma atividade econômica importante em países tropicais como o Brasil. Contudo, a atividade tem enfrentado recorrentes surtos de epizootias, causando grandes perdas para o setor. Os problemas têm sido provocados principalmente pela disseminação de vírus. O vírus da mionecrose infecciosa (IMNV) é um destes agentes epizoóticos que afetam a produção de camarão no Brasil. Na principal região brasileira de produção de camarão, sabe-se que uma redução nos índices de salinidade dos viveiros de cultura causa o aparecimento de doenças virais, ou seja, quando coincide com o início da estação chuvosa de cada ano. Porém, não há no mercado fármacos disponíveis para tratar ou prevenir os surtos recorrentes de viroses em fazendas. Diante disso, o objetivo dessa tese foi estudar a replicação do IMNV *in vivo* e *in vitro* e testar a potencial atividade antiviral do Ctn[15-34]. Para isso, foi realizado um estudo da replicação do IMNV em níveis controlados de salinidade durante as primeiras 12 horas de infecção *in vivo*, bem como, o desenvolvimento de um sistema de ensaio *in vitro*, utilizando hémocitos de *Litopenaeus vannamei* em cultivo, com o objetivo de obter a concentração inibitória (CI_{50}) do vírus nessas células, aliado a busca de substâncias antivirais. Utilizando PCR quantitativa em tempo real e análise estatística, verificamos que a baixa salinidade facilita a replicação e proliferação de IMNV *in vivo*, diminuindo o tempo de geração de 57,4 min (a 35 g L^{-1}, salinidade ideal) para 25,2 min a (5 g L^{-1}, concentração estressante). Da mesma forma, foi demonstrada uma correlação positiva entre a diminuição da salinidade e a redução do tempo de geração de outro vírus – necrose infecciosa hipodérmica e hematopoética (IHHNV). O IHHNV apresenta uma alta taxa de prevalência e normalmente coinfecta camarões em viveiros de cultivo, onde o IMNV surge como surtos. Com relação à replicação *in vitro*, ensaios de citotoxicidade, baseado em fluorescência, em combinação com PCR quantitativa, demonstrou, nesse trabalho, que a CI_{50} do IMNV foi de 227 cópias de trancritos de vírus em hemócitos. Esses ensaios foram utilizados para testar um eicosapeptídeo, denominado Ctn[15-34], derivado de uma catelicidina, uma classe de peptídeos antimicrobianos. O peptídeo Ctn[15-34] inibiu a citotoxicidade do IMNV (CI_{50}) com concentrações entre 0,75 e 12,5 μM. Sendo assim, o presente estudo relata pela primeira vez a replicação do IMNV *in vitro* e o emprego de uma metodologia direta para avaliar a viabilidade celular de hemócitos que, por sua vez, suportam a replicação viral, bem como, aplica um sistema para a triagem de substâncias com atividade antiviral e citoprotetora. Ademais, este trabalho demonstra a atividade

antiviral do eicosapeptídeo Ctn[15-34], de utilidade para o desenvolvimento de análogos, bem como para compor formulações contra doenças epizoóticas de natureza viral que afetam o camarão marinho e a aquicultura.

Palavras-chaves: *Litopenaeus vannamei. IMNV. Replicação viral. Diagnóstico molecular. Peptídeo antiviral. Eicosapeptídeo derivado de catelicidina. Ensaio de viabilidade celular/citotoxicidade por fluorescência. Cultura primária de hemócitos.*

ABSTRACT

Shrimp farming has become a very representative industry worldwide, dealing with over millions of tons of products. It is an important economic activity in tropical countries like Brazil. However, the activity has been facing recurrent outbreaks of epizootics, causing heavy losses for the sector. The problems have been mainly caused by the spread of viruses. Infectious myonecrosis virus (IMNV) is one of these epizootic agents that affect shrimp production in Brazil. In the main Brazilian region of shrimp production, it is known that a reduction in the salinities of cultured farms causes viral diseases, that is, when the rainy season of each year begins. However, there are no drugs on the market available to treat or prevent recurrent outbreaks of farm viruses. Therefore, the objective was to study a replication of the IMNV *in vivo* and *in vitro* and to test the potential antiviral activity of the Ctn [15-34]. In view of this, a study of the replication of IMNV at controlled salinity levels during the first 12 hours of *in vivo* infection was carried out, as well as the development of an *in vitro* assay system using *Litopenaeus vannamei* hemocytes in culture with the objective to obtain the inhibitory concentration (IC_{50}) of the virus in these cells, together with the search for antiviral substances. Using real-time quantitative PCR and statistical analysis, we found that low salinity facilitates the replication and proliferation of IMNV *in vivo*, reducing the generation time from 57.4 min (at 35 g L^{-1}, ideal salinity) to 25.2 min to (5 g L^{-1}, stress concentration). Likewise, a positive correlation between the decrease in salinity and the reduction in the time of generation of another virus, hypodermic and hematopoietic infectious necrosis virus (IHHNV) was demonstrated. The IHHNV has a high prevalence rate and normally co-infects shrimps in nurseries where the IMNV emerges as outbreaks. With respect to *in vitro* replication, fluorescence-based cytotoxicity assays, in combination with quantitative PCR, demonstrated in this work that the IC_{50} of the IMNV was 227 copies of virus transcripts in hemocytes. Theses assays were then used to test an eicosapeptide, named Ctn[15-34], derived from a cathelicidin, a class of antimicrobial peptides. The peptide Ctn[15-34] inhibited IMNV cytotoxicity (IC_{50}) with concentrations between 0.75 and 12.5 μM. Thus, the present study reports for the first time the replication of the IMNV *in vitro* and the use of a direct methodology to evaluate the cellular viability of hemocytes that, in turn, support viral replication, as well as apply a system for screening of substances with antiviral and cytoprotective activity. In addition, it demonstrates the antiviral activity of the eicosapeptide Ctn[15-34] for the development of analogues to

compose formulations against epizootic diseases of viral nature that affect marine shrimp and aquaculture.

Key-words: *Litopenaeus vannamei. IMNV. Viral replicaton. Molecular diagnosis. Antiviral peptide. Cathelicidin-derived eicosapeptide. Fluorescence cell viability/cytotoxicity assay. Primary hemocyte culture.*

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1 INTRODUÇÃO

O cultivo de crustáceos compreende uma indústria que apresenta um crescimento econômico global, da ordem de 2,7 milhões de toneladas, em água salobra, da qual 99% envolvem camarões marinhos. Dentre as espécies cultivadas de camarões, o camarão branco do pacífico *Litopenaeus vannamei* (Boone, 1931) representa 71,8% da produção mundial em relação a todas outras as espécies (FAO, 2012). Esta atividade teve um aumento considerável em países tropicais e subtropicais, dentre os quais figura o Brasil (ABCC, 2017b).

No Brasil, a região nordeste concentra praticamente todo o cultivo comercial de camarão do país, devido as excelentes condições climáticas que contribuem para um bom desempenho e uma alta produtividade. Atualmente, camarões *L. vannamei* são a única espécie cultivada comercialmente no Brasil. Os estados do Rio Grande do Norte e Ceará figuram como os maiores produtores nacionais de camarão cultivado (IBAMA, 2007; ABCC, 2013).

No entanto, esta atividade aquícola tem sofrido problemas recorrentes de surtos de epizootias, causando consequentemente grandes perdas econômicas (BACHÈRE, 2000). Os vírus são os agentes que causam as perdas mais significativas no setor. Contudo, não há fármacos disponíveis no mercado para tratamento de viroses. No nordeste brasileiro, o vírus da mionecrose infecciosa (IMNV) vem sendo o principal causador de prejuízo a carcinicultura (LIGHTNER et al., 2012). O estresse causado pela oscilação de fatores físicos (temperatura) e químicos (salinidade) são condições ligadas ao desencadeamento de infecções virais e mortalidades. Nesse sentido o IMNV, tem sido diagnosticado como agente causador de doença em fazendas de camarão do nordeste brasileiro, como verificado, por exemplo, durante a quadra chuvosa do ano de 2010 (TEIXEIRA-LOPES et al., 2011). No entanto, ainda não havia sido demonstrada experimentalmente a ligação do estresse ambiental com o favorecimento do IMNV, como já foi comprovada para o Vírus da Mancha Branca (WSSV), outro vírus que ataca severamente a carcinicultura mundial (OIE, 2016).

Visando encontrar soluções para os problemas de enfermidades virais no cultivo de camarões marinhos de interesse econômico, muitos pesquisadores tem trabalhado no cultivo de células de camarão para estudo da biologia dos principais vírus que atacam a carcinicultura. Porém, os dados da literatura se concentram no estudo do

WSSV. Contudo, até hoje nenhuma linhagem de célula de camarão foi imortalizada. Com relação aos estudos *in vitro* da replicação do IMNV, também ainda não há dados registrados e disponíveis (JAYESH; SEENA; SINGH, 2012).

Portanto, faz-se necessário o estudo da biologia do IMNV *in vivo* e *in vitro*, visando uma melhor compreensão da replicação desse vírus, associados às variações dos parâmetros ambientais ao longo do cultivo, bem como, a descoberta de agentes antivirais que possam ser utilizados no momento de surtos da doença.

Nesse sentido, os objetivos do presente projeto de tese foram determinar o tempo de replicação do IMNV em camarões mantidos em diferentes salinidades ao longo do tempo, determinar a titulação e o tempo de replicação em cultivo primário de hemócitos de camarão em tempos que foram experimentalmente determinados *in vivo*, bem como, determinar a potencial atividade antiviral do peptídeo Ctn[15-34]. Visando encontrar soluções para os problemas de enfermidades com IMNV no cultivo de *L. vannamei* e, consequentemente, aumentar o desempenho e a rentabilidade do setor aquícola, assim como, dispor de métodos alternativos e rápidos para a prospecção de substâncias antivirais de natureza orgânica e peptídica.

Esta tese está dividido em 3 capítulos. Onde o capítulo I contém a revisão de literatura relevante para o tema. O capítulo II, está o artigo relacionado ao estudo do IMNV *in vivo* ao longo do tempo em diferentes salinidades. E o capítulo III, contempla o artigo com cultivo primário de hemócitos para o estudo do IMNV *in vitro*, e a prospecção da atividade antiviral do Ctn[15-34].

2 REVISÃO DE LITERATURA

2.1 Camarão marinho cultivado: *Litopenaeus vannamei*

Em 2011, um alerta da ONU (Organização das Nações Unidas) declarou que o consumo de recursos naturais não renováveis deve triplicar até o ano de 2050. Produtos oriundos da pesca e aquicultura são muito importantes como fonte de alimento, nutrição e renda para milhões de pessoas no mundo. A aquicultura, diretamente inserida nesse contexto, é o setor de produção de alimentos que mais cresce no mundo. É um setor responsável pelo fornecimento de metade de todo o pescado consumido, com 73,8 milhões de toneladas produzidas e receita estimada de 160,2 bilhões de dólares, bem como pelo recorde de 20 kg de consumo *per capita* de pescado em 2014 (FAO, 2016).

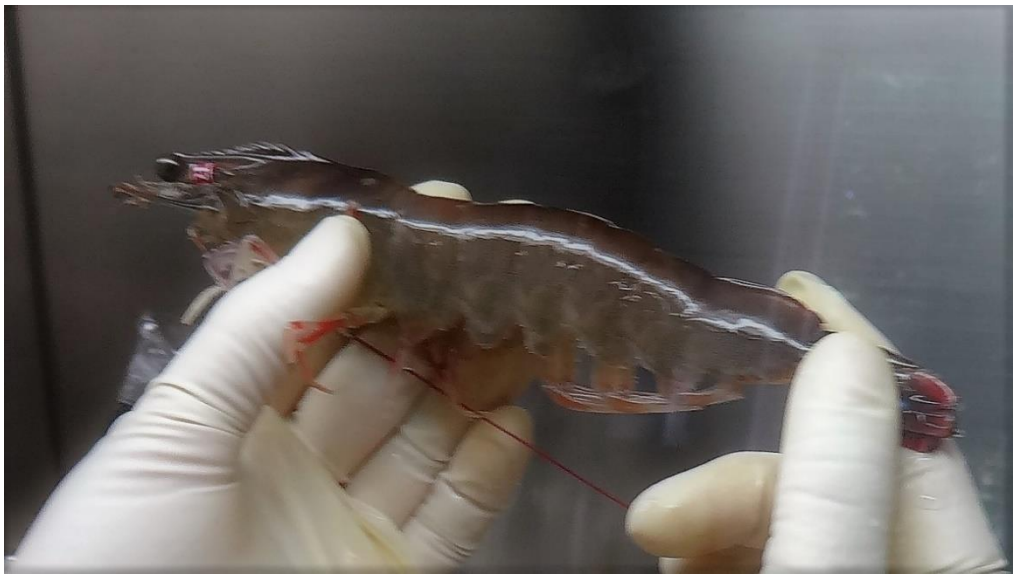
O cultivo de crustáceos é um setor da aquicultura que apresenta um crescimento econômico global da ordem de 2,7 milhões de toneladas em água salobra, da qual 99% envolvem camarões marinhos. Dentre as espécies cultivadas de camarões, representantes da família Penaeidae são os mais cultivados, sendo camarão branco do pacífico *L. vannamei* representante de 71,8% da produção mundial. O aumento considerável desta atividade é proeminente em países tropicais e subtropicais, dentre esses, o Brasil é o terceiro maior produtor aquícola das Américas (FAO, 2012; ABCC, 2017a; ABCC, 2017b).

No Brasil, a produção de camarão marinho alavancou na década de 80 com a introdução da espécie *L. vannamei*, sendo a região nordeste responsável por 99,3% da produção nacional, com destaque para os estados do Ceará e Rio Grande do Norte, correspondendo a 71,5% do total (IBAMA, 2007; ABCC, 2017a; ABCC, 2017b).

Esse camarão é uma espécie exótica da costa do Pacífico, que se distribui desde Sonora (norte do México) até Tumbes (norte do Peru), normalmente em águas tropicais (BARBIERI & OSTRESKY, 2002). Na fase adulta é encontrado em profundidades de até 72 m, em temperaturas de 26-28 °C e salinidade de 3,5 g/L. A fêmea de *L. vannamei* desova no oceano e as larvas, de hábitos planctônicos, migram para a costa onde permanecem de juvenis até tornar-se pré-adultos, que, por sua vez, retornam aos hábitos

de vida mais bentônicos, pois permanecem daí em diante grande parte da vida em contato com o substrato oceânico (CHAMBERLAIN; LAWRENCE, 2009; FAO, 2017)

Figura 1 – Reprodutor fêmea de *Litopenaeus vannamei*. Fonte: Arquivo pessoal do autor (2017).



Além disso, características como rusticidade, rápido crescimento, fácil adaptação a rações comerciais e boa tolerância a variações ambientais, fizeram do *L. vannamei* o camarão mais cultivado em todo o mundo (IBAMA, 2007; FAO, 2017). Conhecido como o camarão-branco-do-Pacífico, e popularmente conhecido no Brasil como camarão-cinza (ABCC, 2011), pertence ao reino Animalia, sua classificação taxonômica segue o esquema abaixo:

Filo Arthropoda

Subfilo Crustacea - Brünnich, 1772

Classe Malacostraca - Latreille, 1802

Subclasse Eumalacostraca - Grobben, 1892

Superordem Eucarida - Calman, 1904

Ordem Decapoda - Latreille, 1802

Subordem Dendrobranchiata - Bate, 1888

Superfamília Penaeoidea - Rafinesque, 1815

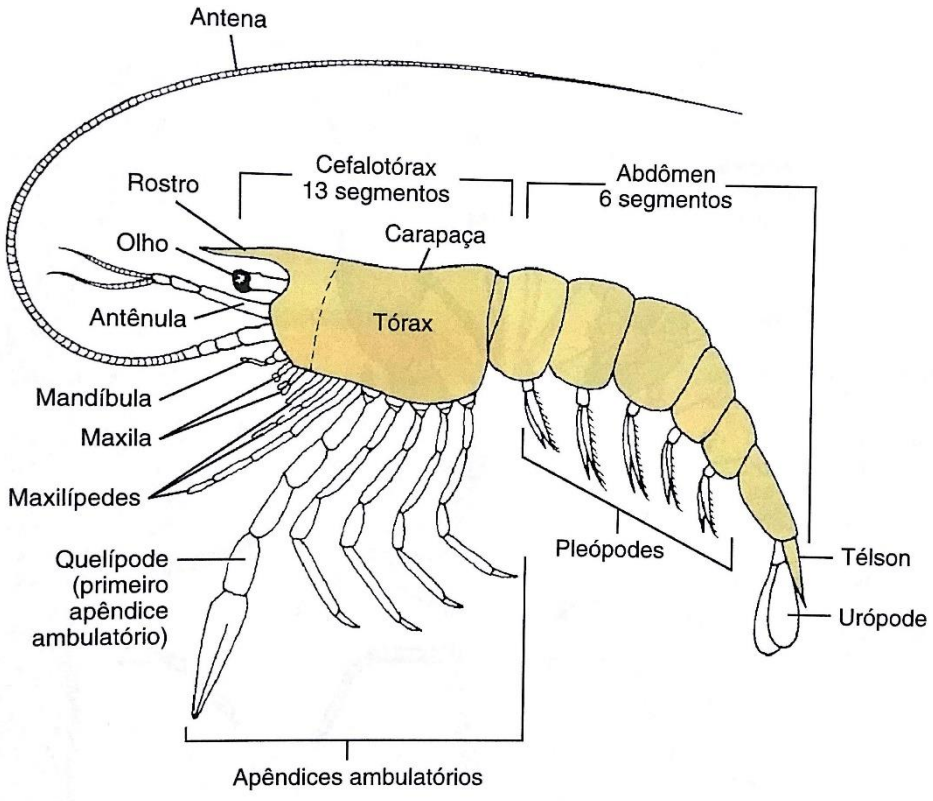
Família Penaeidae - Rafinesque, 1815

Gênero Litopenaeus - Pérez Farfante, 1969

Espécie *Litopenaeus vannamei* - Boone, 1931

A anatomia exterior de um camarão peneídeo é distinguida, estruturalmente, por um cefalotórax com um rostro duro característico e por um abdome segmentado (Figura 2). A maioria dos órgãos, como brânquias, sistema digestivo e coração, estão localizados no cefalotórax, enquanto os músculos se concentram no abdômen. Os apêndices do cefalotórax variam em aparência e função (antenas, antênulas, mandíbulas, maxilípedes e pereiópodes). Na região do abdômen, encontram-se cinco pares de patas modificadas responsáveis pela natação (HICKMAN, ROBERTS; LARSON, 2004).

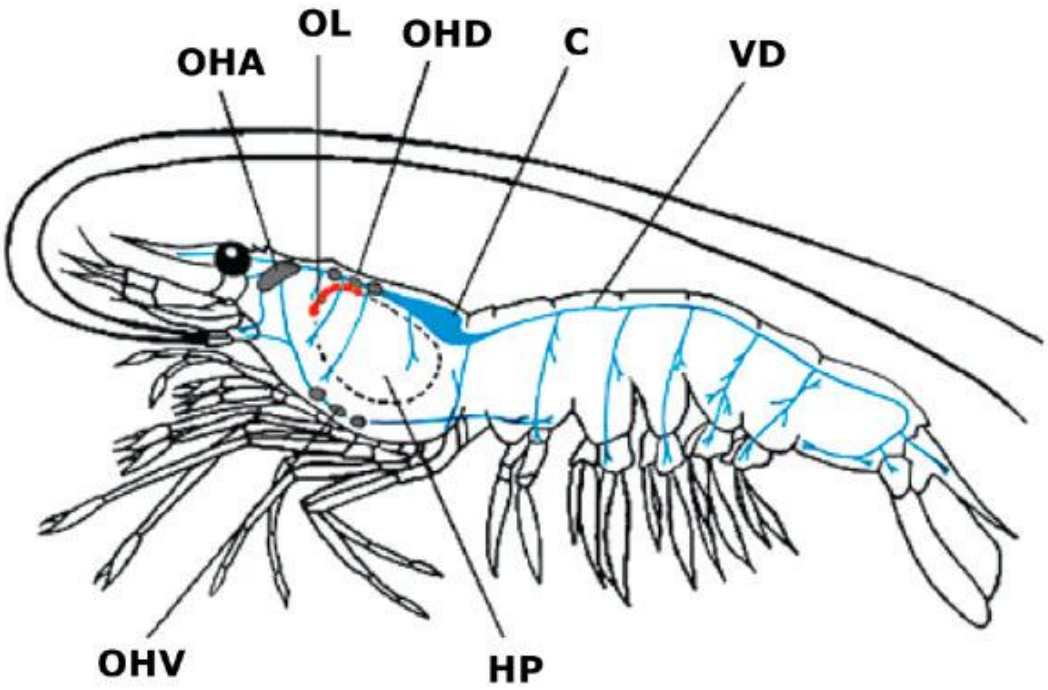
Figura 2 – Morfologia externa de um Malacostraca. Fonte: HICKMAN, 2004.



Internamente, os peneídeos e outros artrópodes têm um sistema circulatório aberto, sendo que, o sangue e as células do sangue são chamados hemolinfa e hemócitos, respectivamente. Crustáceos têm um coração muscular que está dorsalmente localizado no cefalotórax. Os vasos com hemolinfa deixam o coração e se ramificam por seios

espalhados por todo o corpo, para realizar a troca de substâncias. Em seguida, a hemolinfa segue para as brânquias e retorna ao coração. Um dos vasos da hemolinfa que sai do coração termina no órgão linfóide, onde a hemolinfa é filtrada (Figura 3) (RUSAINI, 2010; BARRACCO; PERAZZOLO; ROSA, 2007).

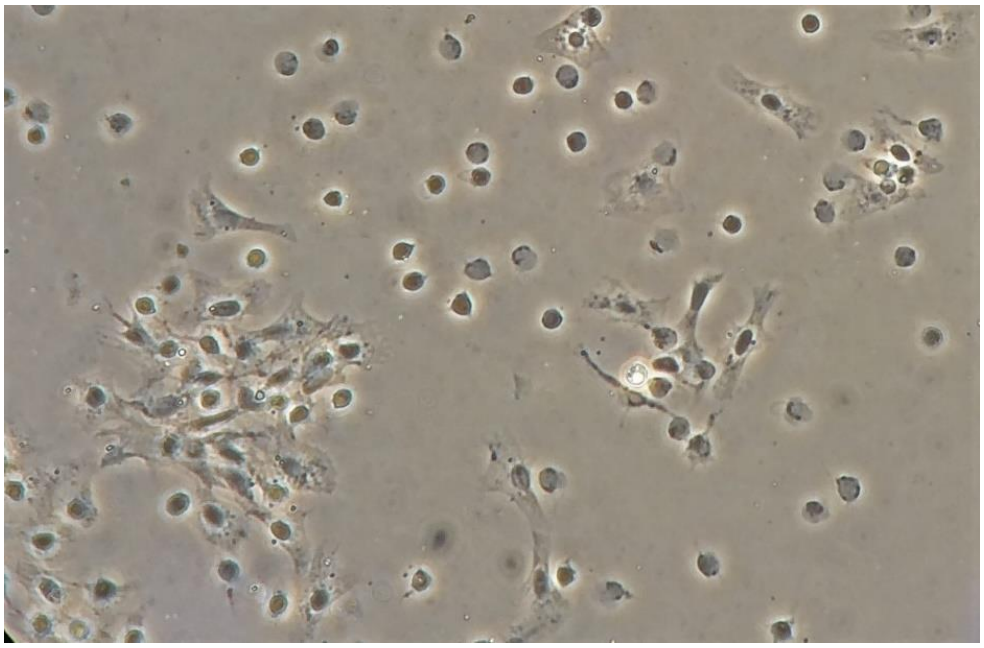
Figura 3 – Sistema circulatório aberto/semi-aberto de camarão e órgãos associados. C: coração; HP: hepatopâncreas; OHA: órgão hematopoiético antenal; OHD: órgão hematopoiético dorsal; OHV: órgão hematopoiético ventral; OL: órgão linfóide; VD: vaso dorsal. Fonte: BARRACCO; PERAZZOLO; ROSA, 2007.



Os hemócitos são as células circulantes imunocompetentes da hemolinfa de camarões produzidos no tecido hematopoiético, que encontra-se disperso no cefalotórax, principalmente em torno do estômago e no início dos maxilípides (Figura 3). Eles são classificados em hemócitos hialinos (menores e sem grânulos), hemócitos semi-granulares ou com pequenos grânulos e hemócitos granulares ou com grandes grânulos (TASSANAKAJON et al., 2013; IWANAGA; LEE, 2005; GROSS et al., 2001). De acordo com alguns autores, os hemócitos hialinos pertenceriam a uma linhagem celular distinta dos hemócitos granulares e estariam essencialmente relacionados aos mecanismos de coagulação (Figura 4). Já os hemócitos granulares (com pequenos e

grandes grânulos) estariam principalmente envolvidos na fagocitose de microrganismos, na formação de nódulos e na produção de moléculas tóxicas e peptídeos antimicrobianos capazes de lisar e/ou degradar os patógenos invasores (BARRACCO; PERAZZOLO; ROSA, 2007; BACHÈRE, 2000).

Figura 4 – Hemócitos de *L. vannamei* em cultura primária em microscopia de luz, em microscópio óptico invertido, objetiva de 10X (Olympus CKX41). Fonte: Arquivo pessoal do autor (2017).



2.2 Doenças virais de camarões marinhos

A carcinicultura tem sofrido com problemas recorrentes de surtos de doenças, assim como outras indústrias de monocultura (LIGHTNER et al., 1997; LIGHTNER; REDMAN, 1998; BACHÈRE, 2000; BROCK; BULLIS, 2001; LIGHTNER 2003). São diversos os fatores que disparam o gatilho para aparecimento de doenças nos viveiros de cultivo (CHAKRABORTI; BANDYAPADHYAY, 2011; TUMBURU et al, 2012; AOKI et al., 2013). Muitas vezes, está relacionado a condições ambientais estressantes, como exemplo, as mudanças bruscas de salinidade que, por sua vez, tem sido associadas como um das mais importantes (RAMOS-CARREÑO et al., 2014; SHEKHAR; KIRUTHIKA; PONNIAH, 2013; VASEEHARAN et al., 2013; LI; YEH; CHEN, 2010). No entanto, as situações se agravam ainda mais quando há um conjunto de fatores tais como, manejo

inadequado, consanguinidade, ausência de medidas de biossegurança e má procedência de pós-larvas (SELVAM; MUJEEB; MOHAMED, 2012; SCHOCK et al., 2013).

As viroses em viveiros de cultivo de camarões marinhos são apontadas como uma das causas que provocaram perdas econômicas recordes nesse setor (JIMENEZ et al., 2000; LIGHTNER, 2011; LIGHTNER et al., 2012; SELVAM; MUJEEB; MOHAMED, 2012). A Organização Mundial de Saúde Animal (do inglês OIE – *Office International des Epizooties*) é o órgão que lista as doenças de camarões em várias regiões do mundo. Os vírus pandêmicos de camarão listados pela OIE (2009) são: Vírus da Mancha Branca (do inglês WSSV - *White spot syndrome virus*), Vírus da Síndrome de Taura (do inglês TSV – *Taura syndrome virus*), Vírus da Cabeça Amarela (do inglês YHV – *Yellow head virus*), Vírus da Necrose Infecciosa Hipodermal e Hematopoiética (do inglês IHHNV - *Infectious Hypodermal and Hematopoietic Necrosis Virus*) e Vírus da Mionecrose Infecciosa (do inglês IMNV - *Infectious myonecrosis virus*).

Os principais vírus que atingem cultivos de camarão no Brasil são o WSSV, IMNV e o IHHNV (LIGHTNER, 2011; LIGHTNER et al., 2012). Dentre estes, os dois últimos constituem-se historicamente os principais causadores de doenças e perdas na produtividade em *L. vannamei* cultivado na região Nordeste do Brasil (TEIXEIRA-LOPES et al., 2011). Embora, recentemente tenha sido registrado o isolamento de vírus da mancha branca no nordeste e perdas econômicas assustadoras, há muitos anos que não se apresentava novos casos da doença nessa região (COSTA et al., 2012; FEIJÓ et al, 2013; PEIXOTO, 2016).

O IHHNV é um vírus importante nos cultivos do nordeste brasileiro por sua alta taxa de prevalência, embora muitos animais são assintomáticos, conferindo um possível caractéristica de latência (BRAZ et al., 2009; TEIXEIRA et al, 2010; TEIXEIRA-LOPES et al., 2011; VIEIRA-GIRÃO et al., 2012). Este é um vírus estável icosaédrico, não-envelopado, com diâmetro de 22 ηm, com material genético composto por DNA genômico de fita simples (ssDNA, do inglês – *sigle strand* DNA) e, de acordo com essas características, pode ser membro da família *Parvoviridae*. O genoma do IHHNV codifica três grandes fases de leitura aberta (do inglês ORFs - *open reading frames*) (MARI; BONAMI; LIGHTNER, 1993). O IHHNV é ubíquo e infecta diversas espécies de camarões peneídeos da Ásia, América Central e América do Sul. Entretanto, o camarão azul do pacífico (*Litopenaeus stylirostris*) é o mais susceptível e a espécie mais

fortemente afetada. No caso do *L. vannamei*, apesar de taxas relativamente baixas de debilidade e mortalidade causadas pelo IHHNV, a manifestação típica da doença inclui retardo de crescimento, que é caracterizado por altos níveis de consumo de energia, e síndrome da deformidade (cutícula e rostro) e do nanismo (KALAGAYAN et al., 1991). Esses sintomas podem ser responsáveis por decréscimos na produção de camarão comercial no mercado e o alto custo de produção com desperdício de nutrientes (ração) para o crescimento do crustáceo (TANG; LIGHTNER, 2006; TANG; NAVARRO; LIGHTNER, 2007). Isso pode ser explicado por esse ser um vírus que causa infecções assintomáticas, ou seja, está se replicando em pequena quantidade nas células, porém não causa morte, mas prejuízo ao metabolismo celular e consequentemente ao animal e a produção. Muitas vezes o IHHNV está integrado ao genoma do camarão e por alguma razão, que ainda não é conhecida, o IHHNV pode começar a replicar-se, assemenlhando-se com o ciclo lisogênico de multipliacação viral (TEIXEIRA et al., 2010; TORTORA; FUNKE; CASE, 2002).

