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DESENVOLVIMENTO DE FERRAMENTAS PARA BIOENSAIOS VIRAIS DO VÍRUS DA MIONECROSE INFECCIOSA (IMNV) PARA O CAMARÃO MARINHO *Litopenaeus vannamei*

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PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA

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Tese julgada adequada para obtenção do título de doutor em Recursos Pesqueiros e Aquicultura. Defendida e aprovada em 25/02/2014 pela seguinte Banca Examinadora.

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Resumo

Nas últimas décadas, o acentuado crescimento da carcinicultura foi responsável por alterações ambientais que tiveram como principal consequência o surgimento e a propagação de doenças virais. No Brasil, o vírus da Mionecrose infecciosa (IMNV) ocasionou uma queda significativa na produção de *Litopenaeus vannamei* em 2004, destacando a necessidade do desenvolvimento de estratégias eficazes para o controle da doença. Diversos aspectos devem ser considerados no controle de doenças, desde a seleção de linhagens resistentes, a validação e padronização de métodos de diagnóstico, a compreensão dos mecanismos de transmissão dos agentes patogênicos, entre outros. Nesta tese, quatro abordagens foram avaliadas: (1) o desenvolvimento de um protocolo experimental de infecção por IMNV em *L. vannamei*, através de dois métodos de exposição, por ingestão (oral) e injeção (inoculação intramuscular); (2) o uso das linhagens celulares de mosquito C6/36 e SF9 para a propagação do IMNV; (3) a determinação da rota de transmissão vertical do IMNV em *L. vannamei* e; (4) a *Artemia* como vetor na transmissão horizontal do IMNV em *L. vannamei*. No primeiro estudo, desafios virais via ingestão mostraram cargas virais indetectáveis, enquanto que os experimentos via injeção intramuscular tiveram as doses infectivas 50% (ID_{50}) determinadas entre valores que indicam a diluição de inoculo viral de $1:10^7$ a 28°C e $1:10^6$ a 26°C como as mais apropriadas. No segundo estudo, linhagens celulares de C6/36 e SF9 foram desafiadas com IMNV e os resultados mostraram que não houve replicação de IMNV nessas células, descartando a possibilidade do uso deste sistema *in vitro* para fins de replicação do vírus. No terceiro estudo, reprodutores desafiados por injeção intramuscular e naturalmente infectados por IMNV mostraram evidências para uma rota de transmissão vertical do IMNV em *L. vannamei*, com maior probabilidade de transmissão atrelada ao parental materno. O quarto estudo comprovou a susceptibilidade de *Artemia* à infecção por IMNV via duas rotas de transmissão (imersão e adesão vírus-fitoplâncton) e seu papel como reservatório ou vetor mecânico na transmissão horizontal deste vírus em *L. vannamei*, sob condições experimentais, enfatizando a contribuição deste microcrustáceo como possível fonte para aumento da incidência de IMNV em unidades de produção.

Palavras-chave: Bioensaio; IMNV; Linhas de célula de mosquito; Transmissão vertical; *Artemia*; *Litopenaeus vannamei*.

Abstract

In the past decades, the marked farmed shrimp industry growth resulted in environmental disturbances, with a major consequence of outbreaking viral diseases. In Brazil, the Infectious myonecrosis virus (IMNV) caused a huge shortfall production in *Litopenaeus vannamei* in 2004, stressing the need of developing effective strategies for disease control. Different aspects have to be considered in disease control, including selective breeding; validation and standardization of viral diagnostic methods, disease transmission mechanisms, among others. In this thesis, four approaches were evaluated: (1) the development of an experimental infection protocol for IMNV in *L. vannamei*, throughout two methods of exposure, by ingestion (oral) and by injection (intramuscular inoculation); (2) the use of C6/36 and SF9 mosquito cell lines for IMNV propagation; (3) the determination of vertical transmission of IMNV in *L. vannamei* and; (4) the assessment of *Artemia* as a vector in IMNV horizontal transmission to *L. vannamei*. In the first study, ingestion bioassay showed undetectable viral loads, whereas injection treatments have determined the median infectious doses (ID_{50}) between values that suggest the dilution of the inoculum in $1:10^7$ at 28°C and in $1:10^6$ at 26°C as the most appropriate infective methods. In the second study, C6/36 and Sf9 cell lines were challenged with IMNV and the results showed that IMNV did not replicate in these cells, thus limiting this *in vitro* system for replication purposes. In the third study, *L. vannamei* broodstock inoculated with IMNV by intramuscular procedure or naturally infected presented evidences for vertical transmission associated to the maternal parent. The fourth study demonstrated the susceptibility of *Artemia* sp. to IMNV by immersion challenge and virus-phytoplankton adhesion route and its role as a reservoir or mechanical vector in IMNV horizontal transmission to *L. vannamei* under experimental conditions, stressing its contribution to virus incidence in aquaculture production units.