Já o WSSV apresenta altas taxas de mortalidade, e é o vírus que mais causou perdas econômicas a nível mundial. Sua primeira ocorrência no Brasil foi em fazendas de cultivo de camarão em novembro de 2004, no estado de Santa Catarina região Sul do país, sendo notificado pela OIE em 20 de janeiro de 2005. Em 2005, o vírus foi relatado em fazendas do Ceará (SEIFFERT; COSTA; MAGGIONI, 2005; CAVALLI et al, 2011), e em 2008 foi identificado o WSSV em fazendas do estado da Bahia, ambos os estados na região Nordeste do Brasil (COSTA et al., 2010; COSTA et al., 2012; MULLER et al., 2010). O WSSV é um vírus de DNA de cadeia dupla (dsDNA, do inglês – *double strand* DNA) com cerca de 305.000 pares de bases. Pertencente ao novo gênero Whispovirus e família Nimaviridae. As partículas virais são de 80-120 × 250- 380 nm de tamanho, em forma elíptica, com apêndices únicos, parecidos com uma cauda, e com um envelope trilaminar. Tais partículas são geradas nos núcleos hipertrofiados de células infectadas sem a formação de corpúsculos de inclusão. Casos da doença foram primeiramente relatados na Ásia, sendo hoje possível obter vários diferentes isolados geográficos com variabilidade genotípica entre eles em todo o mundo. O agente é viável em água do mar (condições de laboratório) por pelo menos 30 dias a 30 °C, e por 3-4 dias em viveiros. Com relação ao ciclo de vida o ciclo de replicação do WSSV é de aproximadamente 20 h a 25 °C. Os animais podem apresentar também letargia, anorexia e manchas brancas, variando de 0,5-2,0 mm de diâmetro na cutícula do cefalotórax ou carapaça, algumas

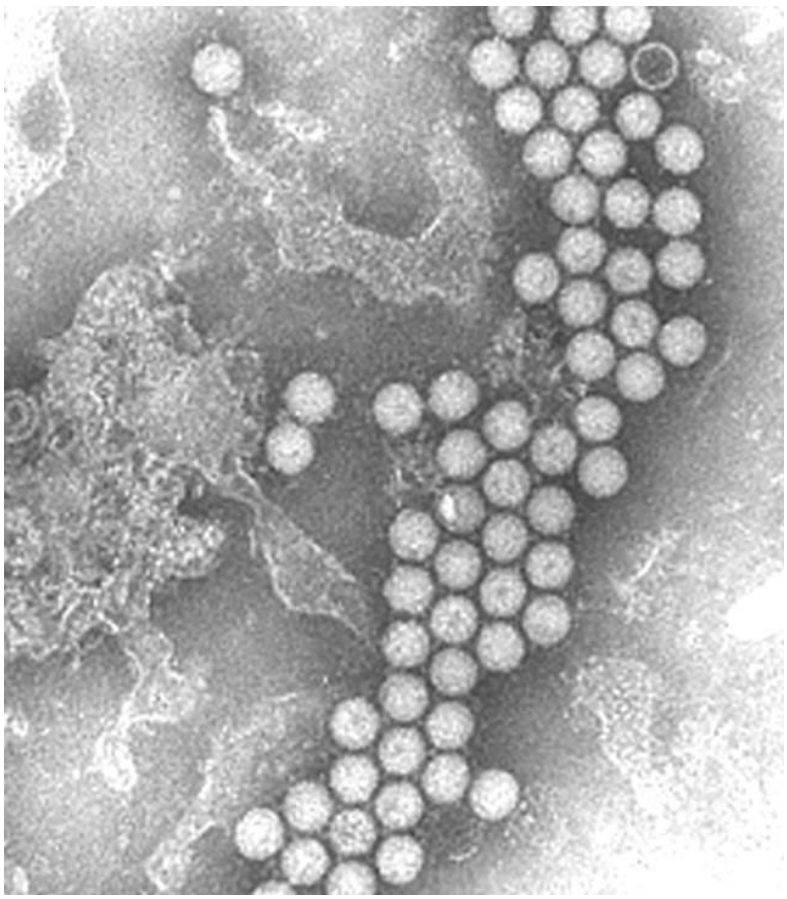
vezes podendo apresentar coloração avermelhada (LIGHTNER et al., 1998; WANG et al., 2000; OIE, 2009; SANCHEZ-PAZ, 2010)

2.2.1 IMNV

O IMNV foi primeiramente identificado em 2002, como o agente causador de uma doença no músculo do camarão cultivado no nordeste do Brasil, e desde então tem sido o vírus que mais afetou os cultivos de camarão nessa região. O vírus se disseminou rapidamente para outras regiões do Brasil e, em 2003, foi responsável por uma perda econômica avaliada em milhões de dólares. Em 2006 a doença se espalhou no sudeste asiático, e em 2007 foram confirmados surtos de IMNV em cultivo de *L. vannamei* na Indonésia (SENAPIN et al., 2007). Os genomas de vírus originários do Brasil e Indonésia foram sequenciados e encontrou-se 99,6% de identidade a nível de nucleotídeo, indicando que a doença foi introduzida do Brasil para a Indonésia (OIE, 2016).

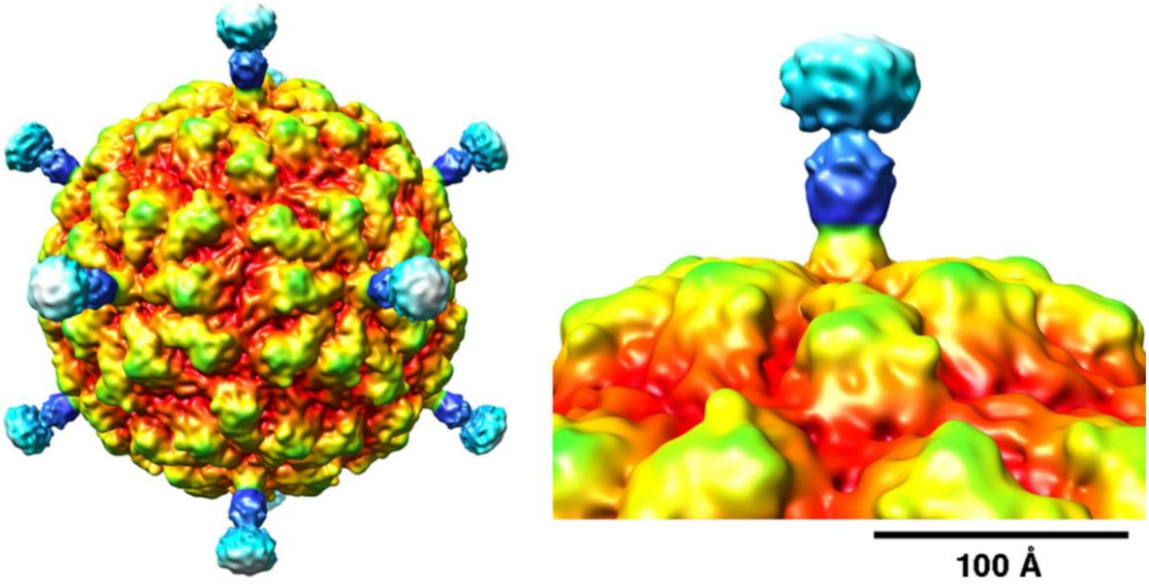
Este vírus apresenta simetria icosaédrica, não-envelopado, 40 ηm de diâmetro e pertence a família Totiviridae, demonstrado por análise filogenética da sequência que codifica a RNA polimerase dependente de RNA (RdRp) estar mais próximo do vírus *Giardia lamblia* da família *Totiviridae* (Figura 5) (JANSSEN et al., 2015). Embora a maioria dos membros dessa família carecem de meios de transmissão extracelular, o IMNV é quase o único a ser transmitido extracelularmente entre as células dos organismos hospedeiros. Provavelmente devido a presença de protusões superficiais, semelhantes a fibras, aliada a proteína majoritária do capsídeo que podem estar envolvidas com a entrada, ligação ao receptor e/ou penetração celular (Figura 6). (TANG et al., 2008). Ao contrário de vários outros membros da família Totiviridae que são associados a infecções latentes e sem virulência, o IMNV causa doença fatal, porém, não tem sido feito estudos mais aprofundados de patogênese desse vírus, pois até então não foram realizados estudos com células *in vitro*.

Figura 5 – Micrografia de transmissão eletrônica de IMNV purificado. Fonte: POULOS et al., 2006.



O seu genoma é composto de RNA dupla fita (dsRNA) com 7561 pb. O sequenciamento do genoma viral indicou a presença de dois ORFs. O ORF1 codifica a proteína do capsídeo e a proteína ligante de dsRNA. Essa última é codificada pela primeira metade do ORF1 (136-4953 nt) que contém o domínio ligante de dsRNA nos primeiros 60 aminoácidos. A segunda metade do ORF1 codifica a proteína do capsídeo, com massa molecular de 106 kDa. O ORF2 (5241-7451 nt) codifica a RdRp (POULOS et al., 2006; TANG et al., 2008; OIE, 2016). Recentemente, o genoma do IMNV foi sequenciado novamente e extendido com mais de 600 nucleotídeos, totalizando, 8230 pb (LOY et al., 2013; LOY et al., 2015; NAIM et al., 2015).

Figura 6 – Estrutura tridimensional de protusões complexas semelhantes a fibras em cinco eixos de uma partícula de IMNV. Fonte: Modificado de TANG et al., 2006.



O sintoma clássico desse vírus é a necrose do músculo estriado do abdômen e cefalotórax. Contudo, compondo o painel de sintomas sugestivos da mionecrose infecciosa, estão a anorexia, a redução no volume do hepatopâncreas, a redução nos lípidios, a natação desorientada, o comprometimento do endurecimento da carapaça e uma mancha esbranquiçada no terceiro segmento abdominal (NUNES; MARTINS; GESTEIRA, 2004) (Figura 7). Além disso, o estresse causado por condições ambientais desfavoráveis parece ser um fator de desencadeamento da infecção tanto aguda como crônica (LIGHTNER et al., 2004). Histologicamente, são caracterizadas lesões por necrose coagulativa no músculo, comumente acompanhado de acúmulo de fluido entre as fibras musculares, infiltração hemocítica, fibroses e a presença típica de esferóides no órgão linfóide. Adicionalmente, corpos de inclusão podem ser observados no citoplasma de células do tecido muscular, conectivo e hemócitos. Sendo esses os tecidos-alvo para esse vírus (POULOS et al., 2006).

O *L. vannamei* em cultivo é a espécie comprovadamente susceptível ao IMNV, sendo os estágios, juvenis e sub-adultos, os mais seriamente afetados, principalmente se cultivados em água de baixa salinidade (NUNES; MARTINS; GESTEIRA, 2004). Temperatura e salinidade são considerados fatores ligados a predisposição para surtos da doença (COSTA et al., 2009). Pode atingir de 40-70% as taxas de mortalidade e o fator de conversão alimentar (FCA = quantidade de ração fornecida/ganho de peso) subir de

~1,5 para 4 ou mais (ANDRADE et al., 2007). Infecções experimentais em *Penaeus stylirostris* e *Penaeus monodon* com IMNV reportaram susceptibilidade, porém não causou mortalidade (TANG et al., 2005). Não existem vectores específicos descritos para esse vírus, porém pela sua estrutura não-envelopada, como o TSV, considera-se que o IMNV pode permanecer infeccioso em intestino e fezes de aves marinhas que se alimentam de camarões moribundos de fazendas de cultivos onde ocorreram surtos de IMN (OIE, 2016).

Além disso, a transmissão da doença se dá por canibalismo, pela água e, provavelmente, por transmissão vertical dos reprodutores para prole (LIGHTNER, 2011; POULOS et al., 2006). Contudo, em nível experimental, o microcrustáceo, *Artemia franciscana* foi considerada um potencial vetor, pois é susceptível ao IMNV e é largamente utilizada como fonte de alimento essencial no cultivo das larvas de camarão (da SILVA et al., 2015).

Figura 7: Juvenil de *L. vannamei* com mionecrose infecciosa (musculatura esbranquiçada). **Fonte**: Arquivo pessoal do autor (2017).

A prevalência do IMNV em locais endêmicos pode chegar a 100% do estoque de camarões. Também foi detectada ocorrência de co-infecção natural com IHHNV e IMNV em *L. vannamei* de fazendas de cultivo no nordeste do Brasil, onde a maioria das amostras

positivas de camarões estavam infectadas, simultaneamente, com ambos os vírus. Porém, o surto e os sintomas resultantes, indicavam que um tipo de vírus proliferava predominantemente em relação ao outro (NUNES; MARTINS; GESTEIRA, 2004; ANDRADE et al., 2007; TEIXEIRA et al., 2010; TEIXEIRA-LOPES et al., 2011).

Medidas de combate ao IMNV em cultivo restringem-se basicamente a prevenção, pois não há agentes terapêuticos disponíveis comercialmente (LIGHTNER, 2003). A desinfecção de ovos e larvas é uma prática recomendada para auxiliar na prevenção da doença, bem como, uma rotina de diagnóstico de reprodutores, ovos e larvas nos plantéis para a produção de animais livres de patógeno específico (SPF, do inglês – *specific pathogen free*), embora não haja dados científicos da rota de transmissão vertical do IMNV (MOSS et al., 2012; LIGHTNER, 2005; PRUDER, 2004; LEE; O'BRYEN, 2003).

Para detecção de IMNV em fazendas de cultivo, um método recomendado é a reação em cadeia da polimerase (PCR, do inglês - *polymerase chain reaction*) precedido de um reação de transcrição reversa (RT-PCR, do inglês – *reverse-transcriptase polymerase chain reaction*). Embora essa seja uma técnica robusta, uma RT-PCR quantititativa (qRT-PCR) é ainda mais recomendada pela sua alta sensibilidade e com isso reduzir os índices de certificações de animais SPF inadequados. Também é utilizado a amplificação isotérmica mediada por *loop* acoplada a transcrição reversa (RT-LAMP, do inglês – *reverse-transcription loop-mediated isothermal amplification*) pela sua praticidade (LIU et al., 2013; TEIXEIRA et al., 2010; TEIXEIRA-LOPES et al., 2011; ANDRADE; LIGHTNER, 2009; PUTHAWIBOOL et al., 2009).

Entretanto, a literatura descreve inúmeros métodos utilizados para diagnosticar IMNV. Dentre eles está a hibridização *in situ* (TANG et al., 2007; TANG et al., 2005), ensaios imunólogicos utilizando anticorpos monoclonais tais como imunocromatografias, imunohistoquímicos (BORSA et al., 2011; SEIBERT et al., 2010), *dot blotting*, *Western blotting* e ELISA (KUNANOPPARAT et al., 2011; MELLO et al., 2011). No mercado existem inúmeros kits de diagnósticos, na sua maioria utilizando PCR, até mesmo para a detecção de mais de dois patógenos simultaneamente (PCR multiplex) (LIFE TECHNOLOGIES, 2015).

2.3 Cultivo de células animais

A cultura de células de animais começou com Harrison em 1907, quando ele cultivou células nervosas de sapo. Em 1912, Alexis Carrel, utilizando informações obtidas por Harrison, desenvolveu um modelo a partir de células cardíacas de embrião de galinha para o cultivo. Seus experimentos foram muito importantes, pois com Carrel descobriu-se a necessidade da troca de fonte de nutrientes contidos nos frascos, permintindo que as células pudessem ser cultivadas por períodos ainda maiores do que os utilizados por Harrison. Após 39 anos (1951), George Grey cultivou células de tecido tumoral humano e estabeleceu a linhagem de célula HeLa, muito utilizada até os dias de hoje (ALVES; GUIMARÃES, 2010; ALBERTS et al., 2009; COOPER; HAUSMAN, 2007).

Atualmente a cultura de células tem sido uma ferramenta de estudo em diversas áreas, não se limitando apenas para o estudo de comportamento de determinado tecido ou célula *in vitro* (LI, 2017; ZHANG; PAVLOVA; THOMPSON, 2017). Por exemplo, o estudo de cultura de células humanas está bastante avançado, tais como a aplicação de terapia celular com o uso de células-tronco, que tem representado um papel importante no desenvolvimento tecnológico mundial (AHARONY; MICHOWIZ; GOLDENBERG-COHEN, 2017; ARRIZABALAGA; NOLLERT, 2017). Por outro lado, o desenvolvimento de cultura de células de invertebrados, tais como crustáceos, ainda tem se desenvolvido lentamente, mesmo com a primeira cultura celular a longo prazo ativa de um crustáceo observada por Quiot, Vago e Luciano (1968).

2.3.1 Cultivo de células de camarão

Dentro do grande grupo de animais invertebrados, está o filo Arthropoda, que são animais caracterizados pela presença de patas articuladas e esqueleto de quitina externo (exoesqueleto) (HICKMAN, ROBERTS; LARSON, 2004). O subfilo Crustacea é contituido de aproximadamente 42.000 espécies, na sua maioria aquáticas (RUPPERT; BARNES, 2005). É um grupo importante pelo seu alto valor comercial. Sendo um

crustáceo da ordem Decapoda, o camarão, responsável por uma grande parcela do valor econômico associado aos crustáceos (FAO, 2016). O principal problema enfrentado hoje pela atividade de cultivo de camarões tem sido as doenças causadas por vírus. Mesmo com inúmeros estudos feitos com vírus de camarão, é necessário o entendimento da patogênese viral bem como o desenvolvimento de uma terapia apropriada (LIGHTNER et al., 2012; LIGHTNER, 2011).

No entanto, esse conhecimento básico sobre vírus de camarão ainda não foi alcançado, pela ausência de linhagens permanentes de células de camarão ou outro crustáceo também susceptível (JAYESH; SEENA; SINGH, 2012; RINKEVICH, 1999). Porém, é inegável que as culturas de células primárias obtidas de órgãos/tecidos representam o primeiro passo rumo ao estabelecimento de linhas celulares e proporcionam informação útil sobre as condições de cultura de células mais adequadas envolvidas na sobrevivência e no desenvolvimento de sistemas para análises citotóxicas dentre outros testes (JOSE et al., 2012; JOSE et al., 2011; JOSE et al., 2010; JOSE, 2009; TIRASOPHON; ROSHORM; PANYIM, 2005).

As primeiras pesquisas com cultura de célula de camarão foram em Taiwan, onde também começaram os surtos de epizootias (CHEN et al., 1986). Esses primeiros estudos foram realizados com a espécie *Penaeus monodon* (THANSA; YOCAWIBUN; SUKSODSAI, 2016), e depois disso várias outras espécies de peneídeos também foram utilizadas em cultivo de células tais como *P. stylirostris* (SHIMIZU et al., 2001), *P. japonicus* (MAEDA et al., 2003, 2004), *P. chinensis* (HU et al., 2008, 2010), *P. penicillatus* (CHEN; WANG, 1999), *P. indicus* (KUMAR et al., 2001) e *P. vannamei* (LI et al., 2015c). Porém, algumas outras espécies de crustáceos também foram utilizados em estudos com cultura de células como o camarão de água doce *Macrobrachium rosenbergii* (GOSWAMI et al., 2010) e o caranguejo *Scylla serrata* (DEEPIKA; MAKESH; RAJENDRAN, 2014).

Os tecidos doadores de células para o desenvolvimento do cultivo primário também são bastante diversos. Sendo o órgão linfóide, óvario e hemócitos os mais escolhidos para estudos (JAYESH; SEENA; SINGH, 2012). As culturas primárias de tecido tem sido utilizadas para testes de susceptibilidade a vírus, porém até a realização do teste propriamente dito, muitas etapas para o isolamento da célula e seu plaqueamento são necessárias, demandando muito tempo e custo (JOSE, 2009). Uma alternativa são os

testes utilizando hemócitos, pois essas células podem ser coletadas e utilizadas diretamente, ou seja, sem os passos inicias de processamento de tecido (DANTAS-LIMA et al., 2012). Além disso, tem sido demonstrada sua susceptibilidade a vírus e outras substâncias testadas *in vitro* (DURESSA; HUYBRECHTS, 2016). Embora os hemócitos sejam células que não se duplicam, são importantes em estudos de resposta imune a diferentes estímulos, e já foi comprovada sua funcionalidade ativa *in vitro* pelo tempo necessário para estudos de processos imunes inatos (DANTAS-LIMA et al., 2012).

Ainda não há um meio de cultivo comercial adequado para células de camarão, apesar de ter sido elaborado um meio de cultivo com uma formulação baseada na composição detalhada da hemolinfa de *P. monodon* (JAYESH et al., 2012). Para realizar o cultivo de células, seja qual for a origem, a necessidade básica é tornar disponível em frascos de cultivo os nutrientes e constituintes do animal de origem (RINKEVICH, 2011; ALVES; GUIMARÃES, 2010). O objetivo é mimetizar o ambiente onde essas células se desenvolvem *in vivo*. Muitos pesquisadores tem concentrado seus esforços para adequar alguns meios de cultivo disponíveis comercialmente para as necessidades das células de camarão, como exemplo, aumentar a osmololidade das soluções de trabalho (JAYESH; PHILIP; SINGH, 2015; JAYESH; SEENA; SINGH, 2012; CLAYDON, 2009).

Os meios para o cultivo de células de insetos tem sido utilizados, tais como, o Meio de cultura de células de Insetos Grace e o Leibovitz's – 15 (L -15) são os mais amplamente escolhidos pelos pesquisadores (JAYESH; SEENA; SINGH, 2012). Algumas modificações foram adotadas ao meio basal de escolha. Por exemplo, a diluição do meio basal é feita em metade do diluente necessário, deixando a concentração dos constituintes do meio dobrada. O mesmo é adotado em alguns suplementos adicionados ao meio, como o soro fetal bovino, que tem sua concentração quase dobrada (JAYESH et al., 2015a; DEEPIKA; MAKESH; RAJENDRAN, 2014; LI et al., 2011; CLAYDON; ROPER; OWENS, 2010; JOSE, 2009; CAIWEN LI, 2007; SHIMIZU et al., 2001). Um outro ponto importante no cultivo de células de camarão é a observância da osmalalidade do meio de cultivo, que deve ficar com uma média da osmolalidade da hemolinfa dos camarões, com cerca de 720 mOsm/kg, a mais vista em publicações com células de camarões. Diferentemente do cultivo de células humanas, por exemplo, que os meios e soluções utilizadas possuem osmolalidade em torno de 300 mOsm/kg (BÜCKLE; BARÓN; HERNÁNDEZ, 2006).

Outras substâncias também foram testadas ao longo dos anos de pesquisa com cultivo de células de camarão. Podendo ser citada algumas principais como, a adição de mais glicose, caldo de triptose fosfato como fontes extra de energia, ou mesmo feniltiouréia para inibir o escurecimento do meio de cultivo, devido ao desencadeamento da cascata enzimática de formação da melanina (JOSE et al., 2010). No sentido de buscar uma melhor proliferação celular ou mesmo uma linhagem imortalizadada de células de camarão, os meios basais tem sido suplementados até com extrato de músculo de camarão, hemolinfa de lagosta, fator de crescimento epidermal e interleucia-2 humana recombinante, dentre outros (JAYESH, 2012).

2.3.1.1 Ensaios com cultivo de células de camarão

Seguindo todas recomendações previamente publicadas, a maioria dos estudos com cultivo de células de camarão se concentram na aplicação de métodos utilizados com outras células eucarióticas (DURESSA; HUYBRECHTS, 2016; JOSE et al., 2011, JOSE, 2009). Nesse sentido, o WSSV tem sido o vírus mais investigado em cultura primária de hemócitos e células linfóides utilizando o método de determinação de viabilidade celular por MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]). O MTT tem sido o único método citado em artigos que avaliam a viabilidade de células de camarão ou outros crustáceos, mesmo havendo a disponibilidade no mercado de técnicas menos laboriosas e mais sensíveis (DEEPIKA; MAKESH; RAJENDRAN, 2014; JOSE et l., 2012, JOSE et al., 2010; MAEDA et al., 2004; WANG et al., 2000; CHEN; WANG, 1999).

O principal objetivo dos programas de desenvolvimento de cultura de células de camarão/crustáceo tem sido o estabelecimento de uma linhagem de células (JAYESH et al., 2015b). Muito já se tem avançado, com relatos de manutenção de células por até 5 meses e um máximo de 44 passagens (LIU et al., 2014; WEST et al., 1999; TAPAY et al., 1995). Também tem relatos de sucesso de transfecção e expressão oncogênica mediada por transdução em células linfóides de *P. monodon*, porém foi detectada a presença de atividade da telomerase nessas mesmas células *in vitro*, o que não é desejado (JAYESH et al, 2015b). Sendo assim, observou-se que os camarões possuem algum mecanismo regulador de carcinoma ou inibidores de telomerase que previnem a transformação de células de camarões *in vitro* (JAYESH, 2012). Ademais, esse fato

poderia fornecer pistas para intervenções sobre anti-envelhecimento e anti-câncer em seres humanos (VOGT, 2012, 2011, 2008).

Diante disso, a maioria das publicações com cultivo de células de diferentes peneídeos tem redirecionado seus objetivos no sentido de desenvolver ensaios utilizando o sistema de cultura primária em investigações sobre susceptibilidade viral, citotoxicidade, expressão de genes do ciclo celular, eventos mitóticos e redes de citoesqueleto (JAYESH; SEENA; SINGH, 2012). Recentemente, os pesquisadores conseguiram, por exemplo, expressão de genes de WSSV (JOSE et al., 2010), determinação de citotoxicidade e genotoxicidade de metais pesados (JOSE, et al., 2011), multiplicação de TSV (GEORGE et al., 2011) e expressão de genes imunes relacionados a infecção por WSSV (JOSE et al., 2012). Outro exemplo, é o trabalho de Li e colaboradores (2015b), que descreve detalhadamente o ciclo de replicação do WSSV em cultura secundária de órgão linfóide de *L. vannamei*. Contudo, não há relatos de estudos *in vitro* com o IMNV.

Desse modo, investigações direcionadas com cultivo de células visam obter um melhor entendimento a respeito das interações patógeno e hospedeiro, pois esse é outro ponto crítico da patogênese de qualquer doença (ROSSI; COLIN, 2017; ROUTHU; BYRAREDDY, 2017). A relação vírus-hospedeiro pode resultar em resposta de defesa contra o invasor, bem como ocasionar mudanças metabólicas e moleculares na maquinaria celular, alterando o nível de expressão de determinados genes que favorecem a replicação viral (LIU; SODERHALL; JIRAVANICHPAISAL, 2009). A esse respeito, Vieira-Girão e colaboradoes (2012) observaram uma expressão diferencial de HSP70 em *L. vannamei* co-infectados com IMNV e IHHNV.

2.3.1.2 Moléculas com atividade antiviral

Doenças causadas por vírus remontam tempos muito antigos, antes de Cristo, datados de 3.600 a.C. no antigo Egito, o primeiro registro descrevia o que hoje conhecemos por poliomielite (ALBERTS et al., 2009). A definição básica de vírus é “Os vírus são parasitas intracelulares obrigatórios, de dimensões submicroscópicas”

(CARTER; SAUNDERS, 2007). Com isso entendemos algo importante sobre o funcionamento dos vírus, que é a necessidade de uma célula (seja qual for a origem, eucariótica ou procariótica), para o vírus realizar sua função de replicação (TORTORA; FUNKE; CASE, 2002).

Em contrapartida, as células possuem uma barreira natural contra os vírus, que são suas membranas celulares, que, nesse caso, atuam como um mecanismo físico de defesa antiviral (TORTORA; FUNKE; CASE, 2002; ALBERTS et al., 2009). Contudo, uma vez dentro da célula, os vírus utilizam a própria maquinaria celular para replicar seu ácido nucléico e produzir suas proteínas, e formar novas partículas virais infectantes que serão liberadas para fora da célula. Como os vírus compatilham muitos processos metabólicos da célula hospedeira, é difícil encontrar fármacos que sejam seletivos para o patógeno. Todavia, existem enzimas que são específicas do vírus, servindo, assim, de alvos potenciais para fármacos (BATISTA, 2011).

Os primeiros relatos para o desenvolvimento de antivirais datam do anos 50, onde os pesquisadores procuravam inibidores da replicação do vírus da varíola (BAUER, 1985). Contudo foi apenas a partir dos anos 60-70 que os primeiros fármacos antivirais foram administrados com sucesso, e a indústria farmacêutica iniciou programas de pesquisa de moléculas com essa propriedade (MITSUYA, H.; BRODER, 1987).

Os agentes antivirais atualmente disponíveis atuam através dos seguintes mecanismos (BATISTA, 2011):

a) Inibição da transcrição do genoma viral:

- inibidores da DNA polimerase;

- inibidores da transcriptase reversa;

- inibidores da protease;

b) Prevenção da entrada na célula do hospedeiro.

Os progressos na terapêutica antiviral surgiram com a descoberta de fármacos que agem inibindo as enzimas codificadas da replicação viral (MITSUYA; BRODER, 1987). Outra fonte potencial para o desenvolvimento de novos agentes antivirais está nas

substâncias endógenas que participam na mediação da imunidade antiviral (STANTON, 1987; MOGENSEN, 1985).

Atualmente existem aproximadamente 50 fármacos antivirais disponíveis para tratamento de vírus que infectam humanos (STRASFELD; CHOU, 2010). A maioria são moléculas com suas estruturas ligeiramente modificadas que atuam inibindo as enzimas que fazem a transcrição dos genomas virais. Basicamente, as estruturas antivirais competem com o substrato das enzimas, sendo, por exemplo o aciclovir, um nucleosídeo de guanina contendo um grupo de açúcar acíclico que age inibindo a DNA polimerase. Já a amantadina é uma amina simétrica derivada do adamantano, que inibe descapsulação dos viriões pelo bloqueio do fluxo de prótons através dos canais iônicos membranares (FIELD; CLERCQ, 2004).

Outro exemplo, são os antivirais que atuam prevenindo a entrada do vírus na célula. Este grupo inclui os antivirais que têm como alvo os receptores das células hospedeiras e as proteínas da superfície viral. Podem ser peptídeos similares aos receptores celulares, bloqueando a ligação do vírus à célula hospedeira (SKALICKOVA et al., 2015; CARTER; SAUNDERS, 2007). Como exemplo, a enfuvirtida que é um peptídeo constituído por 36 aminoácidos que inibe o processo de fusão entre a membrana celular e o invólucro do HIV-1, atuando sobre as proteínas da superfície viral (FIELD; CLERCQ, 2004).

Embora não existam fármacos comercialmente disponíveis para tratar doenças virais em camarão, têm sido dedicados esforços para encontrar novas moléculas com atividade antiviral contra estes agentes patogênicos do camarão. Como exemplo, incluem-se o uso de probióticos (LAKSHMI; VISWANATH; SAI GOPAL, 2013) e pequenos RNAs de interferência (iRNAs, do inglês – *interferences RNAs*) (FEIJO et al., 2015, SHEKHAR; LU, 2009). Bem como, peptídeos antimicrobianos derivados de isoformas de fator anti-lipopolissacarídeo possuem também atividade antiviral (LI et al., 2015a; YANG et al., 2016; METHATHAM et al., 2017). Em nível experimental, por exemplo, Loy e colaboradores (2013) observaram atividade antiviral eficiente de sequências de dsRNA frente à inúmeros vírus dentre eles o IMNV.

Para se defender de patógenos os camarões utilizam sua imunidade natural. O sistema imune inato que é baseado na ação dos componentes celulares (hemócitos) e humorais

(moléculas ativas) do sistema circulatório, os quais interagem para detectar e eliminar microrganismos e parasitas estranhos (BACHERÈ, 2000). No curso de uma infecção, em algumas reações de defesa, várias moléculas tem sua expressão estimulada. Essas moléculas participam do reconhecimento de padrões moleculares, acionando mecanismos de defesa celulares e humorais ou mesmo a resposta antimicrobiana direta. Entre elas estão as lectinas e peptídeos ou proteínas antimicrobianas (PAMs) (HOLMBLAD; SÖDERHÄLL, 1999; THEOPOLD et al., 2004). Desta forma, os PAMs são moléculas que fazem parte do sistema imune inato de vertebrados e invertebrados, possuindo um amplo espectro de atividades contra fungos, bactérias, parasitas e vírus.

As crustinas são um exemplo peptídeos antimicrobianos muito comuns em crustáceos, sendo inicialmente identificada no caranguejo *Carcinus maemas*, chamada de carcinina (SCHNAPP; KEMP; SMITH, 1996). Em seguida, foram encontradas em *L. vannamei* e L. setiferus, obtendo a isoforma LvABP1 (número de acesso no GenBank AF30074) (GROSS et al., 2001). Esses peptídeos são divididos em três tipos: Tipo I, Tipo II e Tipo III (SMITH et al., 2008). As carcininas são crustinas do Tipo I, possuem uma região de comprimento variável rica em cisteína entre a região signal e a porção da proteína do soro acídico (WAP, do inglês - *whey acidic protein*) (SALLENAVE, 2002; HAGIWARA et al., 2003). Já as crustinas do Tipo II, tem uma região rica em glicina e acima de uma rica em cisteína, também entre a porção signal e a região da WAP. As características das crustinas do Tipo III são diferentes das outras duas, possuindo uma pequena região entre porção WAP e signal, constituída de resíduos de prolina e arginina (HAGIWARA et al., 2003).

Dentre os PAMs isolados de crustáceos peneídeos (camarões peneídeos), as peneidinas são caracterizadas por possuírem dois domínios com atividades diferentes, um domínio rico em prolina no N-terminal (PRD) e outro rico em cisteína no C-terminal (CRD) (CUTHBERTSON et al., 2002). Três famílias de peneidinas foram purificadas de *L. vannamei*, e apresentam ação contra bactérias Gram positivas e fungos filamentosos (DESTOUMIEUX et al., 1999; O´LEARY; GROSS, 2006). Com relação à expressão das isoformas de peneidinas em hemócitos de *L. vannamei*, foi observado que a PEN3 demonstrou uma maior expressão se comparado com as PEN2 e 4, utilizando-se o método quantitativo de PCR em tempo real (qRT-PCR) (O´LEARY; GROSS, 2006).

Outro tipo de resposta exibida pelo sistema imune de crustáceos é a produção de "proteínas do estresse", as HSPs (do inglês, *heat shock proteins*), estas moléculas são expressas para auxiliar na reparação de células e tecidos danificados pelo estresse (WELCH, 1992). Algumas classes de HSPs são constitutivas, sendo denominadas de proteínas cognatas, as quais funcionam em condição fisiológica normal. Por exemplo, a HSC70 é uma proteína cognata da classe HSP70 (WELCH, 1993).

Em *L. vannamei* foram estudadas a composição e expressão de HSP60 e HSP70, frente a várias condições de estresse, como infecção bacteriana e choque-térmico, observando-se que essas moléculas estão potencialmente envolvidas na resposta imune desses camarões (ZHOU et al., 2010). A expressão de HSP70 também foi relacionada com a co-infecção IHHNV e IMNV em *L. vannamei*, em situações de estresse ambiental. (VIEIRA-GIRÃO et al., 2012). Com a relação a resposta imunológica de camarões contra vírus, existem relatos da capacidade desses animais desenvolver uma memória específica, por exemplo, em estudos feitos em *L. vannamei* tratados com dsRNA, foi observado um aumento significativo da resistência à infecção por WSSV e TSV (ROBALINO et al., 2004; KURTZ; FRANZ, 2003). Assim como esses, outros relatos tem sido publicados de melhoria da resistência de camarões com vacinas contra vírus, preparadas, por exemplo, a partir de proteínas do capsídeo de vírus, partículas complexadas com vírus ou fragmentos de vírus, vírus inativados, dentre outros (WITTEVELDT et al., 2004ab; BRIGHT et al., 2005; VALDEZ et al., 2014; SOLÍS-LUCERO et al., 2016).

2.3.1.2.1 Peptídeos biologicamente ativos

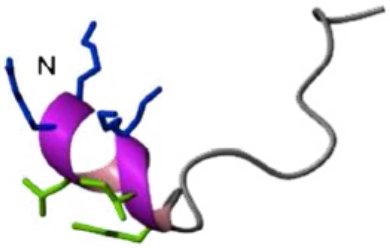
Peptídeos são um grupo de moléculas amplamente estudadas por sua diversidade de atividades biológicas. Como exemplo, estão os peptídeos antimicrobianos (PAMs) que são moléculas constituintes do sistema imune inato de vertebrados e invertebrados, funcionando como barreira química contra a invasão de microorganismos patogênicos. Eles demontram um amplo espectro de atividades contra fungos, bactérias, parasitas e vírus (MISHRA, et al., 2017).

Muitos PAMs foram descritos em diversos grupos de seres vivos, dentre eles moluscos, insetos, crustáceos, plantas, anfíbios, peixes e mamíferos. Seu mecanismo geral de ação é através de interação eletrostática com os fosfolipídios aniônicos das membranas de bactérias, por exemplo. Seguindo a inserção nas membranas de patógenos, os PAMs induzirão a formação de poros e eliminação do micro-organismo (LI et al., 2012; YEUNG; GELLATLY; HANCOCK, 2011). No caso de vírus, os PAMs podem atuar inibindo a adesão e a fusão dos vírus às células hospedeiras, e/ou desorganizando invólucros virais, e/ou inibindo a replicação através da interação com a polimerase viral (SKALICKOVA et al., 2015; JENSSEN; HAMILL; HANCOCK, 2006).

Um exemplo de peptídeos biologicamente ativos são as catelicidinas que estão distribuídas em uma ampla diversidade de animais. Os PAMs relacionados com a catelicidina ou CRAMPs (do inglês, *cathelicidin-related AMPs*) também foram encontrados em venenos de serpentes asiáticas. Recentemente, foram estudados e reportados os precursores de catelicidinas das glândulas de veneno de serpentes da América do Sul da família Viperidae. Estes precursores codificam sequências maduras, semelhantes a catelicidina, denominadas coletivamente de vipericidinas (Catelicidinas de viperídeos), e exibem ampla atividade antibacteriana (FALCAO et al., 2014).

Além disso, um corte estrutural e funcional de crotalicidina (Ctn), que recebeu esse nome por ser isolada de veneno de uma serpente sul-americana, *Crotalus durissus terrificus*, revelou um dos fragmentos, denominado Ctn [15-34] (Figura 8). Esse peptídeo derivado manteve as atividades biológicas antibacteriana, antifúngica e anticancerígena do Ctn parental, porém, com menor toxicidade e maior estabilidade em soro do que a crotalicidina de comprimento total (FALCAO et al., 2015, CAVALCANTE et al., 2016). A potencial atividade antiviral não havia sido investigada, antes do presente trabalho de tese, tornando assim, uma molécula muito promissora para o desenvolvimento de novos fármacos.

Figura 8 – Estrutura secundária do fragmento Ctn[15−34] da Crotalicidina (Ctn).
Fonte: Falcão et al., 2015).



Nos próximos capítulos, são apresentados dois artigos publicados com os resultados do projeto de investigação da tese referentes aos objetivos propostos.

3 LOW SALINITY FACILITATES THE REPLICATION OF INFECTIOUS MYONECROSIS VIRUS AND VIRAL CO-INFECTION IN THE SHRIMP *LITOPENAEUS VANNAMEI*

Artigo submetido ao periódico intitulado, *Journal Aquaculture Research and Development*, em 19 de novembro de 2014, aceito em 10 de dezembro de 2014 e publicado em 12 de janeiro de 2015 (Anexo I):

Todas as normas de redação e citação deste capítulo estão de acordo com as normas estabelecidas pelo periódico supracitado.

Resumo

O camarão branco *Litopenaeus vannamei* é a espécie de camarão cultivado comercialmente mais predominantes no mundo. No entanto, o cultivo intensivo do camarão em todo o mundo propicia surtos de epizootias, principalmente de etiologia viral. Na principal região brasileira de produção de camarão, sabe-se que uma redução nas salinidades dos viveiroa de cultivo causa o aparecimento de doenças virais. No presente trabalho, investigamos a replicação do vírus da mionecrose infecciosa (IMNV) em níveis controlados de salinidade durante as primeiras 12 horas de infecção. Usando PCR quantitativa em tempo real e análise estatística, verificamos que a baixa salinidade facilita positivamente a replicação e proliferação do IMNV, diminuindo o tempo de geração de 57,4 min (a 35 g L^{-1}, salinidade ideal) para 25,2 min a (5 g L^{-1}, concentração estressante). Da mesma forma, demonstrou-se uma relação positiva entre uma diminuição da salinidade e a redução do tempo de geração do vírus da necrose infecciosa persistente hipodermal e hematopoiética, um vírus persistente que geralmente co-infecta o camarão nos viveiros das fazendas.

Palavras-chave: *L. vannamei*; vírus de camarão; IMNV; qPCR; replicação viral; tempo de geração

Low Salinity Facilitates the Replication of Infectious Myonecrosis Virus and Viral Co-Infection in the Shrimp *Litopenaeus Vannamei*

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Abstract

The white leg shrimp *Litopenaeus vannamei* has been converted commercially into the most predominant cultivated shrimp species in the world. However, such shrimp's intensive farming worldwide propitiates outbreaks of epizootic diseases, primarily of viral etiology. In the principal Brazilian region of shrimp production, it is known that a reduction in the salinities of culture ponds causes the appearance of viral diseases. In the present work, we investigate the replication of the infection myonecrosis virus (IMNV) in controlled levels of salinity during the first 12 hours of infection. Using quantitative real-time PCR and statistical analysis, we verify that low salinity positively facilitates IMNV replication and proliferation by decreasing the generation time from 57.4 min (at 35 g L^{-1}, optimum salinity) to 25.2 min at (5 g L^{-1}, stressing concentration). Similarly, a positive relationship was demonstrated between a decrease in salinity and the reduction in the generation time of persistent infectious hypodermal and hematopoietic necrosis virus, a virus that usually co-infects shrimp in farm ponds.

Keywords: *L. vannamei*; Shrimp virus; IMNV; Qpcr; Epizootic agent; Viral replication; Generation time

Introduction

The white leg shrimp *Litopenaeus vannamei* is naturally found along the Pacific coast from the Golf of California to the north littoral of Peru [1]. Due to the rearing performance in shrimp farming, *L. vannamei* has been converted into the most predominant cultivated shrimp species in the world, reaching approximately 2.7 million cubic tons and over 10 billion dollars in sales in the year of 2010 [2]. The characteristics that make this species of shrimp adequate for commercial production, particularly in the Americas, include fast growth, low nutritional requirements, resistance to environmental stress, and a certain osmoregulatory capability for adaptation to a wide range of salinity (from 1 to 50 g L^{-1}) [3]. The intensive rearing techniques required for efficient and cost-effective shrimp farming unintentionally propitiate the outbreaks of epizootic diseases, and severe microbial infection is consequently a recurrent threat in the shrimp industry. Some of the most serious causative infectious agents in shrimp aquaculture are viruses.

The northeastern part of Brazil is the most productive region with a total of 18,500 hectares of shrimp farms that account for approximately 70,000 tons of shrimp, which corresponds to 97% of the national production [4,5]. In this region, two main types of viruses are of great concern: the infection myonecrosis virus (IMNV) and the infectious hypodermal and hematopoietic necrosis virus (IHHNV). Based on their genome organization, the phylogeny of their macromolecular components, the structural characteristics of their capsids and the physical-chemical properties of the viral particles, the first is classified as a member of the Totiviridae family [6,7], and the latter belongs to the Parvoviridae family [8]. As a virus of the Totiviridae family, IMNV is double-stranded RNA virus deprived of an envelope with an isometric capsomer and a genome size of 7560 base pairs encoding two nonoverlapping open reading frames (ORFs), which comprise a predicted RNA-binding protein and a capsid protein [7]. In contrast, IHHNV is a non-enveloped, symmetric icosahedral parvovirus with a singlestranded linear DNA genome composed of 3909 nucleotides and three superposed ORFs [8,9]. When infecting penaeid shrimps, IMNV causes high mortality rates by acutely destroying (via necrosis) the skeletal muscle of distal segments and the tail fan. In contrast, IHHNV causes chronic deformity syndrome and reduced growth and culture performance [10]. In both cases, environmental factors, such as salinity and temperature, appear to trigger viral outbreaks in shrimp culture.

In a previous survey, after an unusual period of rainfall resulting in high mortality and significant economic losses in local shrimp production, we used molecular procedure analysis to find that a high number of samples from extensively farmed L. vannamei developing IHHN or IMN disease were co-infected with both (IHHNV and IMNV) viral agents [11]. In addition, we have shown that the disease symptoms and outcomes found for the co-infected shrimps resulted from reciprocal viral replication, i.e., the proliferation of one type of virus impairs the multiplication of the other. In the same study, we observed that IHHNV appeared to modulate the expression of heat shock protein 70 (HSP70) in IHHNV/IMNV double-infected shrimp.

In aquatic organisms, particularly shrimps, the oscillation provoked by physical (e.g., temperature), chemical (pesticides, pH and salinity) and biological (epibionts, epizootics, enzootics, etc.) insults, which are part of the equivocal strategies of farming management, may independently or cooperatively work to cause outbreaks of severe infections and mortality [12-16]. Moreover, a direct relation between environmental stress, immunity imbalance and development of bacterial and viral infection and disease has been observed at molecular and organism level in shrimp [17,18].

The aim of the present work was to investigate, in controlled laboratory conditions, the influence of low salinity on the replication of IMNV in *L. vannamei*. Additionally, we monitored the proliferation of IHHNV and estimated the generation time (g) of replication for both viruses under the influence of different salinities.

Material and Methods

Shrimp maintenance and experimental viral infection

A total of 150 macroscopically healthy shrimps were obtained from a local shrimp farm (Paraípaba, Ceará - CE, Brazil) and transported to the Laboratory of Aquatic Resources of the Federal University of Ceará (CE-Brazil). The shrimps were acclimated for one week in a 1000 L tank filled with seawater, the salinity of which was similar to that in the collection site (35 g L^{-1}) and controlled through a flow-through system. The shrimp were fed ad libitum a commercial diet (35% crude protein; Nutreco Fri-Ribe®,

Ceará, Brazil). After acclimation, the shrimps (9.3 ± 1.2 g) were captured, anaesthetized with 100 mg L^{-1} benzocaine, randomly selected, weighed, counted and stocked into 30 L aquaria (10 shrimp per aquarium) with three replicate aquaria for each treatment. The experimental procedure was conducted with four different salinities, yielding a control group (35 g L^{-1}) and four treatments (5, 15, 25 and 35 g L^{-1}). Each treatment was operated on a common recirculation system with 20 L mechanical and biological filters and a water exchange of approximately 0.5 L min^{-1} per aquarium. Each aquarium was equipped with an air diffuser to maintain an oxygen concentration in the water close to saturation. Nets to prevent the shrimp from jumping out covered each aquarium. The salinity was gradually reduced by pumping disinfected freshwater at a rate of 0 (control and treatment 1) or 2 g L^{-1} (treatments 2, 3 and 4) per hour until reaching the salinity corresponding to each treatment. The salinity levels were monitored using an optical refractometer. The water in the aquaria were maintained at ambient temperature within a range of 27.5 to 30.5°C and a mean temperature of 29.0°C during the experimental period. The temperature differences among the aquaria never exceeded 0.2°C. A photoperiod of 12 h of light (L)/12 h of darkness (D) was maintained during the experiment.

The IMNV inoculum was obtained from the muscle of IMNV infected shrimp collected during a disease outbreak that occurred in a shrimp cultivation pond in 2011. For viral extract preparation, the muscle from infected shrimp (*L. vannamei*) was homogenized in PBS (0.2 M phosphate buffered saline, pH 7.3) (1:3, w/v). The shrimp extract was centrifuged at 3000×g for 5 min, and the supernatant was used for viral inoculation into healthy shrimps. The concentration of the IMNV stock was quantified by real-time PCR and found to be a target copy number of 10^4 per microliter. For the shrimp challenges, 10 µl of the viral suspension was injected into each shrimp. The shrimp in the negative control group were injected with the same volume of phosphate buffered-saline.

Shrimp tissue processing and total RNA purification

The hepatopancreas from three shrimps in each group was dissected at 0, 1.5, 3, 6 and 12 hours post-injection and transferred to microtubes containing RNA Later solution (Life Technologies, CA, USA) for total RNA purification. The samples were maintained at 4°C until processing, and the total RNA was purified within one week following

collection. The inoculated shrimps were observed from 0 to 48 h post infection for clinical signs of IMN disease, such as anorexia, lethargy and mortality.

The total RNA from the minced hepatopancreas (20 to 30 mg) was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's protocol, which includes a DNase I treatment step. The quality and yield of the total RNA were verified by assessing the integrity of 28S and 18S rRNA and by spectrometrically assessing the 260/280 nm ratio.

cDNA synthesis from viral RNA

For complementary DNA (cDNA) synthesis, up to 1 μg of each DNase I-treated total RNA sample, which was mixed with 500 ng of random primers (Promega, Madison, WI, USA) in a final volume of 10 μl, was heated to 70°C for 10 min and cooled at 4°C. To complete the reverse transcriptase reaction mixture, the following components were mixed with the denatured RNA in a final volume of 20 μl: 100 U of ImProm II reverse transcriptase enzyme (Promega, Madison, WI, USA), 1 mM of each deoxynucleoside triphosphate, 2 mM $MgSO_4$, 1 mM dithiothreitol, and 20 U of RNase inhibitor. The reverse transcription mixture was incubated at 42°C for 90 min and then at 70°C for 15 min. The cDNA was diluted tenfold with TE (10 mM Tris–HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid), and 2 μl aliquots were used for the relative and quantitative real-time PCR (qPCR) experiments.

Quantitative real-time PCR of shrimp virus (IMNV and IHHNV)

For the quantification of the IHHNV and IMNV loads in *L. vannamei*, the absolute quantitative strategy was used. The genes encoding the nonstructural proteins of IMNV (GenBank accession number AAT67231.1) and of IHHNV (GenBank accession number AAF59415.1) were cloned, and serial 10-fold dilutions of each gene were prepared to establish the qPCR standard curves. The standard curve series were constructed in triplicate. The linearity of the qPCR standard curve was expressed as the square of the Pearson correlation coefficient (r^2). The primers for the qPCR detection of IMNV and IHHNV, in addition to those used for the shrimp β-actin gene, are detailed elsewhere [11].

The amplification of all cDNAs in this study was conducted in a Rotor-Gene 3000 system operated with its respective software (version 6.0.19; Corbett Research, Mortlake, Australia). Each reaction, which was conducted in a final reaction volume of 20 μl, consisted of 2.0 μl of the cDNA (~10 ng of reverse-transcribed mRNA), 0.2 μM of each gene specific sense and anti-sense primer, and 10 μl of two-fold concentrated GoTaq qPCR Master Mix (Promega, Madison, WI, USA). The amplification conditions for the viruses were as follows: 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The fluorescence was collected at 494 to 521 nm during the extension phase.

To calculate the copy number in the absolute qPCR experiments, the following equation was used (http://www.uri.edu/research/gsc/resources/cndna.html): Number of copies = [DNA amount (ng) *6.022×1023] / [DNA length (nt)* 1×109 *650]. The threshold and threshold cycle values were automatically determined by the Rotor Gene 6.0.19 software using the default parameters. All of the measurements were obtained as the means of at least nine measurements ± SEM (less than 5% error). The corresponding real-time PCR efficiencies (E) of the cycles in the exponential phase were calculated from the given slopes (k) according to the following equation: $E=10(-1/k)-1$. To normalize the values of the viral load, the mean copy number of β-actin transcripts in each sample, which is equivalent to 1 μg, was determined from at least ten independent experiments ($n \geq 30$); the results of the viral infection and gene expression analyses are denoted as the logarithm of the copy number.

Statistical analysis

The statistical analyses were performed with the BioStat 5.0 software using one-way ANOVA. In the cases in which significant differences were observed, the Least Significant Differences (LSD) test was applied. The positive correlation between the viral loads was expressed as the Pearson coefficient (r).

Results and Discussion

In a recent study, we assessed the expression level of selected gene transcripts (i.e., crustin, peneidin-3a, C-type lectin and HSP70) related to the innate immune systems

of shrimp in response to viral infection caused by IMNV and IHHNV after an unusual period of rainfall in a delimited shrimp production area [11]. Under the natural conditions of shrimp culture, we detected that a high proportion of shrimp samples were positive for both viruses, i.e., they were environmentally co-infected with IMNV and IHHNV. Interestingly, a phenomenon of reciprocal viral replication appeared to occur in this type of coinfection: the species of virus and the viral load at the beginning of the infection determined the disease outcome, i.e., IMN or IHHN disease in the shrimp. Moreover, the level of HSP70, which is a cytoprotective protein, was up-regulated by viral infection and displayed a positive correlation with IHHNV replication. However, these data were obtained from shrimps sampled under natural culture conditions, in which some parameters, such as salinity, temperature and exposition to a complex microbiota (and potential pathogens), were not subjected to a strict control. Therefore, we were compelled to conduct a controlled set of experiments on virus replication in shrimp as a function of different salinities to verify the correlation between salt stress and viral proliferation.

With this aim, a total of 150 asymptomatic adult shrimps were acclimatized in the laboratory to different levels of salinity (5, 15, 25 and 35 g L^{-1}) and intramuscularly inoculated with 100,000 IMNV particles (10^4/μL). Samples of the hepatopancreas were surgically collected from three individual shrimps 0, 1.5, 3, 6 and 12 h post-infection, and the level of viral replication was assessed by qPCR. From the same samples, the number of copies of IHHNV and the number of transcripts of crustacean β-actin were determined. As might be expected, the number of IMNV particles increased steeply in all salinities tested (Figure 9, part A). Unexpectedly, IHHNV was also detected just after the beginning of the experiment and thereafter, even though the only virus that was deliberately inoculated in the study was IMNV (Figure 9B). This finding reflects the nature of IHHNV as a persistent epizootic agent that is present in all stages of the shrimp life cycle, including larvae and asymptomatic adults, and that is capable of invading the germ line and integrating into the host genome. Interestingly, in the first hours post-inoculation (between 1.5 h and 3 h), the high number of IMNV particles suppressed the replication of IHHNV, as observed in Figure 9, in agreement with one of our previous studies. In the subsequent period of infection, IHHNV also proliferated gradually over, particularly at a salinity of 5 g L^{-1}, whereas IMNV replicated exponentially over time and in lower salinities. At a salinity of 35 g L^{-1}, the estimated generation times for IHHNV and IMNV were 37.2 min and 57.4 min, respectively, in contrast to the values of 17.1 min and 25.2

min found at 5 g L^{-1}, respectively. This estimation, which was obtained through the calculation of the viral copy number as a function of the time post-infection, clearly indicates that the generation time for both viruses is practically reduced by two-fold when the salinity is decreased from 35 g L^{-1} to 5 g L^{-1}. Curiously, these values indicate that the persistent IHHN virus detains a lower generation time than IMNV, but due to the experimental design in the aquarium for IMNV replication, as well as the phenomena of reciprocal replication that we had previously observed, the fortuitous number of viral copies of IHHNV was constantly inferior to that of IMNV through the experiment. Moreover, as noted, the replication of IMNV occurs in all levels of salinity and periods of time post-infection, by which were experimentally and statistically tested (coefficient of Pearson, r = 79.65%). The positive correlation between the different salinities and the time of infection for IMNV versus IHHNV was observed with high confidence (Figure 10).

According to our measurements, the peak of IMNV replication reached a maximum in 6 h after infection, suggesting a rapid and efficient mechanism of cell entry and co-option of the molecular machinery of host cells for viral proliferation. In the earlier period of infection (from 0 h to 3 h), the difference in the virus number relative to the preceding hour was not statistically significant, although we observed an increment in the IMNV copy number (Figure 11).

The decrease in the salinity of shrimp culture ponds during the rainy season and the appearance of viral diseases is a known fact of shrimp producers in the northeastern region of Brazil. The scientific reasoning underlying this phenomenon calls for the influence of the osmotic shock response on the components of the crustacean innate immunity system. Thus, a stress caused by osmotic adjustment may trigger the viral replication and the high shrimp mortality observed in the field.

In fact, the maintenance of the isosmotic level of salinity is required to avoid a reduction in the efficiency of innate immunity responses and an increase in the vulnerability of marine shrimps to epizootic agents [19]. The isotonic salinity is a key environmental factor influencing the physiology of numerous species of marine organisms, from algae to fishes. In shrimps in particular, abrupt changes in salinity can affect the metabolic efficiency, the consumption of oxygen, and the rates of growth and survival [20]. Li and collaborators observed that shrimps inoculated with *Vibrio*

alginolyticus and maintained under low salinity displayed, after 6 to 12 h, a significant reduction in immune factors, such as the number of hemocytes and prophenoloxidase activity.

The relationship between fluctuations in salinity and the susceptibility of shrimp to virus infection has been increasingly studied in the case of white spot syndrome virus (WSSV). In a study conducted by Vaseeharan and collaborators [20], the influence of low salinity on the innate immune system of healthy *Fenneropenaeus indicus* challenged with white spot syndrome virus (WSSV) was investigated. These researchers observed a reduction in the shrimp immune competence and an increase in the susceptibility to the virus. In addition, Ramos-Carreño and co-workers studied the susceptibility of *L. vannamei* to WSSV in several levels of salinities and found that the clinical manifestation of viral infection was more severe in condition of hyposmolarity. The low salinity was also verified to contribute to a decrease in the osmoregulation performance of *L. vannamei* and an increase in the replication of WSSV, resulting in higher rates of shrimp mortalities [21].

In the same line, our results demonstrate that the replication rate was higher at a lower salinity (i.e., 5 g L^{-1}) for both viruses, the IMNV and the persistent IHHNV, over a period of 6 to 12 h of infection (Figures 12 and 13).

In summary, we focused our analysis primarily on the replication of IMNV in controlled conditions with different salinities and thus controlled osmotic conditions. Using quantitative real-time PCR data and statistical analysis, we verified that low salinity facilitates the replication of infectious myonecrosis virus by decreasing the generation time. In addition, under conditions of fluctuation of salt content in salt water, we observed the same behavior in the proliferation of the persistent IHHN virus, i.e., a decrease in the time for viral duplication and a positive correlation between a low level of salinity and increased virus multiplication, consequently, facilitating the co-infection in *L. vannamei*.

Acknowledgment

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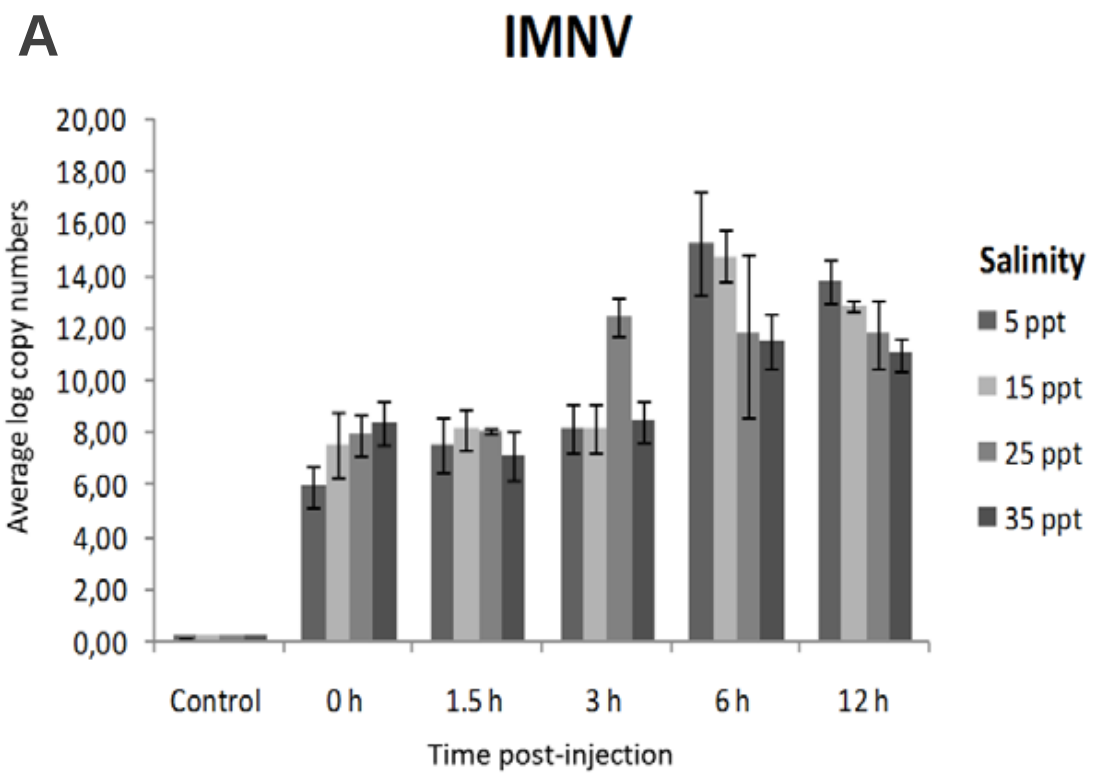
Figures


A
IMNV
Average log copy numbers
20,00
18,00
16,00
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
Control
0 h
1.5 h
3 h
6 h
12 h
Time post-injection
Salinity
5 ppt
15 ppt
25 ppt
35 ppt

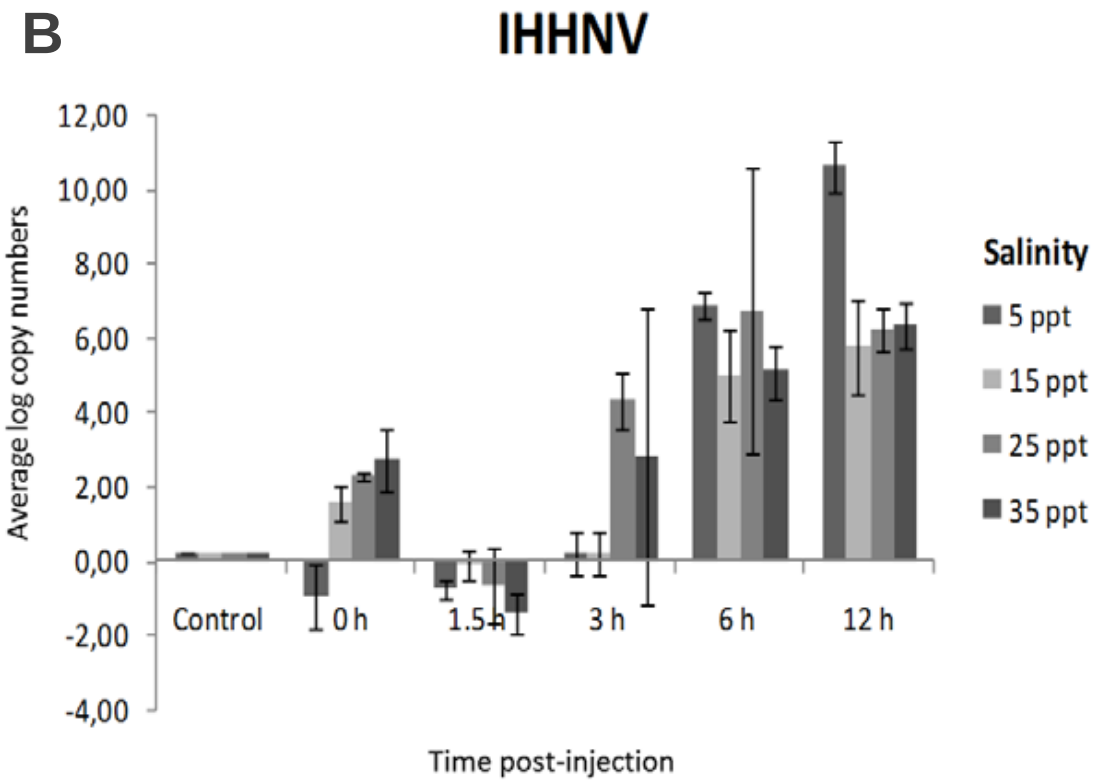

B
IHHNV
Average log copy numbers
12,00
10,00
8,00
6,00
4,00
2,00
0,00
-2,00
-4,00
Control
0 h
1.5 h
3 h
6 h
12 h
Time post-injection
Salinity
5 ppt
15 ppt
25 ppt
35 ppt

Figure 9: Influence of different salinities on the replication of IMNV and IHHNV in L. vannamei. Shrimps were acclimatized in 5, 15, 25 and 35 g L-1 of salinity and experimentally infected with IMNV. The viral replication was assessed 0 h, 1.5 h, 3 h, 6 h and 12 h after virus inoculation (A). The replication of IHHNV was also monitored (B). In each shrimp sample ($n \geq 6$), the actively transcribed genes from IMNV and IHHNV were quantified by absolute qPCR using cDNA as the template and specific primers. The number of viral particles was then normalized in relation to the logarithm (Log10) of the mean copy number of 1 μg of shrimp β-actin transcripts.