Keywords: Bioassay; IMNV; mosquito cell line; Vertical transmission; *Artemia*; *Litopenaeus vannamei*.

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Lista de abreviaturas e siglas

aa - Aminoácidos (Amino acids);

C6/36 - Linha de célula do mosquito *Aedes albopictus* subclone C6/36;

cDNA – DNA complementar (*Complementary DNA*);

CP - Proteína do capsídeo (*Coat protein*);

CPE - Efeito citopático (*Cytopathic Effect*);

CRS - Sistema de recirculação fechado (*Closed Recirculation Systems*);

DEPC – Dietilpirocarbonato;

DSRM - Domínio do motivo da *dsRNA-binding* (*dsRNA-binding motif*);

dsRNA - RNA de dupla fita (*Double-stranded RNA*);

FITC - Isotiocianato de fluoresceína (*Fluorescein isothiocyanate*);

GAV - Vírus gill-associated (*Gill-associated virus*);

GLM - Modelo linear generalizado (*Generalized linear models*);

HPV - Parvovirose hepatopancreática (*Hepatopancreatic parvo-like virus*);

ID_{50} - Dose infectiva 50% (*Median infectious dose*);

IFI - Imunofluorescência indireta (*Indirect Immunofluorescence*);

IHHNV - Vírus da Necrose hipodermal e hematopoiética infecciosa (*Infectious hypodermal and haematopoietic necrosis virus)*;

IMNV – Vírus da Mionecrose infecciosa (*Infectious myonecrosis virus*);

ISH - Hibridização *in situ* (*In Situ Hybridization*);

LB - Meio Luria-Bertani;

kDa - kiloDaltons;

MCMC - Método de Monte Carlo via Cadeias de Markov (*Markov Chain Monte Carlo*);

mRNA – RNA mensageiro (*Messenger RNA)*;

MrNV - Vírus *Macrobrachium rosenbergii nodavirus* (*Macrobrachium rosenbergii nodavirus*);

NHPB - Hepatopancreatite necrosante (*Necrotizing hepatopancreatic bacterium*);

nt - Nucleotídeos;

OIE - Organização Mundial de Saúde Animal (*World Organisation for Animal Health)*;

ORF – Matriz de região de leitura (*Open Reading Frame*);

pb - pares por base;

PCR - Reação em cadeia da polimerase (*Polymerase Chain Reaction)*;

PEMD - Polietileno de média densidade (*Medium Density Polyethylene*);

PES - Membrana polietersulfonica;

PL - Pós-larva;

PMCV - Vírus Piscine myocarditis (*Piscine myocarditis virus*);

PRDV - Vírus penaeid rod-shaped DNA (*penaeid rod-shaped DNA virus*);

RdRp – Enzima RNA-polimerase RNA-dependente (*RNA-dependent RNA polymerase*);

RNA - Ácido ribonucleico (*Ribonucleic Acid*);

RT-PCR - Transcriptase Reversa acoplada à reação em cadeia da polimerase (*Reverse Transcriptase - Polymerase Chain Reaction)*;

SF9 - linha de célula de lepidóptero *Spodoptera frugiperda* subclone SF9;

SPF - Indivíduos livres de patógenos específicos (*Specific Pathogen Free*);

TSV – Vírus da Taura (*Taura Syndrome Virus)*;

UTR - regiões não traduzidas (*Untranslated Region*);

WSSV – Vírus da Mancha Branca (*White Spot Syndrome Virus)*;

WTD - Doença da cauda branca (*White Tail Disease*);