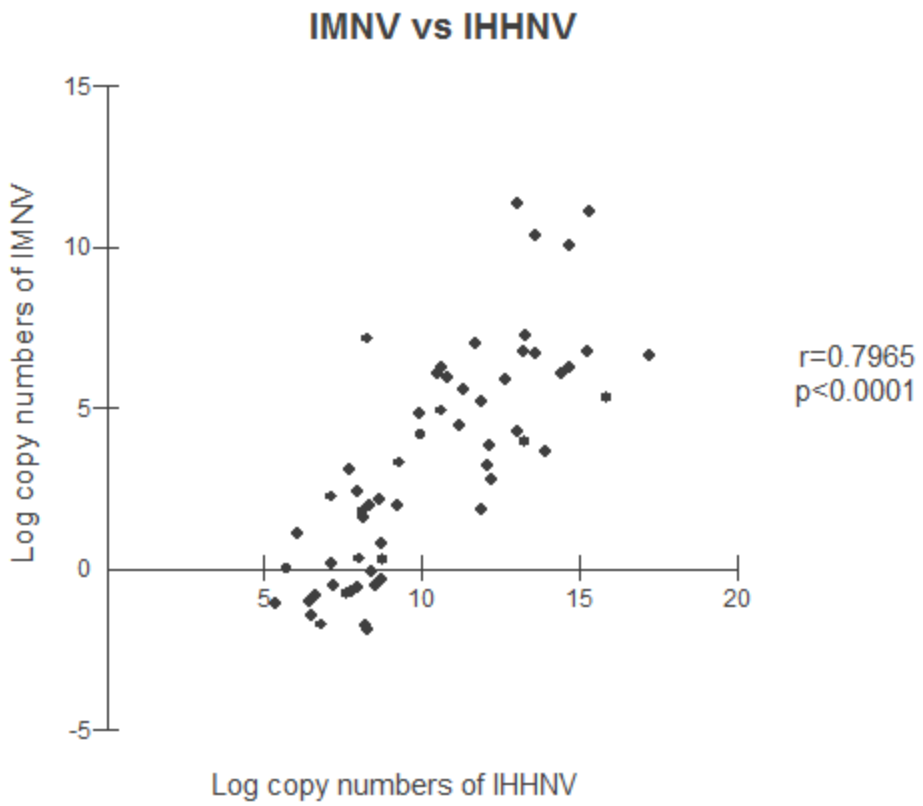
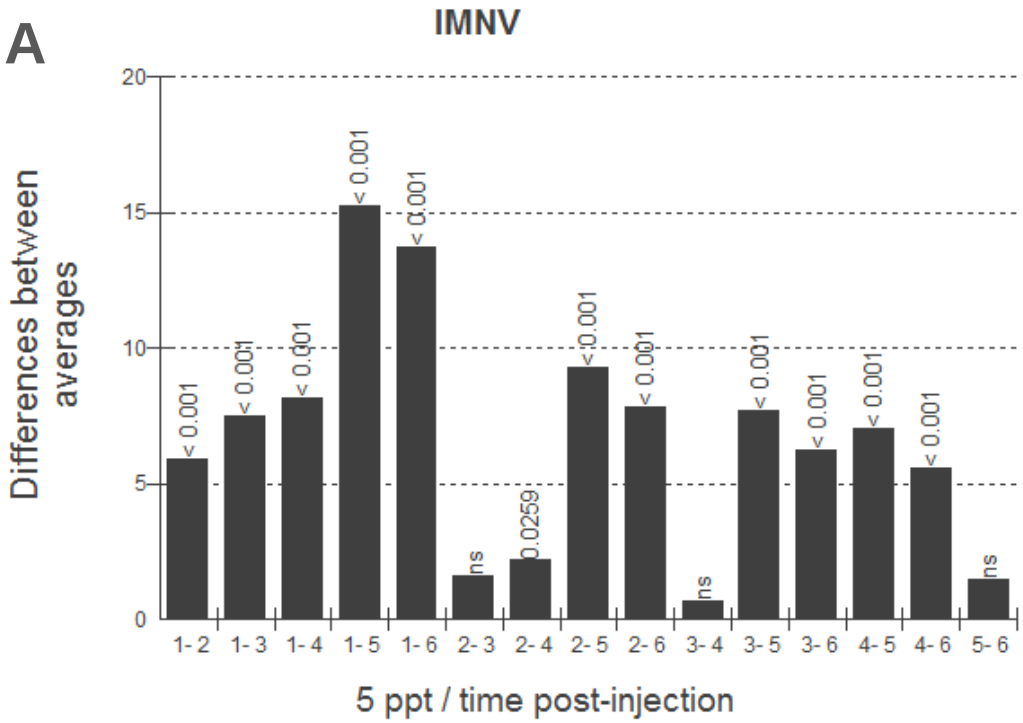
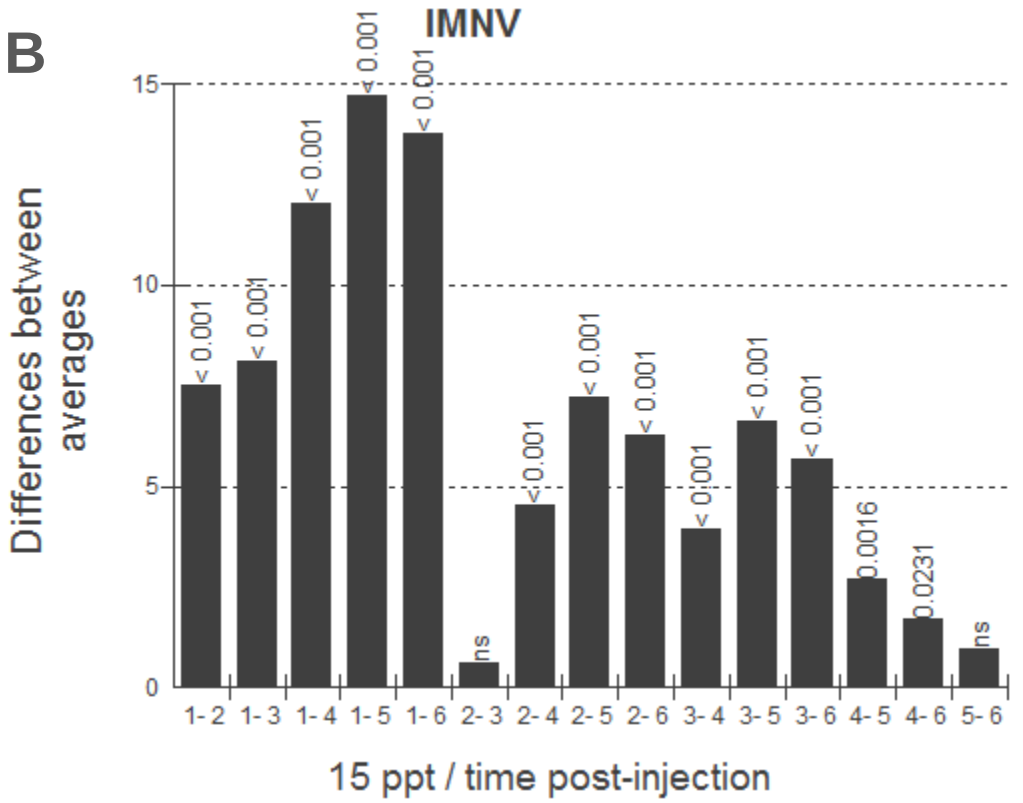
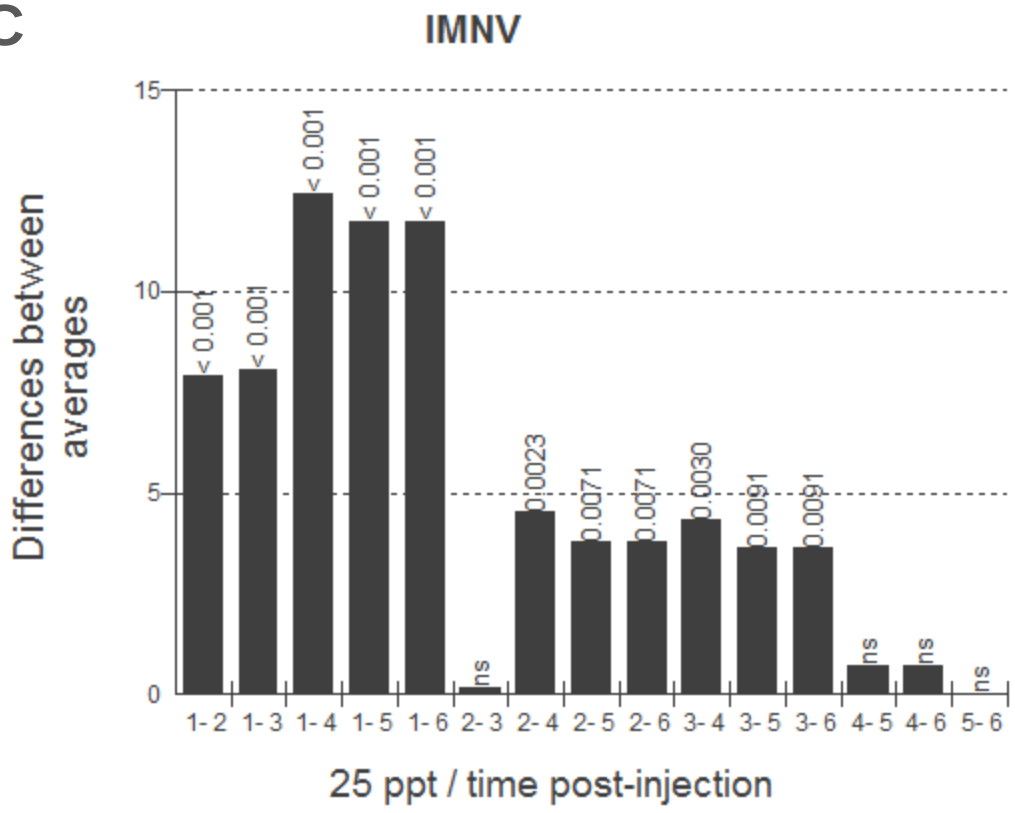


Figure 10: Linear correlation between IMNV and IHHNV replication in shrimp. Plot (coordinate pairs) of IMNV vs. IHHNV proliferation in different salinities and at different times post-infection. The quantitative PCR data were obtained and statistically analyzed as described in the text. The values of the Pearson coefficient (r) and probability (p) are shown in the plots.


A
IMNV
Differences between averages
20
15
10
5
0
< 0.001
< 0.001
< 0.001
< 0.001
< 0.001
ns
0.0259
< 0.001
< 0.001
ns
< 0.001
< 0.001
< 0.001
< 0.001
ns
1-2 1-3 1-4 1-5 1-6 2-3 2-4 2-5 2-6 3-4 3-5 3-6 4-5 4-6 5-6
5 ppt / time post-injection


B
IMNV
Differences between averages
15
10
5
0
< 0.001
< 0.001
< 0.001
< 0.001
< 0.001
ns
< 0.001
< 0.001
< 0.001
< 0.001
< 0.001
< 0.001
0.0016
0.0231
ns
1-2 1-3 1-4 1-5 1-6 2-3 2-4 2-5 2-6 3-4 3-5 3-6 4-5 4-6 5-6
15 ppt / time post-injection

C


IMNV
Differences between averages
15
10
5
0
< 0.001
< 0.001
< 0.001
< 0.001
< 0.001
ns
0.0023
0.0071
0.0071
0.0030
0.0091
0.0091
ns
ns
ns
1-2 1-3 1-4 1-5 1-6 2-3 2-4 2-5 2-6 3-4 3-5 3-6 4-5 4-6 5-6
25 ppt / time post-injection

D

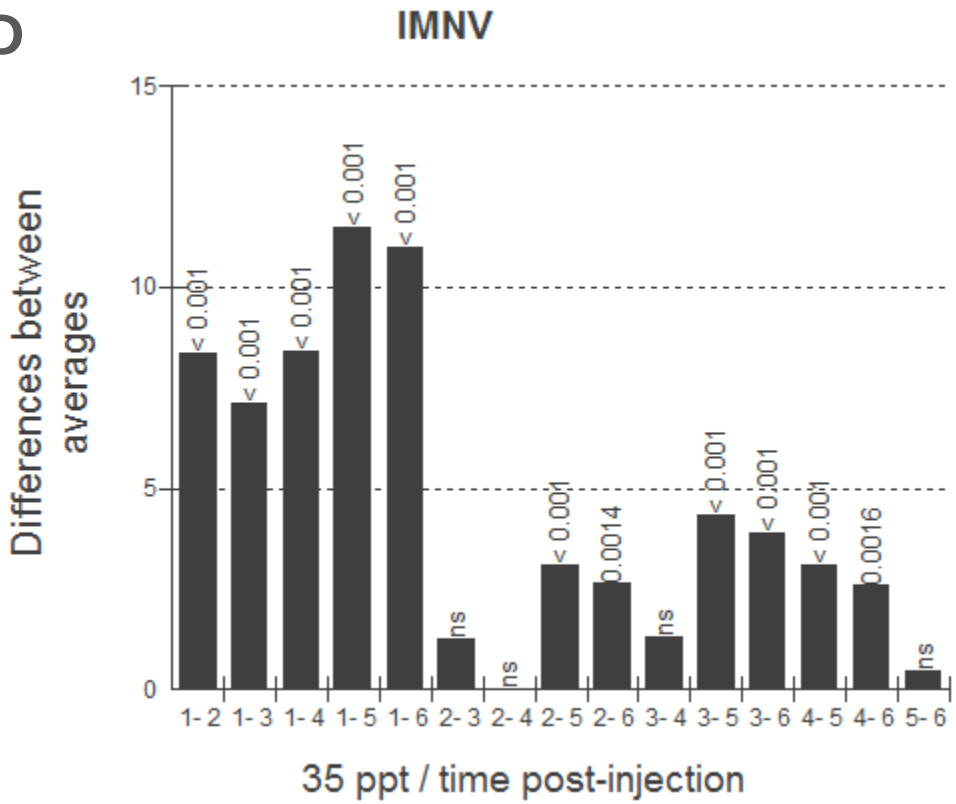
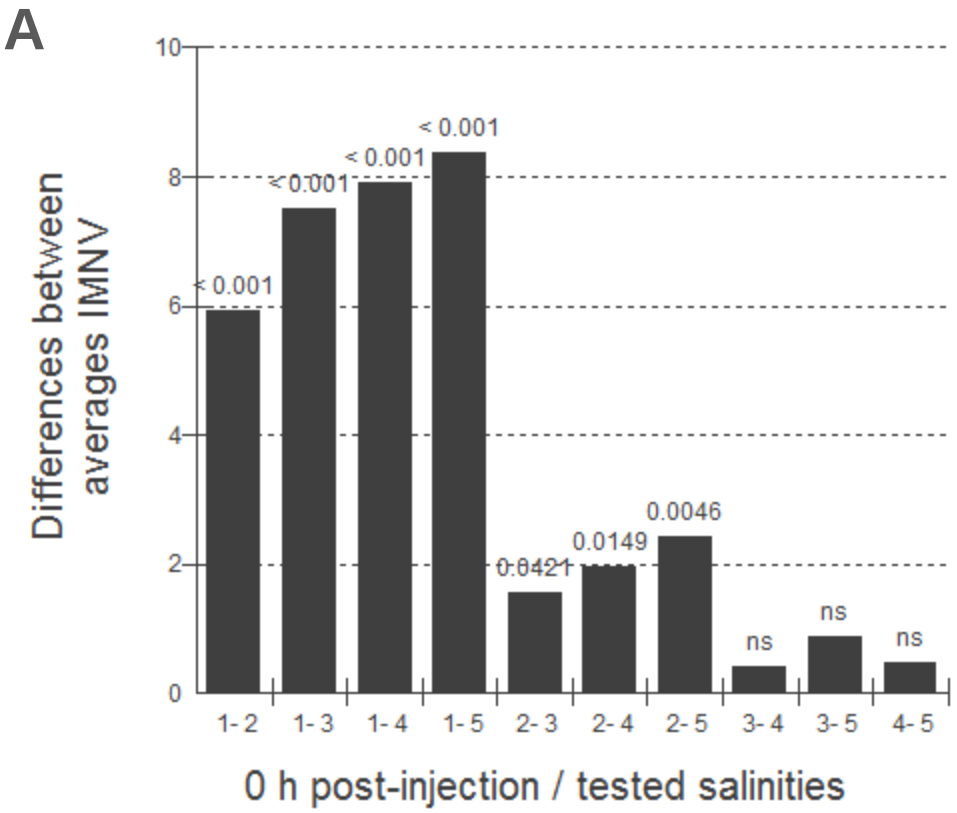
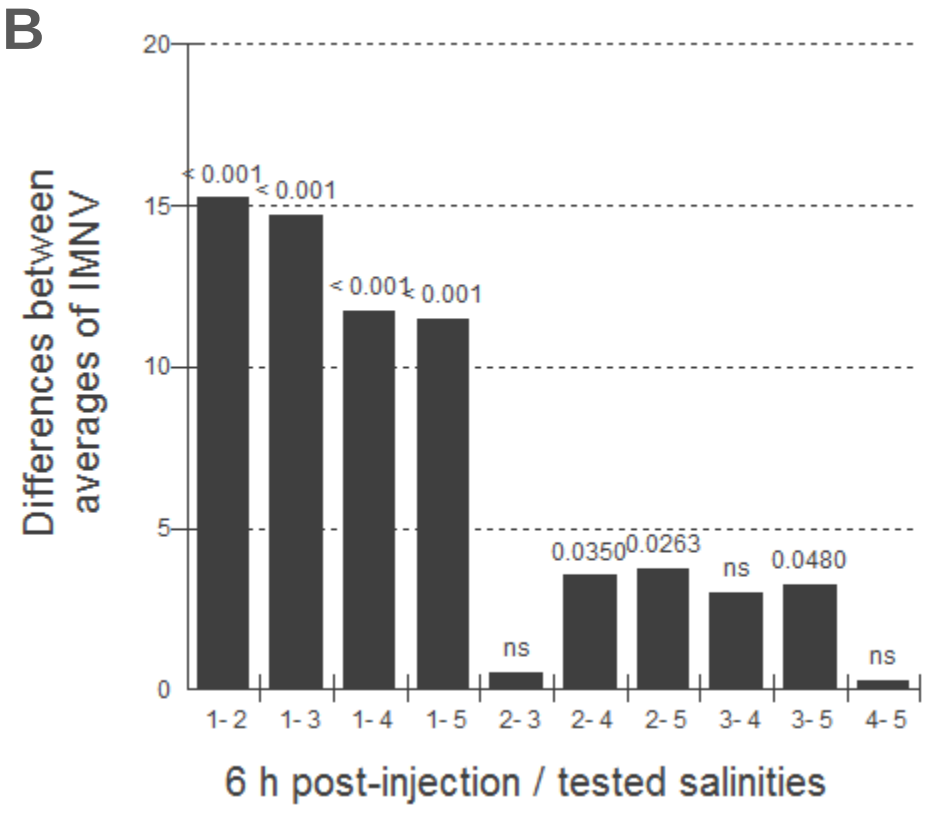

IMNV
Differences between averages
15
10
5
0
< 0.001
< 0.001
< 0.001
< 0.001
< 0.001
ns
ns
< 0.001
0.0014
ns
< 0.001
< 0.001
< 0.001
0.0016
ns
1-2 1-3 1-4 1-5 1-6 2-3 2-4 2-5 2-6 3-4 3-5 3-6 4-5 4-6 5-6
35 ppt / time post-injection

Figure 11: Significant differences in the replication of IMNV in infected shrimp over time. Differences between the logarithm of the mean copy number of IMNV in control shrimps (uninfected, numbered #1) and post-infected individuals at time 0 h (#2), 1.5 h (#3), 3 h (#4), 6 h (#5) and 12 h (#6). The significance level is indicated on top of the bar. The abbreviation “ns” denotes “no statistically significant difference”.


A
Differences between averages IMNV
10
8
6
4
2
0
< 0.001
< 0.001
< 0.001
< 0.001
0.0421
0.0149
0.0046
ns
ns
ns
1-2
1-3
1-4
1-5
2-3
2-4
2-5
3-4
3-5
4-5
0 h post-injection / tested salinities


B
Differences between averages of IMNV
20
15
10
5
0
< 0.001
< 0.001
< 0.001
< 0.001
ns
0.0350
0.0263
ns
0.0480
ns
1-2
1-3
1-4
1-5
2-3
2-4
2-5
3-4
3-5
4-5
6 h post-injection / tested salinities

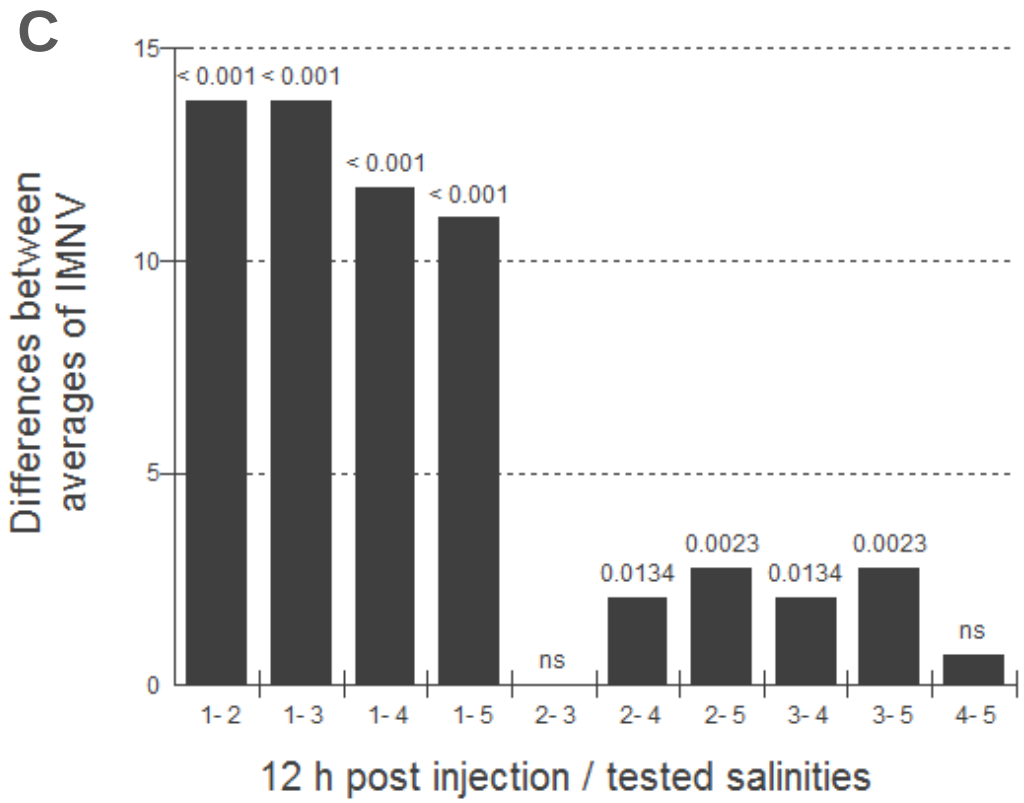
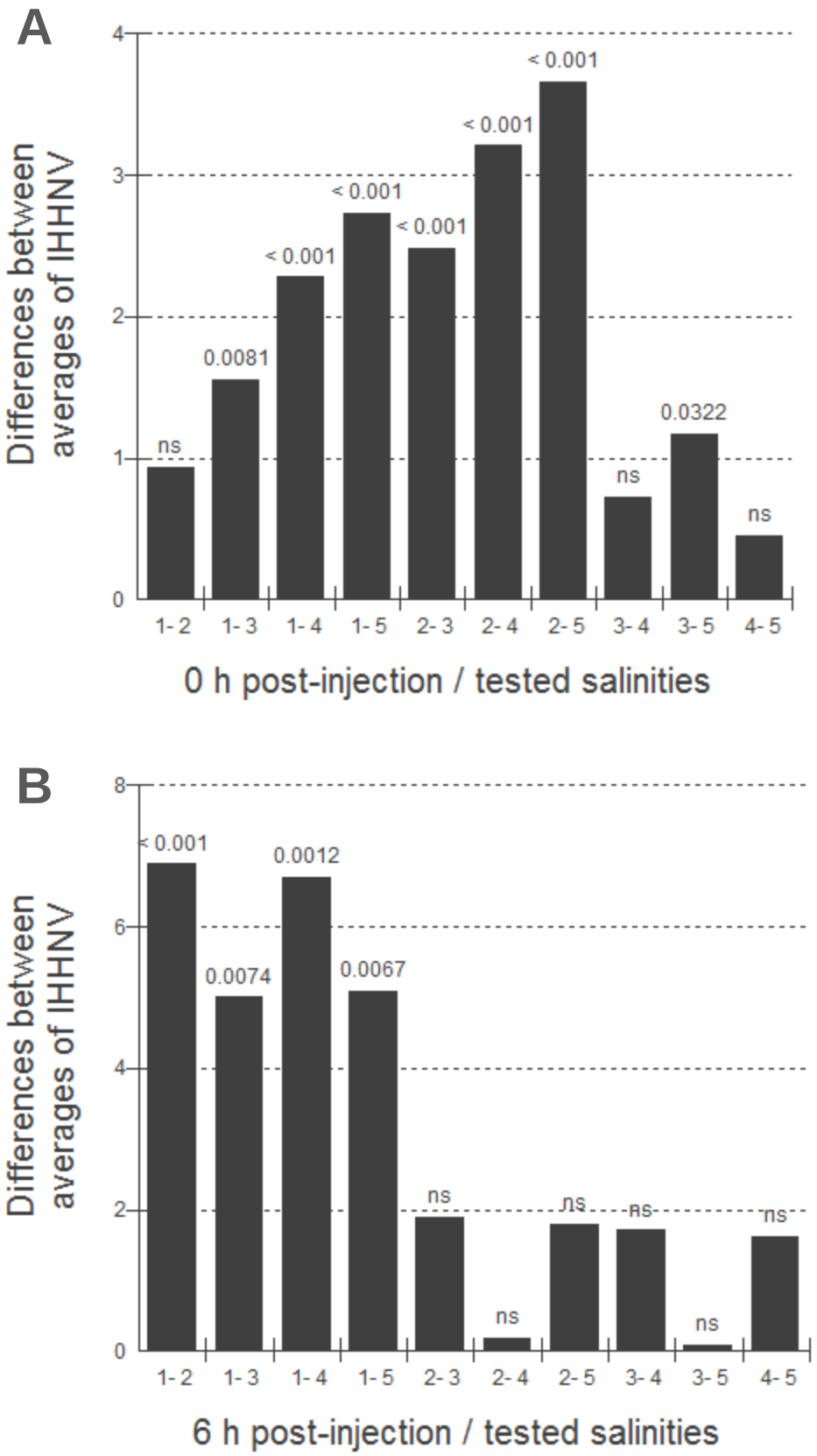


Figure 12: Significant differences in the replication of IMNV in infected shrimp in relation to the salinity and time. Differences between the logarithm of the mean copy number of IMNV in control shrimps acclimated to a salinity of 35 g L-1 (numbered #1) and infected individuals at a salinity of 5 g L-1 (#2), 15 g L-1 (#3), 25 g L-1 (#4), or 35 g L-1 (#5) were monitored at time 0 h (A), 6 h (B) and 12 h (C). As mentioned in the legend of figure 3, the significance level is indicated on top of the bar, and "ns" denotes "no statistically significant difference".


A
Differences between averages of IHHNV
0
1
2
3
4
ns
0.0081
< 0.001
< 0.001
< 0.001
< 0.001
ns
0.0322
ns
1- 2
1- 3
1- 4
1- 5
2- 3
2- 4
2- 5
3- 4
3- 5
4- 5
0 h post-injection / tested salinities
B
Differences between averages of IHHNV
0
2
4
6
8
< 0.001
0.0074
0.0012
0.0067
ns
ns
ns
ns
ns
1- 2
1- 3
1- 4
1- 5
2- 3
2- 4
2- 5
3- 4
3- 5
4- 5
6 h post-injection / tested salinities

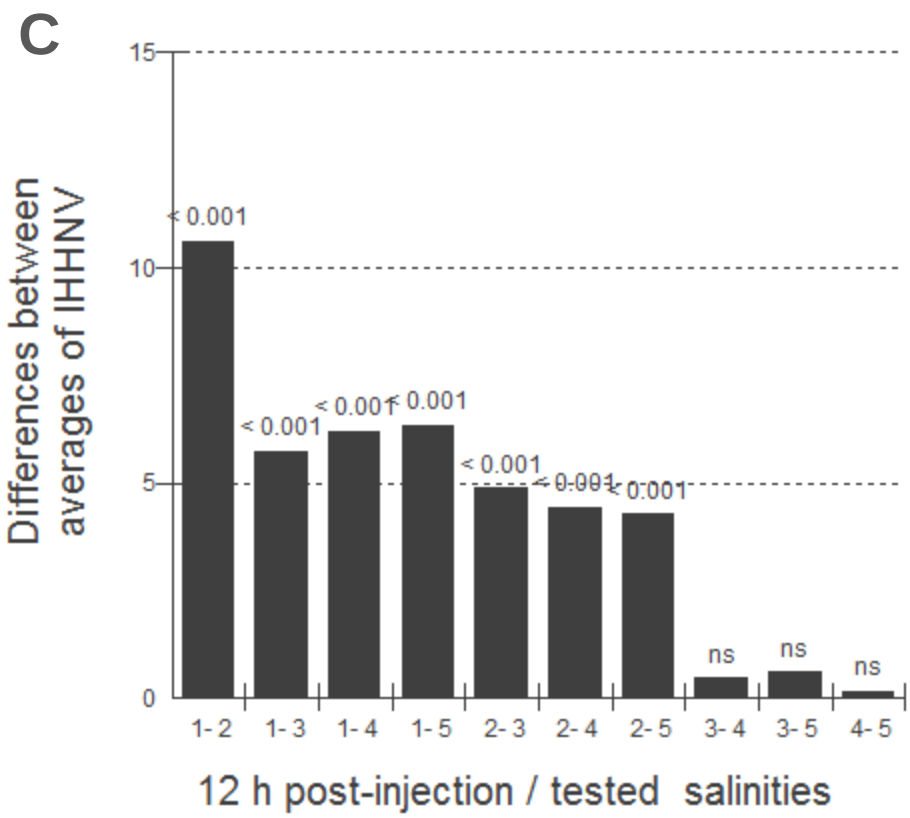


Figure 13: Significant differences in the replication of IHHNV in infected shrimp in relation to the salinity and time. Differences between the logarithm of the mean copy number of IHHNV in control shrimps at a salinity of 35 g L-1 (numbered #1) and infected individuals adapted to a salinity of 5 g L-1 (#2), 15 g L-1 (#3), 25 g L-1 (#4), or 35 g L-1 (#5) were monitored at time 0 h (A), 6 h (B) and 12 h (C). As mentioned, the significance level is indicated on top of each bar, and "ns" denotes "no statistically significant difference".