XSV - Vírus *extra small* (*Extra Small Virus*);

YHV – Vírus da Cabeça Amarela (*Yellow Head Virus)*;

Sumário

Página

1- Introdução

Atualmente, a produção aquícola mundial de crustáceos é composta por cerca de 70% de espécies marinhas, sendo o camarão peneídeo *Litopenaeus vannamei* a espécie predominante, representando 71,8% da produção total de camarões marinhos cultivados em 2010, dos quais 22,1% foram produzidos nas Américas (FAO, 2012). No Brasil, a carcinicultura marinha da espécie *L. vannamei* foi responsável por 78% da produção aquícola marinha nacional, com uma produção de 65.670,6t em 2011 (BRASIL, 2013).

Apesar deste volume de produção, surtos de doenças de etiologia viral têm sido apontados como um dos principais entraves à sustentabilidade e a rentabilidade da indústria do camarão marinho cultivado (LOY et al., 2012). Aproximadamente 60% das perdas por doenças na carcinicultura marinha mundial podem ser atribuídas a enfermidades virais (FLEGEL, 2012), estimando-se no período entre 1994 e 2009, prejuízos anuais em nível mundial superiores a US$ 3 bilhões de dólares (WALKER e MOHAN, 2009).

No Brasil, o vírus da Mionecrose infecciosa (*Infectious myonecrosis virus* – IMNV) resultou em mortalidades acumulativas de até 70% em *L. vannamei* cultivado (NUNES et al., 2004) e perda econômica estimada em US$ 100 milhões de dólares para o período compreendido entre 2002 e 2006 (LIGHTNER, 2011). Embora o impacto econômico do IMNV tenha sido significativo para o Brasil, existem poucas informações disponíveis a respeito das consequências desta infecção viral sobre a imunologia e fisiologia, em especial aos aspectos reprodutivos e de desenvolvimento pré e pós-embrionário de animais infectados.

A resposta fisiológica a doenças em camarões ainda é pouco conhecida (BENZIE, 1998), e apesar dos crustáceos apresentarem uma resposta imune mais eficiente para doenças a que já tenham sido expostos, eles não possuem uma resposta imune celular de "memória" como os vertebrados (imunidade adaptativa) (WU et al., 2002; LUO et al., 2003).

Embora a resposta imune antimicrobiana (antibacteriana e antifúngica) tenha sido caracterizada em hemócitos de *L. vannamei* (DESTOUMIEUX et al., 1997), os mecanismos sobre a resposta imune viral ainda são pouco conhecidos (ROJTINNAKORN et al., 2002). Estes fatos evidenciam a necessidade de se desenvolver ferramentas rápidas para diagnosticar e controlar patógenos, bem como programas nacionais de biosseguridade. Contudo, em longo prazo, a única solução para se garantir altos níveis de produtividade frente aos surtos viróticos é o desenvolvimento de linhagens resistentes a patógenos.

Neste sentido, laboratórios comerciais de camarão têm adotado a estratégia de construção de populações resistentes a doenças, tendo como base que altas taxas de sobrevivência podem estar associadas a menores cargas virais em famílias selecionadas (MOSS et al., 2005).

Em um programa de seleção, a escolha de animais resistentes requer o uso de ensaios de desafio experimental no qual famílias de irmãos-completos são expostas ao agente patogênico em questão sob condições controladas (FJALESTAD et al., 1993). Assim, técnicas de inoculação são primordiais (GITTERLE et al., 2006) e bioensaios *in vivo* continuam a ser uma importante ferramenta de avaliação de infecciosidade e identificação de animais susceptíveis, infectados e resistentes.

O cultivo de linhagens de célula é uma ferramenta primordial nos bioensaios envolvendo vírus, por estes apresentarem replicação intracelular. Contudo, até o momento, não há relatos de um cultivo contínuo de linhas de célula em crustáceos. Recentemente, vários trabalhos obtiveram sucesso na multiplicação do vírus da Cabeça amarela (*Yellow Head Virus* - YHV), da Taura (*Taura Syndrome Virus* - TSV) e da Parvovirose hepatopancreática (*Hepatopancreatic parvo-like virus* - HPV) em linhas de célula de mosquito *Aedes albopictus* subclone C6/36 e de lepidóptero *Spodoptera frugiperda* subclone SF9, seguidos de estudos de infecciosidade em *Penaeus monodon* e *L. vannamei*, abrindo novas possibilidades para isolamento de outros tipos de vírus de camarão (GANGNONNGIW et al., 2010; ARUNRUT et al., 2011; MADAN et al., 2013).