4 ANTIVIRAL ACTIVITY OF CTN[15-34], A CATHELICIDIN-DERIVED EICOSAPEPTIDE, AGAINST INFECTIOUS MYONECROSIS VIRUS IN *LITOPENAEUS VANNAMEI* PRIMARY HEMOCYTE CULTURES

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Todas as normas de redação e citação deste capítulo estão de acordo com as estabelecidas pelo periódico supracitado.

Resumo

A carcinicultura tem se tornado em uma indústria de aquicultura experiente lidando com milhões de toneladas métricas de *commodities* processadas. No entanto, as produções globais de camarão são constantemente ameaçadas por surtos de doenças, principalmente desencadeados pela disseminação rápida de vírus. O vírus da mionecrose infecciosa (IMNV) é um desses agentes epizoóticos que afetam a produção de camarão no Brasil, dos quais não existe tratamento. Aqui, foi demonstrada a atividade antiviral contra IMNV de um eicosapeptídeo, denominado Ctn [15-34], derivado de um membro da família de pepídeos antimicrobianos de catelicidina. Foram estabelecidas culturas de hemócitos de *Litopenaeus vannamei* que suportam replicação de IMNV e titulação de infecciosidade. O efeito citotóxico do IMNV em cultura e a atividade anti-IMNV *in vitro* de Ctn [15-34] foram avaliados utilizando um método de base fluorescente altamente sensível em combinação com PCR quantitativa. O Ctn [15-34] (<12,5 μM) neutralizou os efeitos tóxicos do IMNV em cargas suficientes para matar 50% dos hemócitos de camarão. Este estudo relatou pela primeira vez a replicação do IMNV *in vitro* e o emprego de uma metodologia direta para avaliar a viabilidade celular e as atividades virais / antivirais. Além disso, forneceu a base para o desenvolvimento do anti-infeccioso eicosapepide Ctn [15-34] e análogos como componentes de formulações antivirais contra doenças virais de camarão.

Palavras-chave: *L. vannamei*; vírus da mionecrose infecciosa; doença de camarão; peptídeo antiviral; eicosapeptídeo derivado de catelicidina; cultura primária de hemócitos

Antiviral activity of Ctn[15-34], a cathelicidin-derived eicosapeptide, against infectious myonecrosis virus in *Litopenaeus vannamei* primary hemocyte cultures

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Abstract

The shrimp farming has been converted into a mature aquaculture industry dealing with over millions of metric tonnes of processed commodities. Nevertheless, the global shrimp productions are constantly threatened by disease outbreaks, mainly triggered by rapidly disseminating viruses. Infectious myonecrosis virus (IMNV) is one of these epizootic agents affecting shrimp production in Brazil, of which no treatment exists. Herein, the antiviral activity against IMNV of an eicosapeptide, named Ctn[15-34], derived from a member of the cathelicidin family of antimicrobial peptides, was demonstrated. Cultures of hemocytes from *Litopenaeus vannamei* were established that support IMNV replication and infectivity titration. The cytotoxic effect of IMNV in culture and the in vitro anti-IMNV activity of Ctn[15-34] were assessed using a high sensitive fluorescent-based method in combination with quantitative PCR. The Ctn[15-34] (< 12.5 µM) neutralized the toxic effects of IMNV at loads sufficient to kill 50% of shrimp hemocytes. This study reported for the first time the replication of IMNV in vitro and the employment of a straightforward methodology to assess cell viability and viral/antiviral activities. In addition, it provided the basis for the development of the anti-infective multi-effector Ctn[15-34] eicosapepide and analogues as components of antiviral formulations against shrimp viral diseases.

Keywords: *L. vannamei* aquaculture; IMN virus; shrimp disease; anti-infective peptide; cathelicidin-derived eicosapeptide; fluorescence cell viability/cytotoxicity assay; hemocyte in vitro culture

INTRODUCTION

The shrimp farming has spread out to meet the increasing human food demand and to be converted in a mature and profitable industry. However, this industrial aquaculture sector has constantly suffered from diseases outbreaks, placing the global shrimp farming and trading at risk (Stentiford et al., 2012). The most important shrimp diseases are caused by viruses mainly because of rapid dissemination and the significant economic losses (Lightner et al., 2012, Stentiford et al., 2012). An important viral disease to Americas that is prevalent in the northeast of Brazil is caused by the infectious myonecrosis virus (IMNV). This type of virus belongs to the Totividae family, as first identified at Brazilian farms in 2002 and later at Indonesia farms (Flegel, 2012; Tang et al., 2005). It is a non-enveloped, double-stranded RNA virus, with an isometric capsomer (~ 40nm) and a genome size of 7560 base pairs encoding two overlapping open reading frames (ORFs), ORF1 and ORF2, which comprise the predicted structural proteins and the RNA-dependent RNA polymerase (RdRp), respectively (Lightner, 2011; Liu et al., 2009; Naim et al., 2015).

The Infectious Myonecrosis caused by the IMNV provokes necrosis of the striated muscles of the abdomen and cephalothorax. Suggestive symptoms of the disease include anorexia, reduction in hepatopancreas volume, reduction in lipids, disoriented swimming, impairment in the hardening of the carapace and flexion of the 3rd abdominal segment (Nunes et al., 2004). Histological lesions are characterized by coagulative muscle necrosis, usually accompanied by fluid accumulation between the muscle fibers, hemocytic infiltration, fibrosis and typical presence of spheroids in the lymphoid organ. In addition, inclusion bodies can be observed within the cytoplasm of muscle and connective tissue cells and hemocytes (Poulos et al., 2006).

Over the past few years, scientists have simulated shrimp viral diseases in vitro to understand their pathogeneses, such as host-virus interactions and immune-related gene expressions (Dantas-Lima et al., 2012; Jose et al., 2011; Liu et al., 2007). Since no immortalized shrimp cells lines have been developed thus far, primary tissue cultures from the lymphoid organ, ovaries, as well as hemocytes have been used to study these shrimp viral pathogens (Deepika et al., 2014; Li et al., 2015a; Maeda et al., 2004). These primary cell cultures have also been used to evaluate potential efficacy and toxicity of chemicals used to prevent and/or treat shrimp diseases (Jose et al., 2010). Although there

are no commercially available drugs to treat shrimp viral diseases, research efforts have been dedicated to find new molecules with antiviral activity against these shrimp pathogens. Examples include the use of probiotics (Lakshmi et al., 2013), small-interfering RNAs (siRNAs) (Feijo et al., 2015; Shekhar and Lu, 2009), and antimicrobial peptides (AMPs) derived from anti-lipopolysaccharide factor isoforms (Li et al., 2015a; Yang et al., 2016).

Also called host-defense peptides, AMPs are expressed and used by the innate immunity of plants and animals to fight invading pathogens. Their general mechanism of action is through electrostatic interaction with the anionic phospholipids of bacteria, for example, and insertion in the pathogen membranes, which will induce pore formation and kill the microorganism (Li et al., 2012; Yeung et al., 2011). In the case of viruses, AMPs can act by inhibiting viral adhesion and fusion to host cells, and/or disrupting viral envelopes, and/or inhibiting replication through interaction with the viral polymerase (Jenssen et al., 2006; Skalickova et al., 2015).

In our research group, we have studied the cathelicidins, which are a diverse group of AMPs found from the primitive hagfish to humans (Kosciuczuk et al., 2012). In particular, we have found cathelicidin precursors from the venom gland cDNAs of some South American pit viper snakes. Mature sequences, termed vipericidins, were then synthesized and good antimicrobial activities were found in vitro, especially against Gram-negative bacteria (Falcao et al., 2014). In addition, a structural dissection of crotalicidin, a rattlesnake vipericidin, revealed that one of the fragments, named Ctn[15-34], maintained the antibacterial, antifungal and anticancer activities of the parental peptide with much less toxicity and greater serum stability than full-length crotalicidin (Falcao et al., 2015, Cavalcante et al., 2016).

The present work aimed to investigate the effectiveness of the anti-infective and anti-proliferative intrinsic activities of Ctn[15-34] as an antiviral against IMNV. First, primary hemocyte cultures were established that could respond to different chemical stimuli. Next, the IMNV replication and lethality were verified with these hemocytes. Finally, it was assessed whether treatment with Ctn[15-34] could protect the hemocytes from IMNV.

MATERIALS AND METHODS

Litopenaeus vannamei shrimps

Healthy *L. vannamei* shrimps about 9-10 grams were kindly donated by the Nutrition of Aquatic Organisms Laboratory from the Center of Environmental and Coastal Studies of the Institute of Marine Sciences, at Federal University of Ceara (Brazil). They were kept in sea water (3.5% salinity) tanks with a recirculation system and a daily supply of commercially available pelleted food. These shrimps were used to obtain hemocytes as needed.

Primary hemocyte cultures

The primary hemocyte cultures were established based on a protocol developed by Jose and colleagues (Jose et al., 2010), with some modifications. Shrimps were sacrificed and disinfected by immersion in ice-cold seawater containing 800 mg l^{-1} of sodium hypochlorite for 45 seconds. Next, they were washed with sterile ice-cold seawater, dipped in cold 70% alcohol-sea water solution and rinsed again with sterile ice-cold sea water. Hemolymph was then aseptically withdrawn from the ventral sinus of a shrimp with a sterile 22-gauge needle and syringe containing 200 µl of an anticoagulant solution (0.01M tris-HCl, pH 7.0, 0.25M sucrose and 0.1M sodium citrate). Viable hemocytes were counted by the Tryptan blue (Sigma Chemical, St. Louis, MO) exclusion dye procedure with a hemocytometer. For all experiments, 10^5 hemocytes/well were suspended in 50 µl of a modified 2 x L-15 medium in 96-well plates. That medium consisted of double concentrated Leibovitz's L-15 (2x L-15; Sigma) supplemented with 20% FBS, 2% glucose, MEM vitamins (1x), tryptose phosphate broth (2.95 mg ml^{-1}), 0.2 mM N-phenylthiourea, 0.06 µg ml-1 chloramphenicol, 1x antibiotics solution (100 µg ml-1 streptomycin and 100 IU ml penicillin). Final medium and PBS osmolality were measured with a cryoscopic osmometer, OSMOMAT® 030 (Gonotec, Berlin, Germany), and kept at 720 mOsm/kg. The suspended hemocytes were incubated for one hour at 29 °C before each experiment to allow their attachment to the wells.

Fluorescence Cell Viability/Cytotoxicity assays

The suitability of the primary hemocyte cultures was verified by viability assays using a dye, resazurin (CellTiter-Blue®, Promega, Madison, WI), which is metabolically reduced by live cells to the highly fluorescent resofurin. Hemocytes were treated with 50 µl of the modified 2x L-15 medium containing two-fold serial dilutions of either benzalkonium chloride (Sigma) or chondroitin sulfate (Sigma) at final concentration ranges of 6.25 – 200 µg ml^{-1} and 1.56 – 50 mg ml^{-1}, respectively. After additional 30 min incubation at 29 °C, 30 µl of CellTiter-Blue® (Promega) reagent was added to each well and plates were re-incubated for up to 48 hours. Fluorescent readings were measured at 4, 24 and 48 hours after addition of the dye using a Synergy HT (BioTek, Winooski, VT) multi-detection microplate reader with λex=530 and λem= 590 nm. Relative hemocyte viability was calculated by taking non-treated cells as controls (≈ 100% viability) and experiments were carried out in triplicate. The obtained means ± standard errors of measurement were compared through one-way analysis of variance (ANOVA) and Bonferroni posthoc tests using the software SPSS version 16.0 (IBM Corporation, Somers, NY). Differences were considered statistically significant if $p < 0.05$.

Total RNA isolation and cDNA synthesis from hemolymph and gill tissue extracts of *L. vannamei* shrimps with Infectious Myonecrosis symptoms

L. vannamei shrimps weighting 9-10 g each with opaque, whitish infectious myonecrosis symptoms (Nunes et al., 2004) were kindly donated by the Compescal Fishery Company Ltd (Aracati, Ceara, Brazil). From these shrimps, a pool of approximately 3 g of hemolymph and 4 g of gills was macerated with 50 ml of ice-cold 2x L-15 medium, without serum, on a mortar and pestle kept on an ice bath. The extracts were centrifuged at 10,000g for 10 min at 4°C and the supernatants were passed through 0.22 µm PVDF membranes (Milipore, Bedford, MA). Aliquots of the supernatants were then stored at – 80°C until further use. Total RNA was isolated from 100 µl of the supernatant with the NucleoSpin TriPrep kit (Macherey-Nagel, Bethlehem, PA) and quantified with the RNA HS reagent in a Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA), according to their respective manufacturer´s protocols. Later, single-stranded complementary DNA (ss-cDNA) synthesis was performed starting with

1 µg of total RNA, which was mixed with 0.5 µg of random hexamer primers (Promega, Madison, WI), heated to 70°C for 10 min and quickly cooled to 4°C. Next, the reverse transcription reaction was run at 42°C for 90 min with the denatured RNA and primers, 100 U of ImProm II reverse transcriptase enzyme (Promega, Madison, WI), 1 mM of each dNTP, 5 mM $MgCl_2$, and 20 U of RNase inhibitor, in a final volume of 20 µL. Then, the reverse transcriptase was terminated by heating at 70°C for 15 min, and all individual cDNA synthesis reactions were stored at – 20 °C.

Analyses of IMNV viral loads contained in *L. vannamei* tissue extracts

The amount of IMNV viral particles from the pools of hemolymph and gill tissue extracts were determined by quantitative polymerase chain reaction (qPCR) with the use of the Rotor Gene 3000 and its specific software version 6.0.19 (Corbertt Research, Mortlake, Australia). For each 20 µl reaction, 2 µl of ss-cDNA, prepared as above, were mixed with 10 µl of 2x GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and 0.2 µM of a primer pair, either from IMNV or shrimp β-actin genes, as previously obtained (Vieira-Girão et al., 2012). Then, 45 cycles were run at 95°C for 30 s, at 65°C for 30 s and at 72°C for 30 s each, and fluorescence was detected at 494-521 nm during the extension phase. The Rotor Gene software, running default parameters, automatically provided the threshold and threshold cycle (Ct) values for each reaction, which were used to calculate the IMNV viral loads. Absolute quantification of IMNV viral particles was estimated with the copy numbers of transcript sequence encoding the major capsid protein by the equation: number of transcripts = [DNA amount (ng) x 6.022 x 10^{23}]/ [DNA length (bp) x 10^9 x 650] (http://cels.uri.edu/gsc/cndna.html). The cDNA amounts were found after the Ct values were plotted on previously built standard curves from 10-fold serial dilutions of IMNV and shrimp β-actin gene (Vieira-Girão et al., 2012). The capsid transcript copy numbers used were from triplicates after being normalized against 1µg of shrimp β-actin gene.

Infectivity titration and cytotoxic activity of IMNV in *L. vannamei* primary hemocyte cultures

The capacity of IMNV viral particles from the infected tissue extracts to re-infect and cause cellular damage was verified in vitro using the *L. vannamei* primary hemocyte cultures established as above with two different assays. In the first evaluation, hemocytes (10^5 cells/well) were treated with two-fold serial dilutions of the tissue extracts containing IMNV viral particles (called "IMNV extracts" from now on) at final concentration range of 7 - 227 capsid transcripts (copies) per microliter (copies μl^{-1}) and volume of 100 µl/well of the modified 2x L-15 medium in 96-well plates. Viability was assessed as in section 2.3, and the relative hemocyte viability was calculated with non-treated cells as controls and experiments were carried out in triplicate.

In the second assay, it was verified whether IMNV could replicate in culture in primary hemocytes. In 12-well plates, 1.6 x 10^6 cells per well were seeded with modified 2x L-15 medium and incubated for 1h at 29°C. Then, a diluted IMNV viral extract sufficient to cause 10% hemocyte inhibition (≈ 90% cell viability) was added to the wells with a final volume of 1 ml/well. After different time intervals (2, 4, 6, 10, 12, 24 and 48h) post-inoculation at 29°C, medium was removed, and cells were washed with ice-cold 2.5x phosphate buffered saline (PBS) and harvested. Next, PBS was removed by centrifugation at 10,000g for 5 min at 25°C and 100 µl of RNAlater solution (Invitrogen, Carlsbad, CA, USA) were added to each cell pellet. The re-suspended hemocytes were stored at -20°C until total RNA extraction, cDNA synthesis and qPCR analysis were carried out as described above. Experiments were in triplicate for each time point and controls used were non-treated cells and cells treated with a previously heat-inactivated (95°C for 15 min) diluted extract of IMNV.

The eicosapeptide Ctn[15-34]

Ctn[15-34] (KKRLKKIFKKPMVIGVTIPF-amide, 20-mer, MW = 2371.11 g mol^{-1}) was synthesized and purified according to procedures described in details in previously published work (Falcao et al., 2015). Peptide stock solutions were prepared at 1mM with deionized water as required and stored at 4°C for up to six weeks. Ctn[15-34] toxicity to hemocytes was also evaluated by viability assays as above. A volume of 50 µl of the modified 2x L-15 medium was added to the cells containing 2-fold serial dilutions of the peptide at final concentration range of 6.25 – 200 µM. After 30 min incubation at

29°C, 30 µL of CellTiter-Blue® reagent (Promega) was added to each well and plates were re-incubated for up to 48 hours. Fluorescent readings were at 12, 24 and 48 hours after addition of the dye using a Synergy HT (BioTek) microplate reader with λ_{ex}=530 and λ_{em}= 590 nm. Relative hemocyte viability was calculated by taking non-treated cells as controls and experiments were carried out in triplicate.

Antiviral activity of Ctn[15-34] against IMNV

The potential antiviral properties of Ctn[15-34] against IMNV were next evaluated based on the peptide recently found anti-infective and anti-proliferative properties, such antibacterial, anticancer and antifungal activities (Cavalcante et al., 2016; (Falcao et al., 2015). The Ctn[15-34] capacity to protect *L. vannamei* primary hemocyte cultures from infection by IMNV was assessed by two independent assays, both with IMNV extracts at concentrations sufficient to cause 50% hemocyte inhibition (IC_{50}). In one set of experiments, hemocytes were first incubated with Ctn[15-34] (0.75 – 25 µM concentration range) for 1h at 29°C before inoculation of IMNV extracts into hemocyte culture. In the other set, Ctn[15-34] (0.75 – 200 µM range) was firstly and directly added to IMNV extracts, incubated for 1h at 29°C and then the mixture peptide and viral extract added to hemocytes. In both experiments, after additional incubation of hemocytes with Ctn[15-34] and IMNV extracts for 30 min at 29 °C, 30 µl of CellTiterBlue® reagent (Promega) was added to each well and plates were re-incubated for up to 48 hours. Fluorescence readings were measured again at 24 and 48 hours after addition of the dye using a Synergy HT (BioTek) microplate reader with λ_{ex} = 530 and λ_{em} = 590 nm. Relative hemocyte viability was calculated with non-treated cells as controls and experiments were carried out in triplicate.

Finally, it was also verified whether Ctn[15-34] had a direct destructive action on IMNV viral particles. Thus, 25 µl of Ctn[15-34] were added to microcentrifuge tubes containing 100 µl suspensions of IMNV extracts and a 1x protease inhibitor cocktail (Serva, Heidelberg, Germany). Final concentrations were: peptide at 6.25 µM and IMNV viral extract at hemocyte IC_{50} (but without the cells). After incubation periods of 3, 6 and 24 h at 29 °C, 175 µl of a lysis buffer (Promega) were added to the suspensions and samples were stored at -80°C until RNA extraction, cDNA synthesis and qPCR analysis

as described above. Experiments were carried out in triplicate for each time point and IMNV extracts without Ctn[15-34] were used as controls.

RESULTS

L. vannamei primary hemocyte cultures and fluorescence cell viability assays

Hemocytes were obtained from the ventral sinus of *L. vannamei* shrimps. They were seeded on 96-well plates with a modified 2x L-15 medium. These hemocytes remained viable for at least 8 days with a slight increase in viability on that period and no signs of culture contamination. In addition, it seemed that the viability reagent CellTiter-Blue added right after the 1h incubation period and kept in the cell culture for the 8-day time course had no toxicity to hemocytes (Figure 14).

Later, two chemicals were added separately to the primary hemocyte cultures to verify whether and how the cultured cells could respond to different stimuli with the use of the same CellTiter-Blue viability reagent. The first chemical used was benzalkonium chloride (BKC), which is used in fishery tanks as a preservative. According to Figure 15a, BKC had a late toxic effect because concentrations of as much as 25 µg ml^{-1} were not toxic up to 4 hours of incubation, but were able to cause death in 50% (IC_{50}) of cultured hemocytes in 24 hours.

The second chemical used was chondroitin sulfate, which is a glycosyaminoglycan found in cartilage extracellular matrix. When associated with extracellular proteins, chondroitin sulfate can enhance cell adhesion to surfaces (Frantz et al., 2010). According to Figure 15b, chondroitin sulfate also had a late toxic effect because nontoxic concentrations of 12.5 and 25 mg ml^{-1} up to 4 hours incubation reduced hemocyte viabilities by 50% after 24 hours ($IC_{50} \approx 25$ mg ml^{-1}). Interestingly, lower concentrations of as much as 6.25 mg ml^{-1} incubated for up to 24 hours had a significant increase ($p < 0.02$) in hemocyte viability, which had a direct relationship with increased metabolic activity. Hemocyte viability patterns were mainly the same between 24 and 48 hours incubation for each chemical tested.

Infectivity titration of IMNV extracts in *L. vannamei* primary hemocyte cultures

After the primary hemocyte cultures were established, our next step was to evaluate whether and how these cells could respond to increased doses of IMNV, a shrimp viral pathogen known to cause the infectious myonecrosis disease (Lightner et al., 2012). Thus, IMNV extracts were obtained and quantified as described above and tested with the hemocytes through 2-fold serial dilutions to obtain a dose-response curve. Results are presented in Figure 16.

In Figure 16, one can observe that the IMNV extracts were infective and cytotoxic to hemocytes, in a concentration-dependent manner. In addition, the IMNV toxicity to hemocytes increased over time because there was a clearly observable further decrease in viability between 24 and 48 hours incubation at each concentration. The concentration to inhibit 50% of the hemocytes (IC_{50}) was ~ 227 copies of capsid transcripts μl^{-1}, which was approximately one hundred-fold dilution of the obtained IMNV extracts.

Moreover, in this set of experiments, it was also assessed the capacity of IMNV particles from the viral extracts to replicate in the hemocytes. The variation of the number of viral particles, represented as normalized copies of viral capsid transcript μg^{-1} of shrimp β-actin gene, during the hours post-inoculation is shown in Figure 17.

During the first six hours post-inoculation, the numbers of IMNV particles were lower, with means between $7x\ 10^{10}$ and $5\ x\ 10^{11}$ copies μg^{-1} of β-actin gene, than by 12 hours, when, IMNV particles reached their replication peak with $4\ x\ 10^{12}$ copies μg^{-1}. From 12 to 24 hours, there was a decrease in the number of particles ($1\ x\ 10^{12}$ copies μg^{-1}) most probably because viral shedding released IMNV particles to the medium, and quantification was only performed from the collected hemocytes. The later increase in particle number by 48 hours ($4\ x\ 10^{12}$ copies μg^{-1}) provided a hint that the virus was going through one more replication cycle. Similar findings were previously reported for Taura Syndrome Virus (TSV) inoculated in other shrimp primary hemocyte cultures (George et al., 2011).

Assessment of Ctn[15-34] antiviral activity against IMNV in *L. vannamei* primary hemocyte cultures

In a quest to find possible antiviral substances to prevent and/or treat viral diseases in shrimp farming, our next step was to evaluate the whether recently designed and developed, Ctn[15-34] eicosapeptide, could protect the primary hemocyte cultures from the IMNV in viral extracts. Initially, we performed a cytotoxicity assay, similar to the previous experiments with BKC and chondroitin sulfate, to know the maximal doses that could be used and would not harm the hemocytes. Results are displayed in Figure 18.

According to Figure 18, Ctn[15-34] had a late toxic effect because non-toxic concentrations, such as 50 µM, up to 12 hours of incubation able to kill 50% (IC_{50}) of the cells when hemocytes were exposed longer (after 24 hours) to the peptide. Similar to BKC and chondroitin sulfate, the toxicity pattern of Ctn[15-34] to hemocytes did not change from 24 to 48 hours incubation.

Then, Ctn[15-34] antiviral activity against the IMNV in extracts was estimated in the primary hemocyte cultures using two different experimental conditions, but the same viability test readout, that is, with CellTiter-Blue reagent and fluorescent intensity measurements. In the first assay, hemocytes were incubated with Ctn[15-34] at concentrations up to 25 µM, half of its IC_{50}, for one hour. Subsequently, the IMNV extracts were added to the cultures at a fixed concentration of 227 copies μl^{-1} (IMNV IC_{50}). Hemocyte viability results from 24 to 48 hours incubation are shown in Figure 16b.

Figure 19a shows that the peptide Ctn[15-34] at 0.75 - 12.5 µM concentration range greatly reduced IMNV viral extract lethality since there was only a maximum 10% reduction of hemocyte viability up to 48 hours incubation. Although the IMNV action in the extract was reduced, the decrease in viability by 30% with Ctn[15-34] at 25 µM indicated that the peptide reached toxic concentrations to the cells.

In the second assay, Ctn[15-34] antiviral activity was confirmed when the peptide at 0.75 - 200 µM concentration range was previously incubated with the IMNV extracts at their IC_{50} for one hour before adding to the hemocytes. Cell viability results are displayed in Figure 19b from 24 to 48 hours incubation.

In Figure 19b, although it was observed a general 20% reduction in cell viability at all tested peptide concentrations, there was no further increase in toxicity to the hemocytes. Again, up to 48 hours, Ctn[15-34] did not allow the IMNV extracts to kill 50% of the hemocytes. Interestingly, it seemed that not only Ctn[15-34] interfered with

IMNV activity but also the viral extract reduced the peptide toxicity because concentrations over its IC_{50} reduced hemocyte viability by no more than 20% when firstly incubated with the IMNV extracts.

Finally, we also investigated whether Ctn[15-34] could directly destroy the IMNV particles contained in the viral extracts. Ctn[15-34] at 6.25µM was incubated with a total of 227 copies μl^{-1} of IMNV (equivalent to kill 50% of hemocytes but non-inoculated to the cells) and qPCR analysis was performed after 3, 6 and 24 h incubation. Figure 20 shows the variation of the number of viral particles, represented as copies μg^{-1} of β-actin gene. The results in Figure 20 indicates that Ctn[15-34] apparently did not directly damage the IMNV because the number of virus were mainly the same between treated and non-treated (IMNV viral extract only) groups at all time points.

DISCUSSION

Shrimp primary tissue and hemocyte cultures have been used as *in vitro* platforms to study viral pathogens, especially the white spot syndrome virus (WSSV), because no immortalized shrimp cell line has been established yet (Jayesh et al., 2012). In our studies, we used primary hemocyte cultures because they could be directly seeded in plates without further tissue processing steps (George et al., 2011; Jose et al., 2011; Jose et al., 2010). Moreover, circulating hemocytes are the shrimp primary sentinel immune cells. These cells have similar roles as the macrophages in mammals, which include phagocytosis and lysis of foreign cells (Wynn et al., 2013). Therefore, an entire shrimp organism can be affected if viral pathogens can multiply inside these cells.

Hemocytes from *L.vannamei* shrimps were suspended in a modified 2x L-15 medium and allowed to attach for one hour before each experiment. That period was enough for cells to acquire the same characteristics as in a live organism (Deepika et al., 2014), and the modified 2x L-15 medium kept the cells alive for at least 8 days (Figure 1), similarly as previously demonstrated for cultures of *P. monodon* hemocytes (Jose et al., 2011; Jose et al., 2010). While most of shrimp cell, including experiments with primary tissue cultures, viability assays used the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) (Jayesh et al., 2015; Jayesh et al., 2012; Jose et al., 2012; Jose et al., 2010), we used the resazurin reagent (CellTiter-Blue®, Promega). Although

both reagents are dependent on the metabolism of live cells, resofurin (resazurin product) fluorescence can be directly measured on each well without the need to remove medium and solubilize crystals as the MTT-formazan products (Rampersad, 2012). Thus, resazurin viability assay is non-destructive and readout can be continuously monitored without the need to prepare numerous well plates for different time points in the same experiment. To validate the application of resazurin-based fluorescence cell viability assay, we firstly confirmed that *L.vannamei* hemocytes sensibly responded to different chemical stimuli, and dose-response curves could be determined. BKC and chondroitin sulfate were toxic to hemocytes as their concentrations increased, especially after 4 hours of incubation. However, when chondroitin sulfate was used at low concentrations (up to 6.25 mg ml^{-1}) by 24 hours, an increase in viability could be observed which represented an increase in metabolic activity compared to the non-treated groups (Figures 15a and 15b). Therefore, the addition of chondroitin sulfate at these low concentrations can be beneficial for primary hemocyte cultures because it is proposed that such glycosaminoglycan can help cells enhance their adhesion to the well surfaces (Frantz et al., 2010).

The primary cultures of *L. vannamei* hemocytes were also susceptible to the infectious myonecrosis virus (IMNV) extracts (Figure 16). Viral extract titration revealed that was necessary a load equivalent to 227 copies of capsid transcript μl^{-1} to kill 50% of the hemocyte culture. Similar studies with WSSV incubated in primary hemocyte cultures either from *P. monodon* (Jose et al., 2010) or from the mud crab Scylla serrata (Deepika et al., 2014) only showed IC_{50s} based on dilution factors from the viral extracts.

Moreover, it was here demonstrated that IMNV could replicate in *L. vannamei* primary hemocyte cultures (Figure 17). Although previous studies found that other viruses could multiplicate in hemocytes, such as WSSV (Jiang et al., 2006; Wang et al., 2002) and TSV (George et al., 2011), this is the first time that IMNV replication *in vitro* is reported. Inside hemocytes, IMNV particles reached their replication peak by 12 hours, which was similar to the multiplication of TSV found in a previous study (George et al., 2011). In the case of WSSV, Li and co-workers (Li et al., 2015b) found that it was necessary 18 hours for WSSV to reach their maximum number of particles in lymphoid cells. It is believed that it took longer for WSSV to reach its replication peak than IMNV because the former virus has a bigger DNA-based genome than the latter that has a RNA-based genome (Lightner et al., 2012; Poulos et al., 2006).