Os bioensaios também são importantes instrumentos subsidiários à elucidação do mecanismo de transmissão do agente patogênico. A transmissão vertical pode ser um fator crucial na prevalência de doenças virais, além de contribuir para o subsequente aumento da probabilidade de infecção decorrente de transmissão horizontal em sistemas de cultivo (MOTTE et al., 2003). Para o IMNV, a determinação dos mecanismos de transmissão viral foi demonstrada para a transmissão horizontal, sendo provável a veiculação por meio da água e a transmissão vertical, embora neste último caso não tenha sido estabelecido (OIE, 2009).

A criação de um protocolo de infecção experimental constituirá uma etapa imprescindível não só para a compreensão de diversos aspectos que envolvem a Mionecrose infecciosa, tais como avaliação de virulência, estudos de patogenicidade, determinação da rota de infecção e dose de inóculo, mecanismos de transmissão, identificação de vetores, avaliação de medidas profiláticas e estudos de mecanismo de

resposta imune a infecções, mas também fornecerá bases sólidas para o melhoramento genético em camarões cultivados decorrente da padronização de métodos de desafios a doenças a serem empregadas nos laboratórios.

2- Revisão de literatura

2.1- Carcinicultura marinha mundial e brasileira

Embora o cultivo de crustáceos represente 46,4% da produção mundial, a cultura de peneídeos foi responsável por 73,3% da produção total em 2008, decorrente principalmente, da inserção da China, da Tailândia, da Indonésia e do Vietnã na produção do camarão marinho *L. vannamei*, resultando em uma taxa média de crescimento anual de 15% entre 2000 e 2008 (FAO, 2010).

Similarmente a outros setores da agricultura, a introdução de espécies exóticas tem desempenhado um papel importante na produção da aquicultura mundial, em particular na Ásia. Com a entrada da espécie exótica *L. vannamei* no sudeste asiático houve uma drástica mudança na carcinicultura naquela região, com a quase completa substituição da produção da espécie nativa *P. monodon* na última década (FAO, 2010). Assim, o cultivo de *L. vannamei* atingiu, em 2008, um total de 1,8 milhões de toneladas fora da América, representado 80,7% da produção mundial desta espécie e 40,7% da produção de todos os crustáceos cultivados fora da América (FAO, 2010).

No Brasil, embora iniciativas de cultivo de camarões marinhos datem da década de 1970 com a produção de espécies nativas (*Litopenaeus schmitti*, *Farfantepenaeus brasiliensis* e *Farfantepenaeus paulensis*) e exóticas (*L. vannamei* vinda da Flórida - EUA) através da parceria de empresas privadas e órgãos governamentais no Rio de

Janeiro, Pernambuco, Rio Grande do Norte e Santa Catarina, apenas entre os anos de 1993 e 1998, houve a consolidação da atividade, com o monocultivo de *L. vannamei* (NUNES et al., 2011). Tal consolidação deu-se tanto pelo aprimoramento de técnicas de cultivo semi-intensivo, emprego de rações balanceadas nacionais e controle de manejo alimentar (NUNES et al., 2011), como pela distribuição comercial de pós-larvas e a intensificação das validações tecnológicas nas fazendas de camarão (ROCHA, 2001).

Em 1999, uma combinação de surtos epidêmicos do vírus da Mancha branca (White Spot Syndrome Virus - WSSV) em países da América Central (México e Equador), que resultaram em queda da oferta do camarão no mercado internacional, atrelada ao consequente aumento de preços neste produto fez com que o Brasil atingisse ao final de 2003, uma ampliação de área de viveiros da ordem de 243%, passando de 4.320 ha para 14.824 ha e um aumento na produção anual de 7.250t para 90.190t, alcançando um recorde histórico de US$ 220 milhões de dólares em exportações (NUNES et al., 2011).