The search for therapeutic agents to prevent and/or treat shrimp viral diseases have received attention lately, but few studies have been published so far (Yang et al., 2016). Among them, some proteins such as C-type lectins and Pmfortilin had activity against WSSV (Tonganunt et al., 2008; Zhao et al., 2009). Also, the laminin receptor in shrimps was recently found to interact with WSSV, IMNV, TSV and YHV (Busayarat et al., 2011; Liu et al., 2016). Thus, this receptor can be used to develop antiviral drugs that bind the receptor (drug target) and avoid virus-receptor interactions and/or to design leads that can directly bind and neutralize the viral particles. In addition, peptides that have shown activity against shrimp viruses, such as WSSV, include fragments of anti-lipopolysaccharide factor isoforms from *Fenneropenaeus chinensis* (Li et al., 2015a).

In our studies, we used the antibacterial, antifungal and antitumor Ctn[15-34] (Falcao et al., 2015, Cavalcante et al., 2016) to evaluate whether this peptide could also have antiviral activity against IMNV. The first antiviral assay (Figure 19a) demonstrated that Ctn[15-34] protected the hemocytes from viral cytotoxicity with concentrations (up to 12.5 µM) at least 4-fold lower than the peptide IC_{50} to hemocytes (50 µM – Figure 5). Hence, it has the potential to be administered with commercial pelleted food at low doses to prevent infectious myonecrosis, especially during seasonal disease outbreaks. The second assay (Figure 19b) confirmed hemocyte protection with Ctn[15-34] from IMNV and also indicated an interaction between the peptide and the virus before their addition to hemocytes because none of the tested concentrations (up to 200µM of peptide and viral extract at IC_{50}) were severely toxic to the cells. Therefore, although the interaction mechanism remains to be further investigated, it seems that the structural active domains involved in Ctn[15-34]-IMNV association are primarily the same needed by the peptide and the virus for each interaction with hemocytes. Even though Ctn[15-34] could not reduce the number of IMNV particles by 24 hours (Figure 20), the peptide-virus association looked stable because hemocytes remained with the same viability at least up to 48 hours with Ctn[15-34]-IMNV particles (Figure 19a). Noteworthy, although antimicrobial peptides activity can be greatly reduced with high salt and serum concentrations (Chu et al., 2013; Maisetta et al., 2008), Ctn[15-34] exhibited antiviral effects against IMNV in a medium with elevated salinity and 20% FBS. Since these medium requirements are needed to culture hemocytes because they simulate *in vivo* conditions, Ctn[15-34] therapeutic use is promising against IMNV infections in shrimp aquaculture.

Taken together, we established a *L. vannamei* primary hemocyte culture that responded to chemical stimuli and were sensitive to IMNV in viral extracts. In addition, IMNV particle replication inside these hemocytes can be used as a platform for further viral pathogenesis studies. Moreover, the employment of a straightforward methodology to assess cell viability and viral activity based on fluorescence was demonstrated. Remarkably, Ctn[15-34], a recently developed eicosapeptide with antibacterial, antifungal and anticancer properties, also suppressed IMNV infection in hemocytes and, thus, can be developed into a multi-effector compound and component of antiviral formulations against shrimp viral diseases.

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Figures

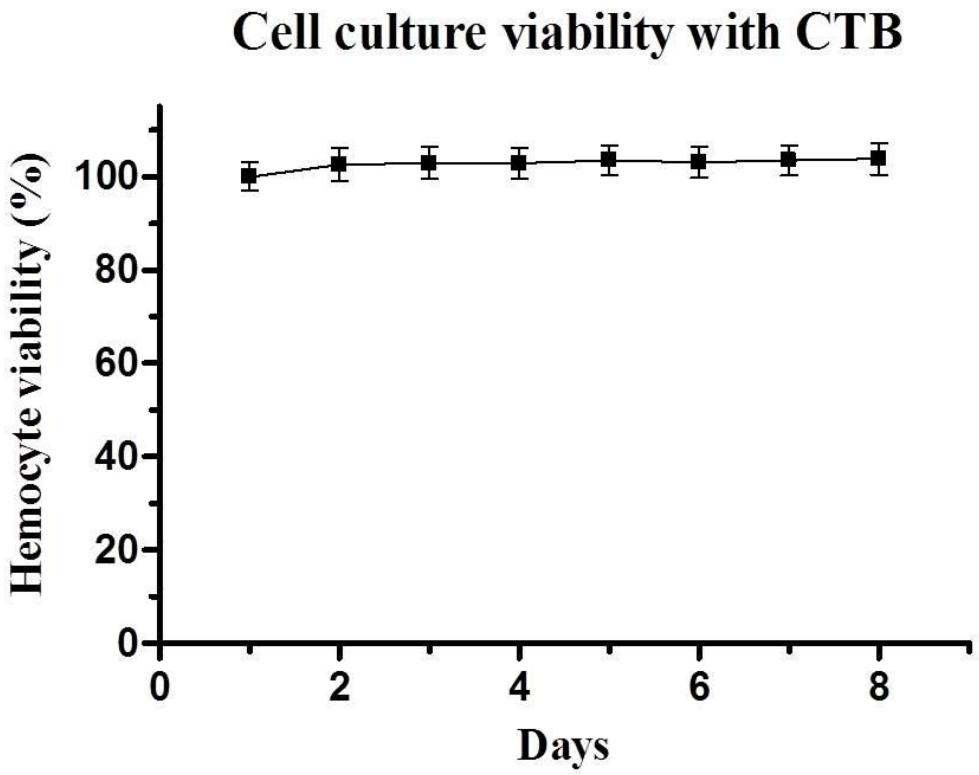

Figure 14: Primary hemocyte culture viability (%) in the modified 2x L-15 medium (see Materials and Methods – section 2.2) up to 8 days (mean ± SEM, with n=3).

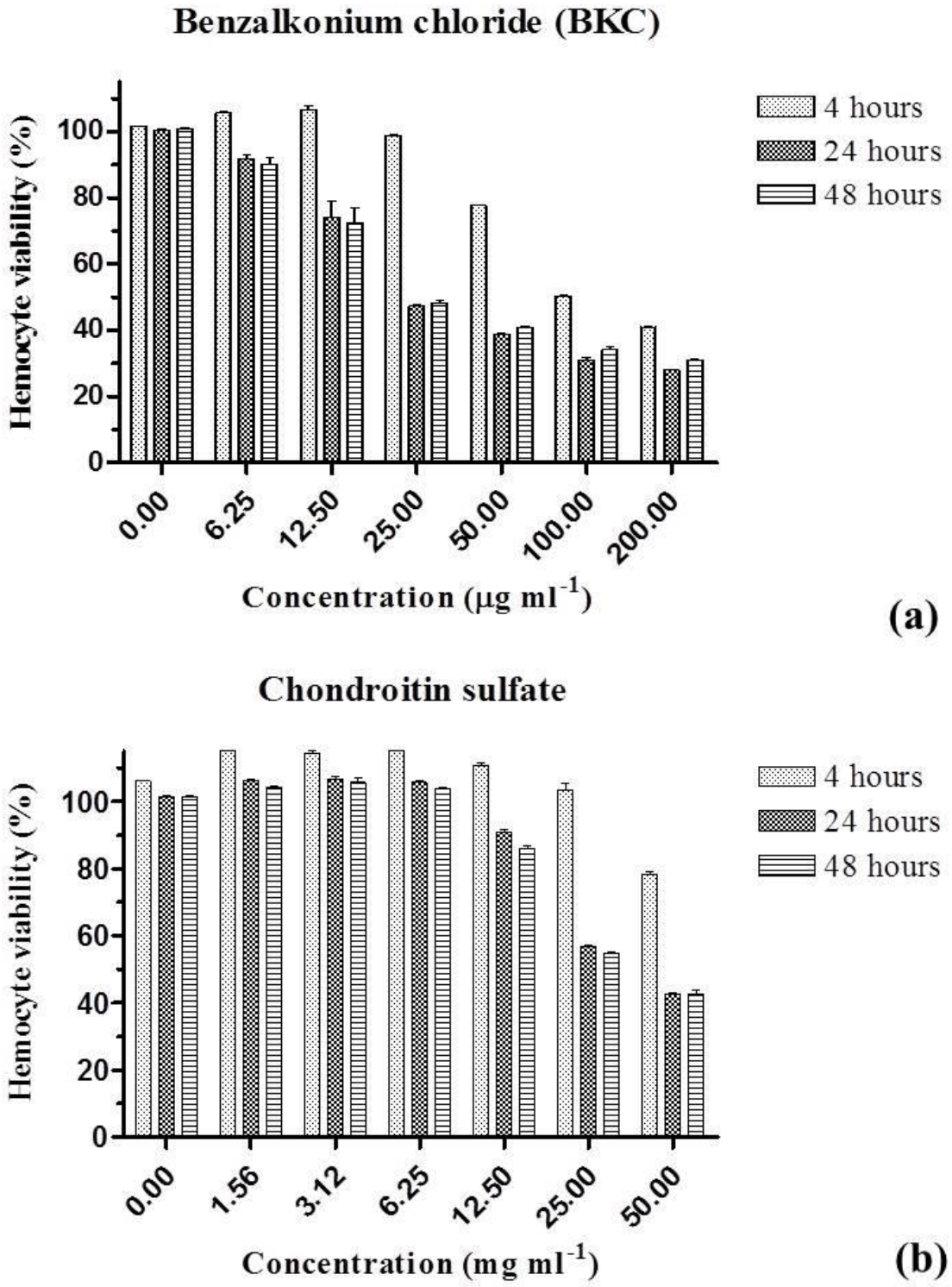

Figure 15: Hemocyte viability (%) after treatment with (a) BKC and (b) chondroitin sulfate (mean ± SEM, with n=3) for 4, 24 and 48 hours.

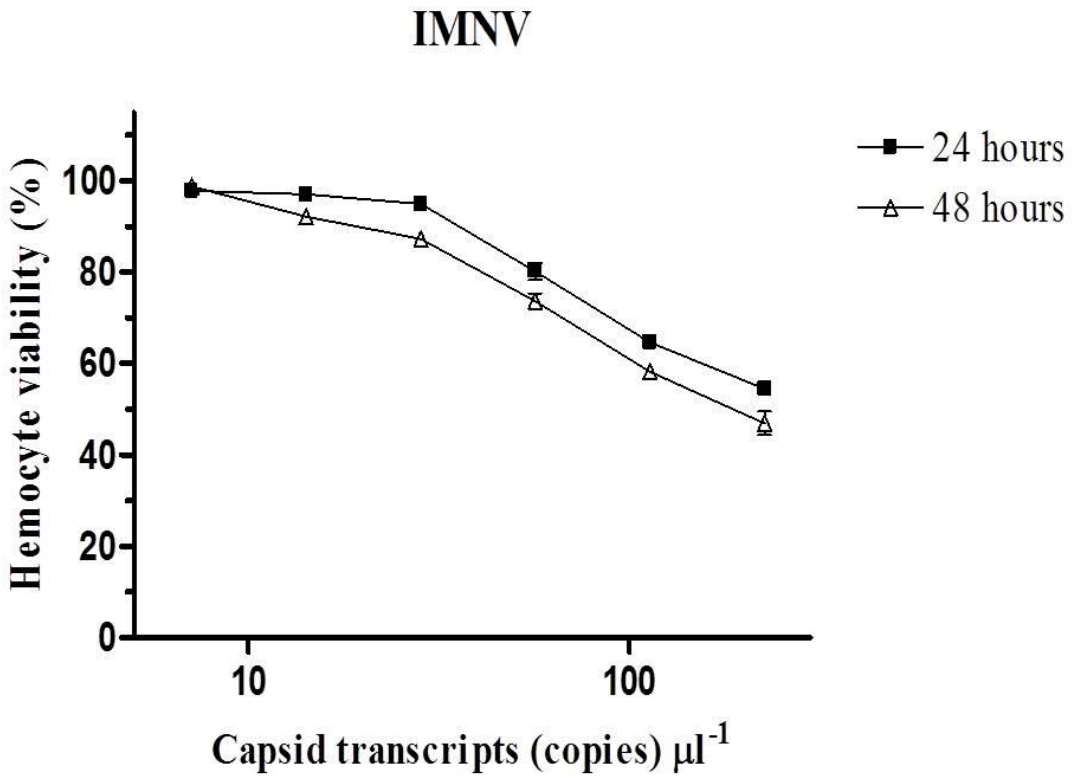

Figure 16: Hemocyte viability (%) after treatment with IMNV extracts (mean ± SEM, with n=3) for 24 and 48 hours.

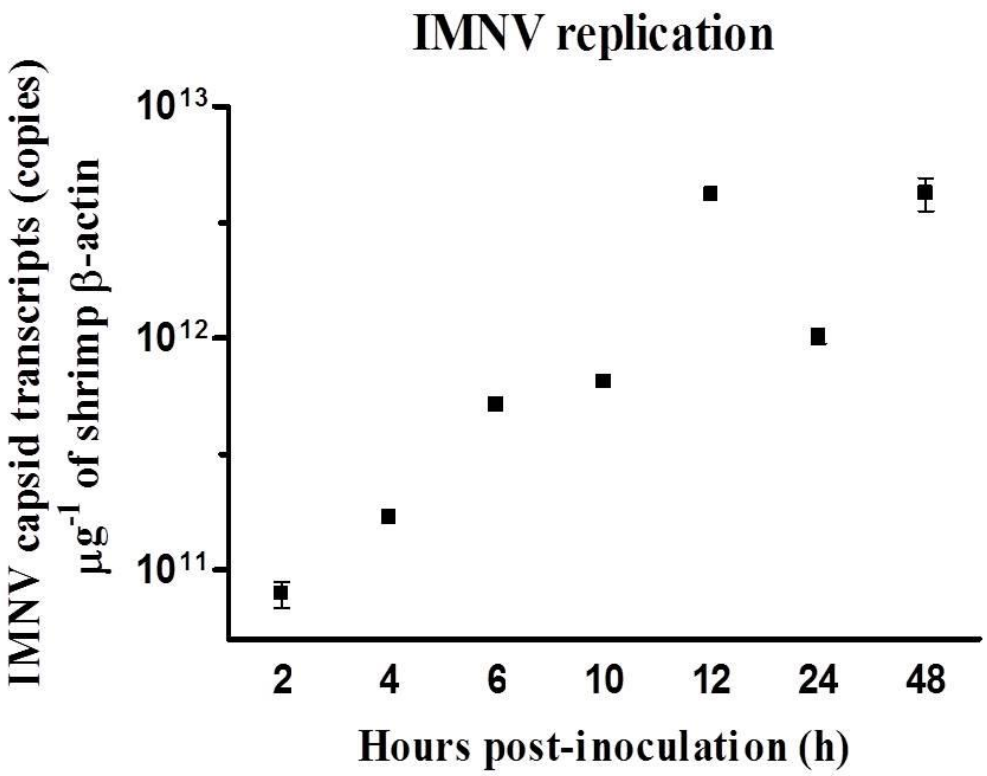

Figure 17: IMNV replication capacity, estimated by capsid transcript copies (mean ± SEM, with n=3), in L. vannamei primary hemocyte cultures up to 48 hours post-inoculation.

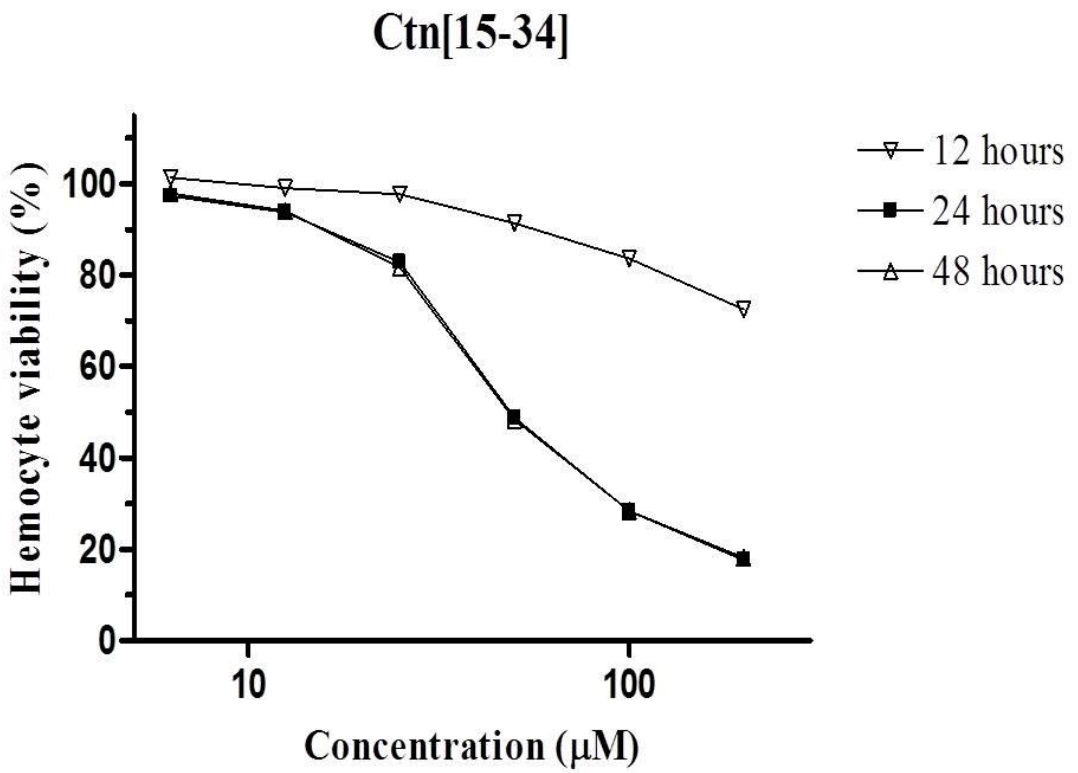

Figure 18: Hemocyte viability (%) after treatment with Ctn[15-34] (mean ± SEM, with n=3) for 12, 24 and 48 hours.

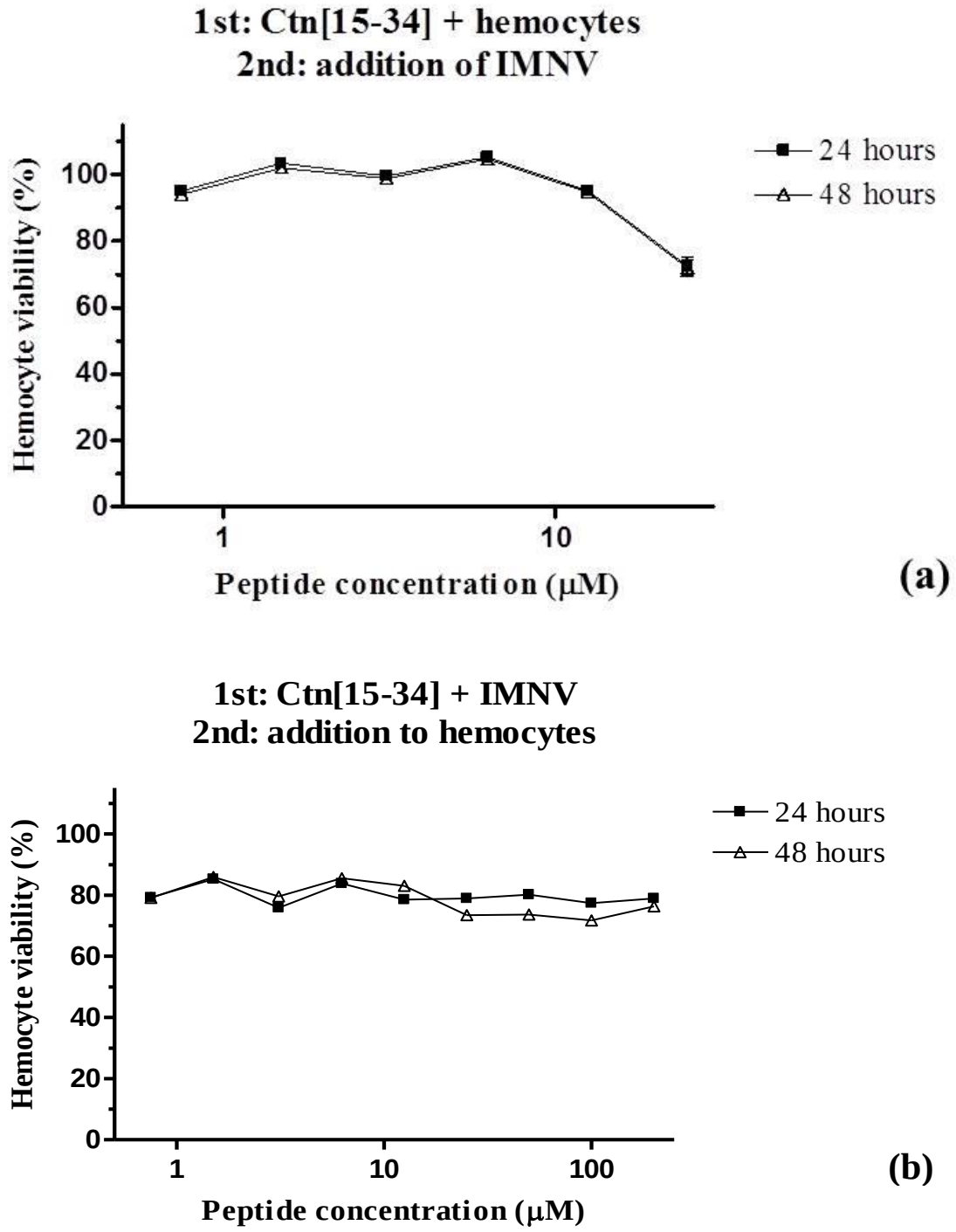

Figure 19: Hemocyte viability (%) after treatment with Ctn[15-34] and IMNV extracts at their IC50 (mean ± SEM, with n=3) for 24 and 48 hours: (a) hemocytes treated with Ctn[15-34] before addition of IMNV; (b) IMNV extracts treated with Ctn[15-34] before addition to hemocytes.

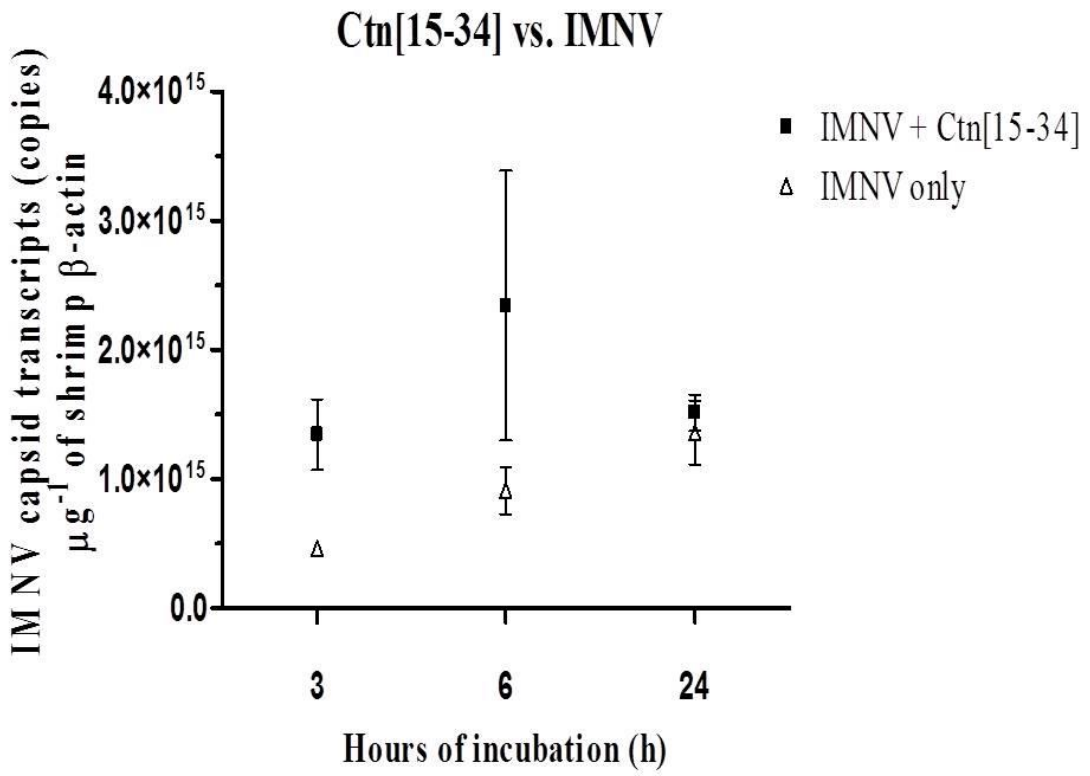

Figure 20: Ctn[15-34] damage capacity to IMNV, estimated by capsid transcript copies (mean ± SEM, with n=3), after treatment for 3, 6 and 24 hours.

4 CONCLUSÕES GERAIS

O presente trabalho teve o objetivo de determinar o número de cópias de IMNV em camarões em ambientes com diferentes salinidades, quantificar e titular o vírus em cultivo primário de hemócitos, bem como avaliar o potencial de atividade antiviral de Ctn[15-34] frente ao IMNV. Diante dos resultados obtidos podemos concluir que:

- O estresse salino em ambientes de cultivo de camarões (chuvas) favorece a infecção pelo IMNV, pois esse vírus se prolifera em maior quantidade e mais rapidamente quando os animais estão em ambientes com salinidades mais baixas do que em salinidade de conforto.

- A diminuição da salinidade em cultivos de camarões é um fator de disparo para proliferação do IHHNV latente, mesmo com a presença majoritária de IMNV, já que o IHHNV não estava presente no inóculo utilizado no estudo, e mesmo assim, esse também apresentou seu número de cópias aumentado e em um tempo mais rápido.

- A cultura primária de hemócitos manteve-se viável e respondendo a estímulos *in vitro* por tempo suficiente para a realização de estudos com vírus, além de ser capaz de suportar a replicação do IMNV, pois o número de cópias do vírus aumentou com o passar do tempo. De modo que esses resultados são importantes para encontrar soluções para os problemas de enfermidades com IMNV no cultivo de *L. vannamei.*

- O ensaio com CellTiter-Blue® mostrou ser rápido e prático podendo ser um método alternativo em estudos com cultivo primário de células de camarão.

- O Ctn[15-34] apresentou atividade antiviral frente ao IMNV em cultivo primário de hemócitos de camarão, mesmo na presença de uma IC_{50} do vírus, em meio com alta concentração de FBS. Esse resultado foi observado tanto em incubação prévia do vírus com o peptídeo, como com incubação prévia dos hemócitos antes da inoculação do vírus, mesmo nas concentrações tóxicas do Ctn[15-34] para os hemócitos.

- O Ctn[15-34] demonstrou ser um candidato seguro para o desenvolvimento de um antiviral contra mionecrose infecciosa em camarões podendo ajudar a aumentar o desempenho e a rentabilidade da carcinicultura.

6 PERSPECTIVAS

A partir desses resultados espera-se que ocorra o desenvolvimento de estudos visando desenvolver um fármaco antiviral derivado do peptídeo Ctn[15-34] contra IMNV, bem como a prospecção de potencial atividade contra outros vírus importantes em cultivos de camarões.

Outro trabalho importante está em desvendar o mecanismo de ação do peptídeo Ctn[15-34] com a realização de estudos de microscopia para determinar onde se localiza o peptídeo nas células com e sem a presença de vírus.

Além disso, estudos de toxicidade e atividade *in vivo* são necessários para o IMNV, bem como para outros vírus que afetam camarões. Uma vez que o peptídeo suportou *in vitro* alta osmolalidade do meio de cultivo e concentração de FBS. Com a realização de histologia dos tecidos, podem ser úteis também para as respostas sobre o mecanismo de ação.

Para conhecer como o peptídeos, e também possivelmente o IMNV, entram nas células são necessários estudos de interação proteína-proteína e *docking* molecular, para descobrir qual(is) é(são) receptor(es) celular(es) para o peptídeo ou os receptores do vírus para o peptídeo.

Como peptídeos são moléculas imunomoduladoras é interessante a realização de estudos imunológicos, como a determinação de perfil de expressão de genes imunes após tratamento com Ctn[15-34] tanto *in vitro* como *in vivo*, a fim de determinar relações, como melhoramento da resposta imune em camarões tratados, além da atividade contra o vírus propriamente dito.

Estudo de desenvolvimento de vacina antiviral através de uma formulação formada de um complexo entre Ctn [15-34] e vírus ou partes do vírus, para quaisquer vírus que possam interagir com o peptídeo.

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Anexo A

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Research Article Open Access

Low Salinity Facilitates the Replication of Infectious Myonecrosis Virus and Viral Co-Infection in the Shrimp *Litopenaeus Vannamei*

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Abstract

The white leg shrimp *Litopenaeus vannamei* has been converted commercially into the most predominant cultivated shrimp species in the world. However, such shrimp's intensive farming worldwide propitiates outbreaks of epizootic diseases, primarily of viral etiology. In the principal Brazilian region of shrimp production, it is known that a reduction in the salinities of culture ponds causes the appearance of viral diseases. In the present work, we investigate the replication of the infection myonecrosis virus (IMNV) in controlled levels of salinity during the first 12 hours of infection. Using quantitative real-time PCR and statistical analysis, we verify that low salinity positively facilitates IMNV replication and proliferation by decreasing the generation time from 57.4 min (at 35 g L^{-1}, optimum salinity) to 25.2 min at (5 g L^{-1}, stressing concentration). Similarly, a positive relationship was demonstrated between a decrease in salinity and the reduction in the generation time of persistent infectious hypodermal and hematopoietic necrosis virus, a virus that usually co-infects shrimp in farm ponds.

Keywords: *L. Vannamei*; Shrimp virus; IMNV; Qpcr; Epizootic agent; Viral replication; Generation time

Introduction

The white leg shrimp *Litopenaeus vannamei* is naturally found along the Pacific coast from the Golf of California to the north littoral of Peru [1]. Due to the rearing performance in shrimp farming, *L. vannamei* has been converted into the most predominant cultivated shrimp species in the world, reaching approximately 2.7 million cubic tons and over 10 billion dollars in sales in the year of 2010 [2]. The characteristics that make this species of shrimp adequate for commercial production, particularly in the Americas, include fast growth, low nutritional requirements, resistance to environmental stress, and a certain osmoregulatory capability for adaptation to a wide range of salinity (from 1 to 50 g L^{-1}) [3]. The intensive rearing techniques required for efficient and cost-effective shrimp farming unintentionally propitiate the outbreaks of epizootic diseases, and severe microbial infection is consequently a recurrent threat in the shrimp industry. Some of the most serious causative infectious agents in shrimp aquaculture are viruses. The northeastern part of Brazil is the most productive region with a total of 18,500 hectares of shrimp farms that account for approximately 70,000 tons of shrimp, which corresponds to 97% of the national production [4,5]. In this region, two main types of viruses are of great concern: the infection myonecrosis virus (IMNV) and the infectious hypodermal and hematopoietic necrosis virus (IHHNV). Based on their genome organization, the phylogeny of their macromolecular components, the structural characteristics of their capsids and the physical-chemical properties of the viral particles, the first is classified as a member of the Totiviridae family [6,7], and the latter belongs to the Parvoviridae family [8]. As a virus of the Totiviridae family, IMNV is double-stranded RNA virus deprived of an envelope with an isometric capsomer and a genome size of 7560 base pairs encoding two non-overlapping open reading frames (ORFs), which comprise a predicted RNA-binding protein and a capsid protein [7]. In contrast, IHHNV is a non-enveloped, symmetric icosahedral parvovirus with a single-stranded linear DNA genome composed of 3909 nucleotides and three superposed ORFs [8,9]. When infecting penaeid shrimps, IMNV causes high mortality rates by acutely destroying (via necrosis) the skeletal muscle of distal segments and the tail fan. In contrast, IHHNV causes chronic deformity syndrome and reduced growth and culture performance [10]. In both cases, environmental factors, such as salinity and temperature, appear to trigger viral outbreaks in shrimp culture.

In a previous survey, after an unusual period of rainfall resulting in high mortality and significant economic losses in local shrimp production, we used molecular procedure analysis to find that a high number of samples from extensively farmed *L. vannamei* developing IHHN or IMN disease were co-infected with both (IHHNV and IMNV) viral agents [11]. In addition, we have shown that the disease symptoms and outcomes found for the co-infected shrimps resulted from reciprocal viral replication, i.e., the proliferation of one type of virus impairs the multiplication of the other. In the same study, we observed that IHHNV appeared to modulate the expression of heat shock protein 70 (HSP70) in IHHNV/IMNV double-infected shrimp.

In aquatic organisms, particularly shrimps, the oscillation provoked by physical (e.g., temperature), chemical (pesticides, pH and salinity) and biological (epibionts, epizootics, enzootics, etc.) insults, which are part of the equivocal strategies of farming management, may independently or cooperatively work to cause outbreaks of severe infections and mortality [12-16]. Moreover, a direct relation between environmental stress, immunity imbalance and development of bacterial and viral infection and disease has been observed at molecular and organism level in shrimp [17,18].

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The aim of the present work was to investigate, in controlled laboratory conditions, the influence of low salinity on the replication of IMNV in *L. vannamei*. Additionally, we monitored the proliferation of IHHNV and estimated the generation time (*g*) of replication for both viruses under the influence of different salinities.

Material and Methods

Shrimp maintenance and experimental viral infection

A total of 150 macroscopically healthy shrimps were obtained from a local shrimp farm (Paraípaba, Ceará - CE, Brazil) and transported to the Laboratory of Aquatic Resources of the Federal University of Ceará (CE-Brazil). The shrimps were acclimated for one week in a 1000-L tank filled with seawater, the salinity of which was similar to that in the collection site (35 g L^{-1}) and controlled through a flow-through system. The shrimp were fed *ad libitum* a commercial diet (35% crude protein; Nutreco Fri-Ribe®, Ceará, Brazil). After acclimation, the shrimps (9.3 ± 1.2 g) were captured, anaesthetized with 100 mg L^{-1} benzocaine, randomly selected, weighed, counted and stocked into 30-L aquaria (10 shrimp per aquarium) with three replicate aquaria for each treatment. The experimental procedure was conducted with four different salinities, yielding a control group (35 g L^{-1}) and four treatments (5, 15, 25 and 35 g L^{-1}). Each treatment was operated on a common recirculation system with 200-L mechanical and biological filters and a water exchange of approximately 0.5 L min^{-1} per aquarium. Each aquarium was equipped with an air diffuser to maintain an oxygen concentration in the water close to saturation. Nets to prevent the shrimp from jumping out covered each aquarium. The salinity was gradually reduced by pumping disinfected freshwater at a rate of 0 (control and treatment 1) or 2 g L^{-1} (treatments 2, 3 and 4) per hour until reaching the salinity corresponding to each treatment. The salinity levels were monitored using an optical refractometer. The water in the aquaria were maintained at ambient temperature within a range of 27.5 to 30.5°C and a mean temperature of 29.0°C during the experimental period. The temperature differences among the aquaria never exceeded 0.2°C. A photoperiod of 12 h of light (L)/12 h of darkness (D) was maintained during the experiment.

The IMNV inoculum was obtained from the muscle of IMNV-infected shrimp collected during a disease outbreak that occurred in a shrimp cultivation pond in 2011. For viral extract preparation, the muscle from infected shrimp (*L. vannamei*) was homogenized in PBS (0.2 M phosphate buffered saline, pH 7.3) (1:3, w/v). The shrimp extract was centrifuged at 3000× g for 5 min, and the supernatant was used for viral inoculation into healthy shrimps. The concentration of the IMNV stock was quantified by real-time PCR and found to be a target copy number of 10^4 per microliter. For the shrimp challenges, 10 µl of the viral suspension was injected into each shrimp. The shrimp in the negative control group were injected with the same volume of phosphate buffered-saline.

Shrimp tissue processing and total RNA purification

The hepatopancreas from three shrimps in each group was dissected at 0, 1.5, 3, 6 and 12 hours post-injection and transferred to microtubes containing RNA Later solution (Life Technologies, CA, USA) for total RNA purification. The samples were maintained at 4°C until processing, and the total RNA was purified within one week following collection. The inoculated shrimps were observed from 0 to 48 h post infection for clinical signs of IMN disease, such as anorexia, lethargy and mortality.

The total RNA from the minced hepatopancreas (20 to 30 mg) was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the manufacturer's protocol, which includes a DNase I treatment step. The quality and yield of the total RNA were verified by assessing the integrity of 28S and 18S rRNA and by spectrometrically assessing the 260/280 nm ratio.

cDNA synthesis from viral RNA

For complementary DNA (cDNA) synthesis, up to 1 µg of each DNase I-treated total RNA sample, which was mixed with 500 ng of random primers (Promega, Madison, WI, USA) in a final volume of 10 µl, was heated to 70°C for 10 min and cooled at 4°C. To complete the reverse transcriptase reaction mixture, the following components were mixed with the denatured RNA in a final volume of 20 µl: 100 U of ImProm II reverse transcriptase enzyme (Promega, Madison, WI, USA), 1 mM of each deoxynucleoside triphosphate, 2 mM $MgSO_4$, 1 mM dithiothreitol, and 20 U of RNase inhibitor. The reverse transcription mixture was incubated at 42°C for 90 min and then at 70°C for 15 min. The cDNA was diluted tenfold with TE (10 mM Tris–HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid), and 2-µl aliquots were used for the relative and quantitative real-time PCR (qPCR) experiments.

Quantitative real-time PCR of shrimp virus (IMNV and IHHNV)

For the quantification of the IHHNV and IMNV loads in *L. vannamei*, the absolute quantitative strategy was used. The genes encoding the nonstructural proteins of IMNV (GenBank accession number AAT67231.1) and of IHHNV (GenBank accession number AAF59415.1) were cloned, and serial 10-fold dilutions of each gene were prepared to establish the qPCR standard curves. The standard curve series were constructed in triplicate. The linearity of the qPCR standard curve was expressed as the square of the Pearson correlation coefficient (r^2). The primers for the qPCR detection of IMNV and IHHNV, in addition to those used for the shrimp β-actin gene, are detailed elsewhere [11].

The amplification of all cDNAs in this study was conducted in a Rotor-Gene 3000 system operated with its respective software (version 6.0.19; Corbett Research, Mortlake, Australia). Each reaction, which was conducted in a final reaction volume of 20 µl, consisted of 2.0 µl of the cDNA (~10 ng of reverse-transcribed mRNA), 0.2 µM of each gene specific sense and anti-sense primer, and 10 µl of two-fold concentrated GoTaq qPCR Master Mix (Promega, Madison, WI, USA). The amplification conditions for the viruses were as follows: 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The fluorescence was collected at 494 to 521 nm during the extension phase.

To calculate the copy number in the absolute qPCR experiments, the following equation was used (http://www.uri.edu/research/gsc/resources/cndna.html): Number of copies=[DNA amount (ng) $*6.022\times10^{23}$]/[DNA length (nt)* 1×10^9 *650]. The threshold and threshold cycle values were automatically determined by the Rotor Gene 6.0.19 software using the default parameters. All of the measurements were obtained as the means of at least nine measurements ± SEM (less than 5% error). The corresponding real-time PCR efficiencies (E) of the cycles in the exponential phase were calculated from the given slopes (k) according to the following equation: $E=10^{(-1/k)}-1$. To normalize the values of the viral load, the mean copy number of β-actin transcripts in each sample, which is equivalent to 1 µg, was determined from at least ten independent experiments ($n \geq 30$); the results of the viral infection and gene expression analyses are denoted as the logarithm of the copy number.

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Statistical analysis

The statistical analyses were performed with the BioStat 5.0 software using one-way ANOVA. In the cases in which significant differences were observed, the Least Significant Differences (LSD) test was applied. The positive correlation between the viral loads was expressed as the Pearson coefficient (r).

Results and Discussion

In a recent study, we assessed the expression level of selected gene transcripts (i.e., crustin, peneidin-3a, C-type lectin and HSP70) related to the innate immune systems of shrimp in response to viral infection caused by IMNV and IHHNV after an unusual period of rainfall in a delimited shrimp production area [11]. Under the natural conditions of shrimp culture, we detected that a high proportion of shrimp samples were positive for both viruses, i.e., they were environmentally co-infected with IMNV and IHHNV. Interestingly, a phenomenon of reciprocal viral replication appeared to occur in this type of co-infection: the species of virus and the viral load at the beginning of the infection determined the disease outcome, i.e., IMN or IHHN disease in the shrimp. Moreover, the level of HSP70, which is a cytoprotective protein, was up-regulated by viral infection and displayed a positive correlation with IHHNV replication. However, these data were obtained from shrimps sampled under natural culture conditions, in which some parameters, such as salinity, temperature and exposition to a complex microbiota (and potential pathogens), were not subjected to a strict control. Therefore, we were compelled to conduct a controlled set of experiments on virus replication in shrimp as a function of different salinities to verify the correlation between salt stress and viral proliferation.

With this aim, a total of 150 asymptomatic adult shrimps were acclimatized in the laboratory to different levels of salinity (5, 15, 25 and 35 g L^{-1}) and intramuscularly inoculated with 100,000 IMNV particles (10^4/µL). Samples of the hepatopancreas were surgically collected from three individual shrimps 0, 1.5, 3, 6 and 12 h post-infection, and the level of viral replication was assessed by qPCR. From the same samples, the number of copies of IHHNV and the number of transcripts of crustacean β-actin were determined. As might be expected, the number of IMNV particles increased steeply in all salinities tested (Figure 1, part A). Unexpectedly, IHHNV was also detected just after the beginning of the experiment and thereafter, even though the only virus that was deliberately inoculated in the study was IMNV (Figure 1B). This finding reflects the nature of IHHNV as a persistent epizootic agent that is present in all stages of the shrimp life cycle, including larvae and asymptomatic adults, and that is capable of invading the germ line and integrating into the host genome. Interestingly, in the first hours post-inoculation (between 1.5 h and 3 h), the high number of IMNV particles suppressed the replication of IHHNV, as observed in Figure 1, in agreement with one of our previous studies. In the subsequent period of infection, IHHNV also proliferated gradually over, particularly at a salinity of 5 g L^{-1}, whereas IMNV replicated exponentially over time and in lower salinities. At a salinity of 35 g L^{-1}, the estimated generation times for IHHNV and IMNV were 37.2 min and 57.4 min, respectively, in contrast to the values of 17.1 min and 25.2 min found at 5 g L^{-1}, respectively. This estimation, which was obtained through the calculation of the viral copy number as a function of the time post-infection, clearly indicates that the generation time for both viruses is practically reduced by two-fold when the salinity is decreased from 35 g L^{-1} to 5 g L^{-1}. Curiously, these values indicate that the persistent IHHN virus detains a lower generation time than IMNV, but due to the experimental design in the aquarium for IMNV

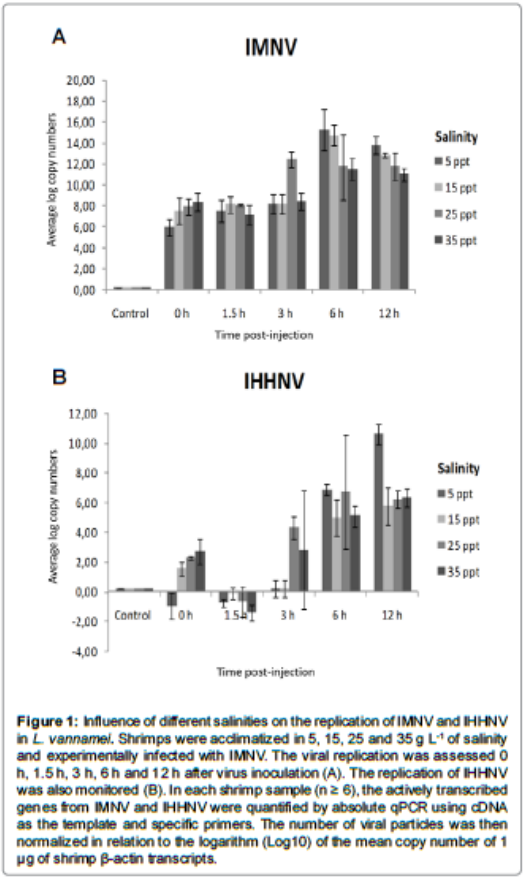

Figure 1: Influence of different salinities on the replication of IMNV and IHHNV in *L. vannamei*. Shrimps were acclimatized in 5, 15, 25 and 35 g L^{-1} of salinity and experimentally infected with IMNV. The viral replication was assessed 0 h, 1.5 h, 3 h, 6 h and 12 h after virus inoculation (A). The replication of IHHNV was also monitored (B). In each shrimp sample ($n \geq 6$), the actively transcribed genes from IMNV and IHHNV were quantified by absolute qPCR using cDNA as the template and specific primers. The number of viral particles was then normalized in relation to the logarithm (Log10) of the mean copy number of 1 µg of shrimp β-actin transcripts.

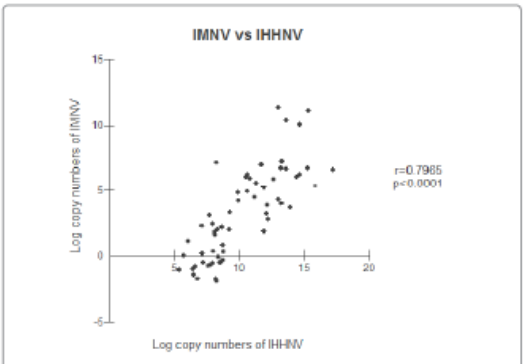

Figure 2: Linear correlation between IMNV and IHHNV replication in shrimp. Plot (coordinate pairs) of IMNV vs. IHHNV proliferation in different salinities and at different times post-infection. The quantitative PCR data were obtained and statistically analyzed as described in the text. The values of the Pearson coefficient (*r*) and probability (*p*) are shown in the plots.

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replication, as well as the phenomena of reciprocal replication that we had previously observed, the fortuitous number of viral copies of IHHNV was constantly inferior to that of IMNV through the experiment. Moreover, as noted, the replication of IMNV occurs in all levels of salinity and periods of time post-infection, by which were experimentally and statistically tested (coefficient of Pearson, r=79.65%). The positive correlation between the different salinities and the time of infection for IMNV versus IHHNV was observed with high confidence (Figure 2).

According to our measurements, the peak of IMNV replication reached a maximum in 6 h after infection, suggesting a rapid and efficient mechanism of cell entry and co-option of the molecular machinery of host cells for viral proliferation. In the earlier period of infection (from 0 h to 3 h), the difference in the virus number relative to the preceding hour was not statistically significant, although we observed an increment in the IMNV copy number (Figure 3).

The decrease in the salinity of shrimp culture ponds during the rainy season and the appearance of viral diseases is a known fact of shrimp producers in the northeastern region of Brazil. The scientific reasoning underlying this phenomenon calls for the influence of the osmotic shock response on the components of the crustacean innate immunity system. Thus, a stress caused by osmotic adjustment may trigger the viral replication and the high shrimp mortality observed in the field.

In fact, the maintenance of the isosmotic level of salinity is required to avoid a reduction in the efficiency of innate immunity responses and an increase in the vulnerability of marine shrimps to epizootic agents [19]. The isotonic salinity is a key environmental factor influencing the physiology of numerous species of marine organisms, from algae to fishes. In shrimps in particular, abrupt changes in salinity can affect the metabolic efficiency, the consumption of oxygen, and the rates of growth and survival [20]. Li and collaborators observed that shrimps inoculated with *Vibrio alginolyticus* and maintained under low salinity displayed, after 6 to 12 h, a significant reduction in immune factors, such as the number of hemocytes and prophenoloxidase activity.

The relationship between fluctuations in salinity and the susceptibility of shrimp to virus infection has been increasingly studied in the case of white spot syndrome virus (WSSV). In a study conducted by Vaseeharan and collaborators [20], the influence of low salinity on the innate immune system of healthy *Fenneropenaeus indicus* challenged with white spot syndrome virus (WSSV) was investigated. These researchers observed a reduction in the shrimp immune competence and an increase in the susceptibility to the virus. In addition, Ramos-Carreño and co-workers studied the susceptibility of *L. vannamei* to WSSV in several levels of salinities and found that the clinical manifestation of viral infection was more severe in condition of hyposmolarity. The low salinity was also verified to contribute to a decrease in the osmoregulation performance of *L. vannamei* and an increase in the replication of WSSV, resulting in higher rates of shrimp mortalities [21].

In the same line, our results demonstrate that the replication rate was higher at a lower salinity (i.e., 5 g L^{-1}) for both viruses, the IMNV and the persistent IHHNV, over a period of 6 to 12 h of infection

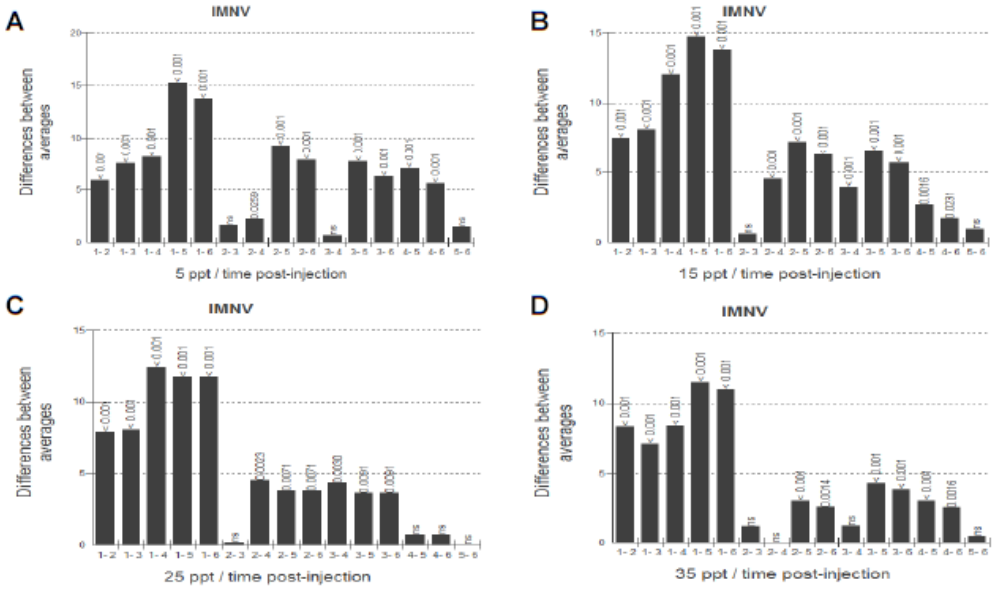

Figure 3: Significant differences in the replication of IMNV in infected shrimp over time. Differences between the logarithm of the mean copy number of IMNV in control shrimps (uninfected, numbered #1) and post-infected individuals at time 0 h (#2), 1.5 h (#3), 3 h (#4), 6 h (#5) and 12 h (#6). The significance level is indicated on top of the bar. The abbreviation "ns" denotes "no statistically significant difference".

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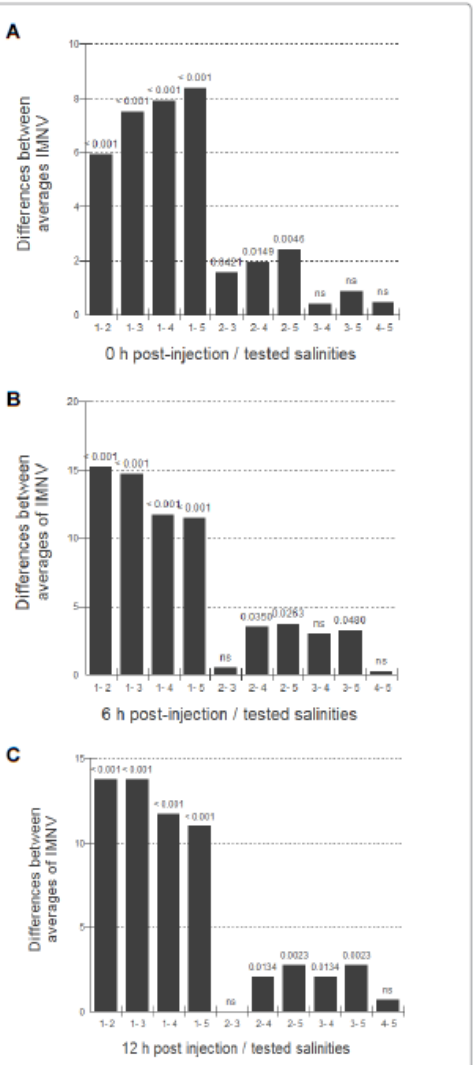

Figure 4: Significant differences in the replication of IMNV in infected shrimp in relation to the salinity and time. Differences between the logarithm of the mean copy number of IMNV in control shrimps acclimated to a salinity of 35 g L^{-1} (numbered #1) and infected individuals at a salinity of 5 g L^{-1} (#2), 15 g L^{-1} (#3), 25 g L^{-1} (#4), or 35 g L^{-1} (#5) were monitored at time 0 h (A), 6 h (B) and 12 h (C). As mentioned in the legend of figure 3, the significance level is indicated on top of the bar, and "ns" denotes "no statistically significant difference".

(Figures 4 and 5).

In summary, we focused our analysis primarily on the replication of IMNV in controlled conditions with different salinities and thus controlled osmotic conditions. Using quantitative real-time PCR data and statistical analysis, we verified that low salinity facilitates the replication of infectious myonecrosis virus by decreasing the generation time. In addition, under conditions of fluctuation of salt content in salt water, we observed the same behavior in the proliferation of the persistent IHHN virus, i.e., a decrease in the time for viral duplication and a positive correlation between a low level of salinity and increased

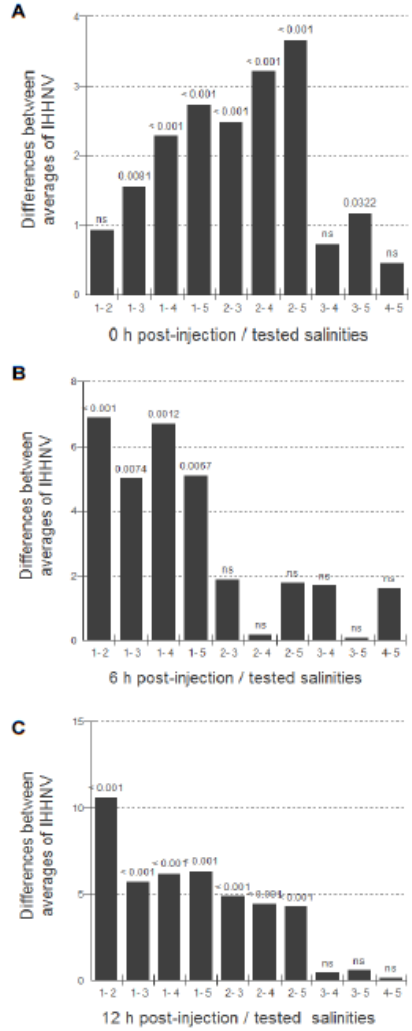

Figure 5: Significant differences in the replication of IHHNV in infected shrimp in relation to the salinity and time. Differences between the logarithm of the mean copy number of IHHNV in control shrimps at a salinity of 35 g L^{-1} (numbered #1) and infected individuals adapted to a salinity of 5 g L^{-1} (#2), 15 g L^{-1} (#3), 25 g L^{-1} (#4), or 35 g L^{-1} (#5) were monitored at time 0 h (A), 6 h (B) and 12 h (C). As mentioned, the significance level is indicated on top of each bar, and "ns" denotes "no statistically significant difference".

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virus multiplication, consequently, facilitating the co-infection in *L. vannamei*.

Acknowledgment

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Anexo B

Food Environ Virol
DOI 10.1007/s12560-017-9285-5

ORIGINAL PAPER

Antiviral Activity of Ctn[15-34], A Cathelicidin-Derived Eicosapeptide, Against Infectious Myonecrosis Virus in *Litopenaeus vannamei* Primary Hemocyte Cultures

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Abstract The shrimp farming has been converted into a mature aquaculture industry dealing with over millions of metric tonnes of processed commodities. Nevertheless, the global shrimp productions are constantly threatened by disease outbreaks, mainly triggered by rapidly disseminating viruses. Infectious myonecrosis virus (IMNV) is one of these epizootic agents affecting shrimp production in Brazil, of which no treatment exists. Herein, the antiviral activity against IMNV of an eicosapeptide, named Ctn[15-34], derived from a member of the cathelicidin family of antimicrobial peptides, was demonstrated. Cultures of hemocytes from *Litopenaeus vannamei* were established that support IMNV replication and infectivity titration. The cytotoxic effect of IMNV in culture and the in vitro anti-IMNV activity of Ctn[15-34] were assessed using a high-sensitive fluorescent-based method in combination with quantitative PCR. The Ctn[15-34] (<12.5 µM) neutralized the toxic effects of IMNV at loads sufficient to kill 50% of shrimp hemocytes. This study reported for the first time the replication of IMNV in vitro and the employment of a straightforward methodology to assess cell viability and viral/antiviral activities. In addition, it provided the basis for the development of the anti-infective multi-effector Ctn[15-34] eicosapeptide and analogs as components of antiviral formulations against shrimp viral diseases.

Keywords *L. vannamei* aquaculture · IMN virus · Anti-infective peptide · Cathelicidin-derived eicosapeptide · Fluorescence cell viability/cytotoxicity assay · Hemocyte in vitro culture

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Introduction

The shrimp farming has spread out to meet the increasing human food demand and to be converted in a mature and profitable industry. However, this industrial aquaculture sector has constantly suffered from diseases outbreaks, placing the global shrimp farming and trading at risk (Stentiford et al. 2012). The most important shrimp diseases are caused by viruses mainly because of rapid dissemination and the significant economic losses (Lightner et al. 2012; Stentiford et al. 2012). An important viral disease to Americas that is prevalent in the northeast of Brazil is caused by the infectious myonecrosis virus (IMNV). This type of virus belongs to the Totividae family, as first identified at Brazilian farms in 2002 and later at Indonesia farms (Flegel 2012; Tang et al. 2005). It is a non-enveloped, double-stranded RNA virus, with an isometric capsomer (~40 nm) and a genome size of 7560 base pairs encoding two overlapping open reading frames (ORFs), ORF1 and ORF2, which comprise the predicted structural proteins and the RNA-dependent RNA polymerase (RdRp),

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respectively (Lightner 2011; Liu et al. 2009; Naim et al. 2015).

The infectious myonecrosis caused by the IMNV provokes necrosis of the striated muscles of the abdomen and cephalothorax. Suggestive symptoms of the disease include anorexia, reduction in hepatopancreas volume, reduction in lipids, disoriented swimming, impairment in the hardening of the carapace and flexion of the 3rd abdominal segment (Nunes et al. 2004). Histological lesions are characterized by coagulative muscle necrosis, usually accompanied by fluid accumulation between the muscle fibers, hemocytic infiltration, fibrosis, and typical presence of spheroids in the lymphoid organ. In addition, inclusion bodies can be observed within the cytoplasm of muscle and connective tissue cells and hemocytes (Poulos et al. 2006).

Over the past few years, scientists have simulated shrimp viral diseases in vitro to understand their pathogeneses, such as host-virus interactions and immune-related gene expressions (Dantas-Lima et al. 2012; Jose et al. 2011; Liu et al. 2007). Since no immortalized shrimp cells lines have been developed thus far, primary tissue cultures from the lymphoid organ, ovaries, as well as hemocytes have been used to study these shrimp viral pathogens (Deepika et al. 2014; Li et al. 2015a; Maeda et al. 2004). These primary cell cultures have also been used to evaluate potential efficacy and toxicity of chemicals used to prevent and/or treat shrimp diseases (Jose et al. 2010). Although there are no commercially available drugs to treat shrimp viral diseases, research efforts have been dedicated to find new molecules with antiviral activity against these shrimp pathogens. Examples include the use of probiotics (Lakshmi et al. 2013), small-interfering RNAs (siRNAs) (Feijo et al. 2015; Shekhar and Lu 2009), and antimicrobial peptides (AMPs) derived from anti-lipopolysaccharide factor isoforms (Li et al. 2015a; Yang et al. 2016).

Also called host-defense peptides, AMPs are expressed and used by the innate immunity of plants and animals to fight invading pathogens. Their general mechanism of action is through electrostatic interaction with the anionic phospholipids of bacteria, for example, and insertion in the pathogen membranes, which will induce pore formation and kill the microorganism (Li et al. 2012; Yeung et al. 2011). In the case of viruses, AMPs can act by inhibiting viral adhesion and fusion to host cells, and/or disrupting viral envelopes, and/or inhibiting replication through interaction with the viral polymerase (Jenssen et al. 2006; Skalickova et al. 2015).

In our research group, we have studied the cathelicidins, which are a diverse group of AMPs found from the primitive hagfish to humans (Kosciuczuk et al. 2012). In particular, we have found cathelicidin precursors from the venom gland cDNAs of some South American pit viper snakes. Mature sequences, termed vipericidins, were then synthesized and good antimicrobial activities were found in vitro, especially against gram-negative bacteria (Falcao et al. 2014). In addition, a structural dissection of crotalicidin, a rattlesnake vipericidin, revealed that one of the fragments, named Ctn[15-34], maintained the antibacterial, antifungal, and anticancer activities of the parental peptide with much less toxicity and greater serum stability than full-length crotalicidin (Falcao et al. 2015; Cavalcante et al. 2016).

The present work aimed to investigate the effectiveness of the anti-infective and anti-proliferative intrinsic activities of Ctn[15-34] as an antiviral against IMNV. First, primary hemocyte cultures were established that could respond to different chemical stimuli. Next, the IMNV replication and lethality were verified with these hemocytes. Finally, it was assessed whether treatment with Ctn[15-34] could protect the hemocytes from IMNV.

Materials and Methods

Litopenaeus vannamei Shrimps

Healthy *L. vannamei* shrimps about 9–10 g were kindly donated by the Nutrition of Aquatic Organisms Laboratory from the Center of Environmental and Coastal Studies of the Institute of Marine Sciences, at Federal University of Ceara (Brazil). They were kept in sea water (3.5% salinity) tanks with a recirculation system and a daily supply of commercially available pelleted food. These shrimps were used to obtain hemocytes as needed.

Primary Hemocyte Cultures

The primary hemocyte cultures were established based on a protocol developed by Jose and colleagues (Jose et al. 2010), with some modifications. Shrimps were sacrificed and disinfected by immersion in ice-cold seawater containing 800 mg l^{-1} of sodium hypochlorite for 45 s. Next, they were washed with sterile ice-cold seawater, dipped in cold 70% alcohol-sea water solution and rinsed again with sterile ice-cold sea water. Hemolymph was then aseptically withdrawn from the ventral sinus of a shrimp with a sterile 22 gauge needle and syringe containing 200 µl of an anticoagulant solution (0.01 M tris–HCl, pH 7.0, 0.25 M sucrose, and 0.1 M sodium citrate). Viable hemocytes were counted by the Trypan blue (SigmaChemical, St. Louis, MO) exclusion dye procedure with a hemocytometer. For all experiments, 10^5 hemocytes/well were suspended in 50 µl of a modified 2x L-15 medium in 96-well plates. That medium consisted of double-concentrated Leibovitz's L-15 (2x L-15; Sigma) supplemented with 20% FBS, 2% glucose, MEM vitamins (1x), tryptose phosphate broth (2.95 mg ml^{-1}), 0.2 mM N-phenylthiourea, 0.06 µg ml^{-1}

chloramphenicol, 1x antibiotics solution (100 µg ml^{-1} streptomycin and 100 IU ml penicillin). Final medium and PBS osmolarity were measured with a cryoscopic osmometer, OSMOMAT® 030 (Gonotec, Berlin, Germany), and kept at 720 mOsm/kg. The suspended hemocytes were incubated for 1 h at 29 °C before each experiment to allow their attachment to the wells.

Fluorescence Cell Viability/Cytotoxicity Assays

The suitability of the primary hemocyte cultures was verified by viability assays using a dye, resazurin (CellTiter-Blue®, Promega, Madison, WI), which is metabolically reduced by live cells to the highly fluorescent resofurin. Hemocytes were treated with 50 µl of the modified 2x L-15 medium containing twofold serial dilutions of either benzalkonium chloride (Sigma) or chondroitin sulfate (Sigma) at final concentration ranges of 6.25–200 µg $ml^{-1}$and 1.56–50 mg ml^{-1}, respectively. After additional 30 min incubation at 29 °C, 30 µl of CellTiter-Blue®(Promega) reagent was added to each well, and plates were re-incubated for up to 48 h. Fluorescent readings were measured at 4, 24, and 48 h after the addition of the dye using a Synergy HT (BioTek, Winooski, VT) multi-detection microplate reader with $\lambda_{ex} = 530$ and $\lambda_{em} = 590$ nm. Relative hemocyte viability was calculated by taking non-treated cells as controls (≈100% viability), and experiments were carried out in triplicate. The obtained means ± standard errors of measurement were compared through one-way analysis of variance (ANOVA) and Bonferroni post hoc tests using the software SPSS version 16.0 (IBM Corporation, Somers, NY). Differences were considered statistically significant if $p < 0.05$.

Total RNA Isolation and cDNA Synthesis from Hemolymph and Gill Tissue Extracts of *L. vannamei* Shrimps with Infectious Myonecrosis Symptoms

Litopenaeus vannamei shrimps weighting 9–10 g each with opaque, whitish infectious myonecrosis symptoms (Nunes et al. 2004) were kindly donated by the Compescal Fishery Company Ltd (Aracati, Ceara, Brazil). From these shrimps, a pool of approximately 3 g of hemolymph and 4 g of gills was macerated with 50 ml of ice-cold 2x L-15 medium, without serum, on a mortar and pestle kept on an ice bath. The extracts were centrifuged at 10000*g* for 10 min at 4 °C and the supernatants were passed through 0.22 µm PVDF membranes (Millipore, Bedford, MA). Aliquots of the supernatants were then stored at −80 °C until further use. Total RNA was isolated from 100 µl of the supernatant with the NucleoSpin TriPrep kit (Macherey–Nagel, Bethlehem, PA) and quantified with the RNA HS reagent in a Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA), according to their respective manufactureŕs protocols. Later, single-stranded complementary DNA (ss-cDNA) synthesis was performed starting with 1 µg of total RNA, which was mixed with 0.5 µg of random hexamer primers (Promega, Madison, WI), heated to 70 °C for 10 min and quickly cooled to 4 °C. Next, the reverse transcription reaction was run at 42 °C for 90 min with the denatured RNA and primers, 100 U of ImProm II reverse transcriptase enzyme (Promega, Madison, WI), 1 mM of each dNTPs, 5 mM $MgCl_2$, and 20 U of RNase inhibitor, in a final volume of 20 µL. Then, the reverse transcriptase was terminated by heating at 70 °C for 15 min, and all individual cDNA synthesis reactions were stored at −20 °C.

Analyses of IMNV Viral Loads Contained in *L. vannamei* Tissue Extracts

The amount of IMNV viral particles from the pools of hemolymph and gill tissue extracts were determined by quantitative polymerase chain reaction (qPCR) with the use of the Rotor Gene 3000 and its specific software version 6.0.19 (Corbertt Research, Mortlake, Australia). For each 20 µl reaction, 2 µl of ss-cDNA, prepared as above, were mixed with 10 µl of 2x GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and 0.2 µM of a primer pair, either from IMNV or shrimp β-actin genes, as previously obtained (Vieira-Girão et al. 2012). Then, 45 cycles were run at 95 °C for 30 s, at 65 °C for 30 s and at 72 °C for 30 s each, and fluorescence was detected at 494–521 nm during the extension phase. The Rotor Gene software, running default parameters, automatically provided the threshold and threshold cycle (Ct) values for each reaction, which were used to calculate the IMNV viral loads. Absolute quantification of IMNV viral particles was estimated with the copy numbers of transcript sequence encoding the major capsid protein by the equation: number of transcripts = [DNA amount (*ng*) × 6.022× 10^{23}]/[DNA length (bp) × 10^9 × 650] (http://cels.uri.edu/gsc/cndna.html). The cDNA amounts were found after the Ct values were plotted on previously built standard curves from tenfold serial dilutions of IMNV and shrimp β-actin gene (Vieira-Girão et al. 2012). The capsid transcript copy numbers used were from triplicates after being normalized against 1 µg of shrimp β-actin gene.

Infectivity Titration and Cytotoxic Activity of IMNV in *L. vannamei* Primary Hemocyte Cultures

The capacity of IMNV viral particles from the infected tissue extracts to re-infect and cause cellular damage was verified in vitro using the *L. vannamei* primary hemocyte

cultures established as above with two different assays. In the first evaluation, hemocytes (10^5 cells/well) were treated with twofold serial dilutions of the tissue extracts containing IMNV viral particles (called "IMNV extracts" from now on) at final concentration range of 7–227 capsid transcripts (copies) per microliter (copies μl^{-1}) and volume of 100 µl/well of the modified 2x L-15 medium in 96-well plates. Viability was assessed as in Fluorescence cell viability/Cytotoxicity assays section, and the relative hemocyte viability was calculated with non-treated cells as controls, and experiments were carried out in triplicate.

In the second assay, it was verified whether IMNV could replicate in culture in primary hemocytes. In 12-well plates, 1.6 x 10^6 cells per well were seeded with modified 2x L-15 medium and incubated for 1 h at 29 °C. Then, a diluted IMNV viral extract sufficient to cause 10% hemocyte inhibition (≈90% cell viability) was added to the wells with a final volume of 1 ml/well. After different time intervals (2, 4, 6, 10, 12, 24, and 48 h) post-inoculation at 29 °C, medium was removed, and cells were washed with ice-cold 2.5 × phosphate buffered saline (PBS) and harvested. Next, PBS was removed by centrifugation at 10000*g* for 5 min at 25 °C, and 100 µl of RNA later solution (Invitrogen, Carlsbad, CA, USA) was added to each cell pellet. The re-suspended hemocytes were stored at −20 °C until total RNA extraction, cDNA synthesis and qPCR analysis were carried out as described above. Experiments were in triplicate for each time point, and controls used were non-treated cells and cells treated with a previously heat-inactivated (95 °C for 15 min) diluted extract of IMNV.

The Eicosapeptide Ctn[15-34]

Ctn[15-34] (KKRLKKIFKKPMVIGVTIPF-amide, 20-mer, MW = 2371.11 g mol^{-1}) was synthesized and purified according to procedures described in details in previously published work (Falcao et al. 2015). Peptide stock solutions were prepared at 1 mM with deionized water as required and stored at 4 °C for up to six weeks. Ctn[15-34] toxicity to hemocytes was also evaluated by viability assays as above. A volume of 50 µl of the modified 2x L-15 medium was added to the cells containing twofold serial dilutions of the peptide at final concentration range of 6.25–200 µM. After 30 min incubation at 29 °C, 30 µl of CellTiter-Blue® reagent (Promega) was added to each well, and plates were re-incubated for up to 48 h. Fluorescent readings were at 12, 24, and 48 h after addition of the dye using a Synergy HT (BioTek) microplate reader with $\lambda_{ex} = 530$ and $\lambda_{em} = 590$ nm. Relative hemocyte viability was calculated by taking non-treated cells as controls, and experiments were carried out in triplicate.

Antiviral Activity of Ctn[15-34] Against IMNV

The potential antiviral properties of Ctn[15-34] against IMNV were next evaluated based on the peptide recently found anti-infective and anti-proliferative properties, such antibacterial, anticancer, and antifungal activities (Cavalcante et al. 2016; Falcao et al. 2015). The Ctn[15-34] capacity to protect *L. vannamei* primary hemocyte cultures from infection by IMNV was assessed by two independent assays, both with IMNV extracts at concentrations sufficient to cause 50% hemocyte inhibition (IC_{50}). In one set of experiments, hemocytes were first incubated with Ctn[15-34] (0.75–25 µM concentration range) for 1 h at 29 °C before inoculation of IMNV extracts into hemocyte culture. In the other set, Ctn[15-34] (0.75–200 µM range) was firstly and directly added to IMNV extracts, incubated for 1 h at 29 °C, and then the mixture peptide and viral extract were added to hemocytes. In both experiments, after additional incubation of hemocytes with Ctn[15-34] and IMNV extracts for 30 min at 29 °C, 30 µl of CellTiterBlue® reagent (Promega) was added to each well, and plates were re-incubated for up to 48 h. Fluorescence readings were measured again at 24 and 48 h after addition of the dye using a Synergy HT (BioTek) microplate reader with λex = 530 and λem = 590 nm. Relative hemocyte viability was calculated with non-treated cells as controls, and experiments were carried out in triplicate.

Finally, it was also verified whether Ctn[15-34] had a direct destructive action on IMNV viral particles. Thus, 25 µl of Ctn[15-34] were added to microcentrifuge tubes containing 100 µl suspensions of IMNV extracts and a 1x protease inhibitor cocktail (Serva, Heidelberg, Germany). Final concentrations were: peptide at 6.25 µM and IMNV viral extract at hemocyte IC_{50} (but without the cells). After incubation periods of 3, 6, and 24 h at 29 °C, 175 µl of a lysis buffer (Promega) were added to the suspensions and samples were stored at −80 °C until RNA extraction, cDNA synthesis, and qPCR analysis as described above. Experiments were carried out in triplicate for each time point, and IMNV extracts without Ctn[15-34] were used as controls.

Results

Litopenaeus vannamei Primary Hemocyte Cultures and Fluorescence Cell Viability Assays

Hemocytes were obtained from the ventral sinus of *L. vannamei* shrimps. They were seeded on 96-well plates with a modified 2x L-15medium. These hemocytes remained viable for at least 8 days with a slight increase in viability on that period and no signs of culture

contamination. In addition, the viability reagent CellTiter-Blue, added right after the 1 h incubation period and kept in the cell culture for the 8-day time course, was not cytotoxic to hemocytes (Fig. 1).

Later, two chemicals were added separately to the primary hemocyte cultures to verify whether and how the cultured cells could respond to different stimuli with the use of the same CellTiter-Blue viability reagent. The first chemical used was benzalkonium chloride (BKC), which is used in fishery tanks as a preservative. According to Fig. 2a, BKC had a late toxic effect because concentrations of as much as 25 μg ml^{-1} were not toxic up to 4 h of incubation, but were able to cause death in 50% (IC_{50}) of cultured hemocytes in 24 h.

The second chemical used was chondroitin sulfate, which is a glycosaminoglycan found in cartilage extracellular matrix. When associated with extracellular proteins, chondroitin sulfate can enhance cell adhesion to surfaces (Frantz et al. 2010). According to Fig. 2b, chondroitin sulfate also had a late toxic effect because non-toxic concentrations of 12.5 and 25 mg ml^{-1} up to 4 h incubation reduced hemocyte viabilities by 50% after 24 h ($IC_{50} \approx 25$ mg ml^{-1}). Interestingly, lower concentrations of as much as 6.25 mg ml^{-1} incubated for up to 24 h had a significant increase ($p < 0.02$) in hemocyte viability, which had a direct relationship with increased metabolic activity. Hemocyte viability patterns were mainly the same between 24 and 48 h incubation for each chemical tested.

Infectivity Titration of IMNV Extracts in *L. vannamei* Primary Hemocyte Cultures

After the primary hemocyte cultures were established, our next step was to evaluate whether and how these cells could respond to increased doses of IMNV, a shrimp viral pathogen known to cause the infectious myonecrosis disease (Lightner et al. 2012). Thus, IMNV extracts were obtained and quantified as described above and tested with the hemocytes through twofold serial dilutions to obtain a dose–response curve. Results are presented in Fig. 3.

In Fig. 3, one can observe that the IMNV extracts were infective and cytotoxic to hemocytes, in a concentration-dependent manner. In addition, the IMNV toxicity to hemocytes increased over time because there was a clearly observable further decrease in viability between 24 and 48 h incubation at each concentration. The concentration to inhibit 50% of the hemocytes (IC_{50}) was ~227 copies of capsid transcripts μl^{-1}, which was approximately one hundred-fold dilution of the obtained IMNV extracts.

Moreover, in this set of experiments, it was also assessed the capacity of IMNV particles from the viral extracts to replicate in the hemocytes. The variation of the number of viral particles, represented as normalized copies of viral capsid transcript μg^{-1} of shrimp β-actin gene, during the hours post-inoculation is shown in Fig. 4.

During the first 6 h of post-inoculation, the numbers of IMNV particles were lower, with means between 7×10^{10} and 5×10^{11} copies μg^{-1} of β-actin gene, than by 12 h, when IMNV particles reached their replication peak with 4×10^{12} copies μg^{-1}. From 12 to 24 h, there was a decrease in the number of particles (1×10^{12} copies μg^{-1}) most probably because viral shedding released IMNV particles to the medium, and quantification was only performed from the collected hemocytes. The later increase in particle number by 48 h (4×10^{12} copies μg^{-1}) provided a hint that the virus was going through one more replication cycle. Similar findings were previously reported for Taura Syndrome Virus (TSV) inoculated in other shrimp primary hemocyte cultures (George et al. 2011).

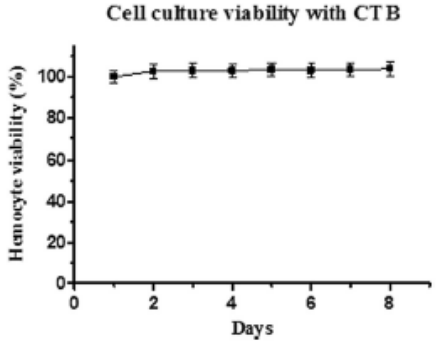

Fig. 1 Primary hemocyte culture viability (%) in the modified 2x L-15 medium (see Materials and Methods—Primary Hemocyte Cultures) sections up to 8 days (mean ± SEM, with $n = 3$)

Assessment of Ctn[15-34] Antiviral Activity Against IMNV in *L. vannamei* Primary Hemocyte Cultures

In a quest to find possible antiviral substances to prevent and/or treat viral diseases in shrimp farming, our next step was to evaluate the whether recently designed and developed, Ctn[15-34] eicosapeptide, could protect the primary hemocyte cultures from the IMNV in viral extracts. Initially, we performed a cytotoxicity assay, similar to the previous experiments with BKC and chondroitin sulfate, to know the maximal doses that could be used and would not harm the hemocytes. Results are displayed in Fig. 5.

According to Fig. 5, Ctn[15-34] had a late toxic effect because non-toxic concentrations, such as 50 μM, up to 12 h of incubation was able to kill 50% (IC_{50}) of the cells when hemocytes were exposed longer (after 24 h) to the peptide. Similar to BKC and chondroitin sulfate, the toxicity pattern of Ctn[15-34] to hemocytes did not change from 24 to 48 h incubation.

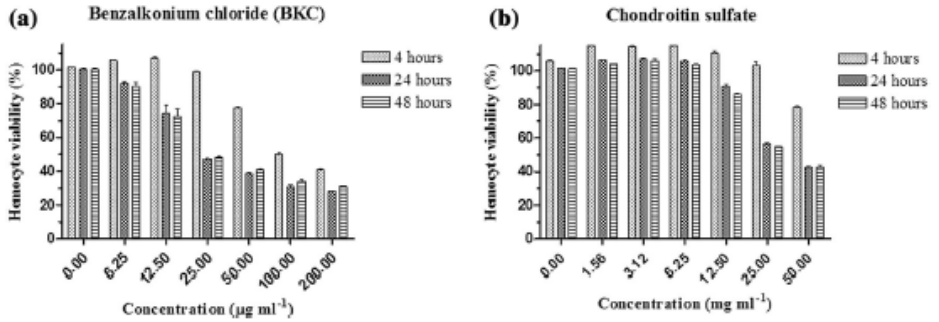

Fig. 2 Hemocyte viability (%) after treatment with (**a**) BKC and (**b**) chondroitin sulfate (mean ± SEM, with $n = 3$) for 4, 24, and 48 h

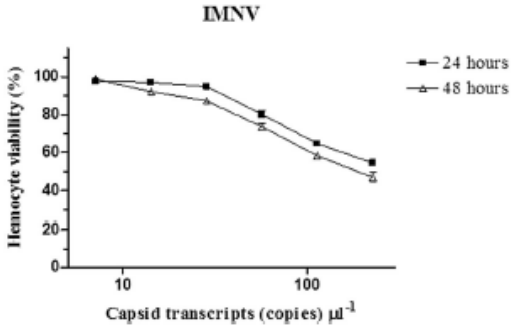

Fig. 3 Hemocyte viability (%) after treatment with IMNV extracts (mean ± SEM, with $n = 3$) for 24 and 48 h

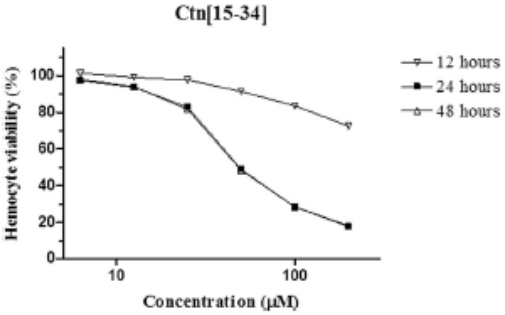

Fig. 5 Hemocyte viability (%) after treatment with Ctn[15-34] (mean ± SEM, with $n = 3$) for 12, 24 and 48 h

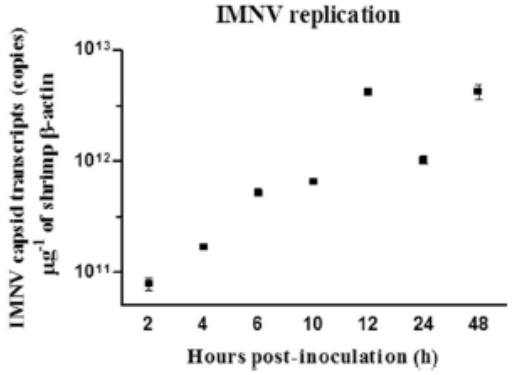

Fig. 4 IMNV replication capacity, estimated by capsid transcript copies (mean ± SEM, with $n = 3$), in *L. vannamei* primary hemocyte cultures up to 48 h post-inoculation

Then, Ctn[15-34] antiviral activity against the IMNV in extracts was estimated in the primary hemocyte cultures using two different experimental conditions, but the same viability test readout, that is, with CellTiter-Blue reagent and fluorescent intensity measurements. In the first assay, hemocytes were incubated with Ctn[15-34] at concentrations up to 25 µM, half of its IC_{50}, for 1 h. Subsequently, the IMNV extracts were added to the cultures at a fixed concentration of 227 copies μl^{-1} (IMNV IC_{50}). Hemocyte viability results from 24 to 48 h incubation are shown in Fig. 3b.

Figure 6a shows that the peptide Ctn[15-34] at 0.75–12.5 µM concentration range greatly reduced IMNV viral extract lethality since there was only a maximum 10% reduction of hemocyte viability up to 48 h incubation. Although the IMNV action in the extract was reduced, the decrease in viability by 30% with Ctn[15-34] at 25 µM indicated that the peptide reached toxic concentrations to the cells.

In the second assay, Ctn[15-34] antiviral activity was confirmed when the peptide at 0.75–200 µM concentration range was previously incubated with the IMNV extracts at their IC_{50} for 1 h before adding to the hemocytes. Cell viability results are displayed in Fig. 6b from 24 to 48 h incubation.

In Fig. 6b, although it was observed a general 20% reduction in cell viability at all tested peptide concentrations, there was no further increase in toxicity to the

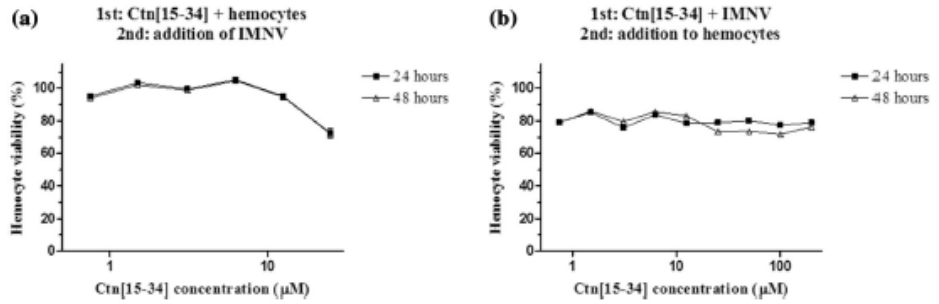

Fig. 6 Hemocyte viability (%) after treatment with Ctn[15-34] and IMNV extracts at their IC_{50} (mean ± SEM, with $n = 3$) for 24 and 48 h: **a** hemocytes treated with Ctn[15-34] before addition of IMNV; **b** IMNV extracts treated with Ctn[15-34] before addition to hemocytes

hemocytes. Again, up to 48 h, Ctn[15-34] did not allow the IMNV extracts to kill 50% of the hemocytes. Interestingly, it seemed that not only Ctn[15-34] interfered with IMNV activity but also the viral extract reduced the peptide toxicity because concentrations over its IC_{50} reduced hemocyte viability by no more than 20% when firstly incubated with the IMNV extracts.

Finally, we also investigated whether Ctn[15-34] could directly destroy the IMNV particles contained in the viral extracts. Ctn[15-34] at 6.25 μM was incubated with a total of 227 copies μl^{-1} of IMNV (equivalent to kill 50% of hemocytes but non-inoculated to the cells) and qPCR analysis was performed after 3, 6, and 24 h incubation. Figure 7 shows the variation of the number of viral particles, represented as copies μg^{-1} of β-actin gene. The results in Fig. 7 indicates that Ctn[15-34] apparently did not directly damage the IMNV because the number of virus were mainly the same between treated and non-treated (IMNV viral extract only) groups at all time points.

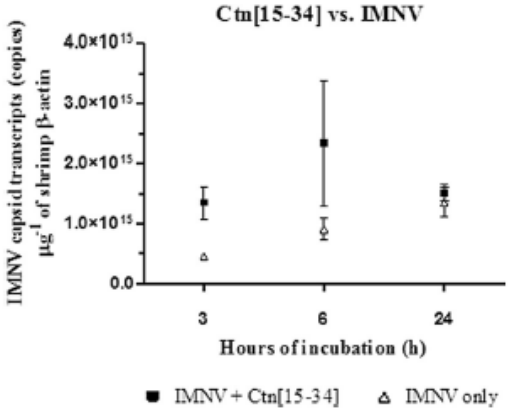

Fig. 7 Ctn[15-34] damage capacity to IMNV, estimated by capsid transcript copies (mean ± SEM, with $n = 3$), after treatment for 3, 6, and 24 h

Discussion

Shrimp primary tissue and hemocyte cultures have been used as in vitro platforms to study viral pathogens, especially the white spot syndrome virus (WSSV), because no immortalized shrimp cell line has been established yet (Jayesh et al. 2012). In our studies, we used primary hemocyte cultures because they could be directly seeded in plates without further tissue processing steps (George et al. 2011; Jose et al. 2011, 2010). Moreover, circulating hemocytes are the shrimp primary sentinel immune cells. These cells have similar roles as the macrophages in mammals, which include phagocytosis and lysis of foreign cells (Wynn et al. 2013). Therefore, an entire shrimp organism can be affected if viral pathogens can multiply inside these cells.

Hemocytes from *L. vannamei* shrimps were suspended in a modified 2x L-15 medium and allowed to attach for 1 h before each experiment. That period was enough for cells to acquire the same characteristics as in a live organism (Deepika et al. 2014), and the modified 2x L-15 medium kept the cells alive for at least 8 days (Fig. 1), similarly as previously demonstrated for cultures of *P. monodon* hemocytes (Jose et al. 2011, 2010). While most of shrimp cell, including experiments with primary tissue cultures, viability assays used the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) (Jayesh et al. 2015, 2012; Jose et al. 2012, 2010), and we used the resazurin reagent (CellTiter-Blue®, Promega). Although both reagents are dependent on the metabolism of live cells, resofurin (resazurin product) fluorescence can be directly measured on each well without the need to remove medium and solubilize crystals as the MTT-formazan products (Rampersad 2012). Thus, resazurin viability assay is non-destructive, and readout can be continuously monitored without the need to prepare numerous well plates for different time points in the same

experiment. To validate the application of resazurin-based fluorescence cell viability assay, we firstly confirmed that *L. vannamei* hemocytes sensibly responded to different chemical stimuli, and dose-response curves could be determined. BKC and chondroitin sulfate were toxic to hemocytes as their concentrations increased, especially after 4 h of incubation. However, when chondroitin sulfate was used at low concentrations (up to 6.25 mg ml^{-1}) by 24 h, an increase in viability could be observed which represented an increase in metabolic activity compared to the non-treated groups (Fig. 2a, b). Therefore, the addition of chondroitin sulfate at these low concentrations can be beneficial for primary hemocyte cultures because it is proposed that such glycosaminoglycan can help cells enhance their adhesion to the well surfaces (Frantz et al. 2010).

The primary cultures of *L. vannamei* hemocytes were also susceptible to the IMNV extracts (Fig. 3). Viral extract titration revealed that was necessary a load equivalent to 227 copies of capsid transcript μl^{-1} to kill 50% of the hemocyte culture. Similar studies with WSSV incubated in primary hemocyte cultures either from *P. monodon*(Jose et al. 2010) or from the mud crab *Scylla serrata* (Deepika et al. 2014) only showed IC_{50s} based on dilution factors from the viral extracts.

Moreover, it was here demonstrated that IMNV could replicate in *L. vannamei* primary hemocyte cultures (Fig. 4). Although previous studies found that other viruses could multiplicate in hemocytes, such as WSSV (Jiang et al. 2006; Wang et al. 2002) and TSV (George et al. 2011), this is the first time that IMNV replication in vitro is reported. Inside hemocytes, IMNV particles reached their replication peak by 12 h, which was similar to the multiplication of TSV found in a previous study (George et al. 2011). In the case of WSSV, Li and co-workers (Li et al. 2015b) found that it was necessary 18 h for WSSV to reach their maximum number of particles in lymphoid cells. It is believed that it took longer for WSSV to reach its replication peak than IMNV because the former virus has a bigger DNA-based genome than the latter that has a RNA-based genome (Lightner et al. 2012; Poulos et al. 2006).

The search for therapeutic agents to prevent and/or treat shrimp viral diseases have received attention lately, but few studies have been published so far (Yang et al. 2016). Among them, some proteins such as C-type lectins and Pmfortilin had activity against WSSV (Tonganunt et al. 2008; Zhao et al. 2009). Also, the laminin receptor in shrimps was recently found to interact with WSSV, IMNV, TSV, and YHV (Busayarat et al. 2011; Liu et al. 2016). Thus, this receptor can be used to develop antiviral drugs that bind the receptor (drug target) and avoid virus-receptor interactions and/or to design leads that can directly bind and neutralize the viral particles. In addition, peptides that have shown activity against shrimp viruses, such as WSSV, include fragments of anti-lipopolysaccharide factor isoforms from *Fenneropenaeus chinensis*(Li et al. 2015a).

In our studies, we used the antibacterial, antifungal, and antitumor Ctn[15-34] eicosapeptide (Falcao et al. 2015, Cavalcante et al. 2016) to evaluate whether this peptide could also have antiviral activity against IMNV. The first antiviral assay (Fig. 6a) demonstrated that Ctn[15-34] protected the hemocytes from viral cytotoxicity with concentrations (up to 12.5 μM) at least 4-fold lower than the peptide IC_{50} to hemocytes (50 μM—Fig. 5). Hence, it has the potential to be administered with commercial pelleted food at low doses to prevent infectious myonecrosis, especially during seasonal disease outbreaks. The second assay (Fig. 6b) confirmed hemocyte protection with Ctn[15-34] from IMNV and also indicated an interaction between the peptide and the virus before their addition to hemocytes because none of the tested concentrations (up to 200 μM of peptide and viral extract at IC_{50}) were severely toxic to the cells. Therefore, although the interaction mechanism remains to be further investigated, it seems that the structural active domains involved in Ctn[15-34]-IMNV association are primarily the same needed by the peptide and the virus for each interaction with hemocytes. Even though Ctn[15-34] could not reduce the number of IMNV particles by 24 h (Fig. 7), the peptide–virus association looked stable because hemocytes remained with the same viability at least up to 48 h with Ctn[15-34]-IMNV particles (Fig. 6a). Noteworthy, although antimicrobial peptides activity can be greatly reduced with high salt and serum concentrations (Chu et al. 2013; Maisetta et al. 2008), Ctn[15-34] exhibited antiviral effects against IMNV in a medium with elevated salinity and 20% FBS. Since these medium requirements are needed to culture hemocytes because they simulate in vivo conditions, Ctn[15-34] therapeutic use is promising against IMNV infections in shrimp aquaculture.

Taken together, we established a *L. vannamei* primary hemocyte culture that responded to chemical stimuli and were sensitive to IMNV in viral extracts. In addition, IMNV particle replication inside these hemocytes can be used as a platform for further viral cytopathogenesis studies. Moreover, the employment of a straightforward methodology to assess cell viability and viral activity based on fluorescence was demonstrated. Remarkably, Ctn[15-34], a recently developed eicosapeptide with antibacterial, antifungal, and anticancer properties, also suppressed IMNV infection in hemocytes and, thus, can be developed into a multi-effector compound and component of antiviral formulations against shrimp viral diseases.

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