Entretanto, em 2004, a carcinicultura viveu um ano atípico, quando a produção registrou uma queda de 15,84%, ocasionada por uma série de fatores, como sanções comerciais movidas por pescadores norte-americanos contra o camarão brasileiro, os preços baixos do camarão no mercado internacional, as enchentes de 2004 e 2008, a desvalorização do dólar e o surto do vírus da Mionecrose infecciosa (MADRID, 2005). Além disso, a confirmação da presença de WSSV em Santa Catarina em 2005 paralisou a atividade produtiva no sul do país (NUNES et al., 2011).

Em 2006, devido à queda nas exportações, o modelo produtivo brasileiro voltou-se ao mercado interno com a redução do tempo de engorda para menos de 90 dias e oferta de camarão de 8g (NUNES et al., 2011). Entre 2008 e 2009, a produção caiu de

70.251,2t para 65.189,0t quando as chuvas causaram enchentes nos estados do Rio Grande do Norte e Ceará, que são os maiores produtores nacionais (BRASIL, 2010).

Atualmente, a carcinicultura nacional encontra-se com a produção estabilizada em 65 mil toneladas, respondendo por 78% da produção aquícola marinha nacional em 2011 (BRASIL, 2013).

2.2 - O vírus da Mionecrose Infecciosa (IMNV)

Primeiramente relatado em 2002, o vírus da Mionecrose infecciosa (*Infectious myonecrosis virus* – IMNV) surgiu em uma fazenda de engorda de camarão marinho *L. vannamei* localizada no município de Parnaíba, estado do Piauí. Os camarões moribundos exibiam uma perda da transparência do músculo abdominal devido a extensas áreas necrosadas esbranquiçadas do músculo esquelético (Figura 1) e uma incidência persistente na mortalidade diária a partir de 7 g (NUNES et al., 2004). Após difundir-se por todo o Nordeste, o IMNV foi responsável por uma perda econômica estimada em US$ 20 milhões de dólares em 2003 (NUNES et al., 2004), embora estimativas mais recentes apontem para prejuízos superiores a US$ 100 milhões de dólares para o período compreendido entre 2002 e 2006 (LIGHTNER, 2011).

Figura 1. Lesões esbranquiçadas no último segmento abdominal (seta) provocada pela infecção por IMNV, obtida a partir da inoculação experimental de vírions purificados em indivíduos livres de patógenos específicos (*Specific Pathogen Free* -SPF) de *L.*

vannamei, e aparência normal, de músculo abdominal translúcido (camarão inferior, controle negativo). (Fonte: POULOS et al., 2006).

Apesar do impacto desta doença na produção, apenas em 2004 foi possível confirmar que o agente etiológico da Mionecrose infecciosa era de origem viral (NUNES et al, 2011) a partir de desafios virais em *L. vannamei* SPF de vírions purificados isolados de animais naturalmente infectados coletados no Brasil durante o surto (POULOS et al., 2006).

O IMNV é um vírus não envelopado e com simetria icosaédrica de 40 ηm de diâmetro, contendo um genoma monopartido de molécula de RNA de dupla fita (dsRNA) com 7560 pb, formados por duas matrizes de região de leitura (ORF1 e ORF2) flanqueado pelas regiões não traduzidas 5'UTR (*Untranslated region*) na extremidade 5' e 3'UTR, na 3', e uma região intergênica de 288 nucleotídeos que separa as ORFs (Figura 2) (POULOS et al., 2006).

A ORF1 (nucleotídeos 136-4953) codifica um polipeptídeo de 1606 aa, com peso molecular de 179 KDa, que inclui a sequência terminal N da principal proteína do capsídeo (*Coat protein* - CP) iniciada no nucleotídeo 2248 e o domínio do motivo da *dsRNA-binding* (*dsRNA-binding motif* - DSRM) na porção extrema da 5’ desta região. A ORF2 (nucleotídeos 5241-7451) codifica uma proteína de 736 aa (85 kDa) que contém as características da RNA-polimerase RNA-dependente (RdRp). As estruturas secundárias (5'UTR e 3'UTR - Regiões não traduzidas) estão relacionadas à separação do dsRNA durante a replicação do vírus (Figura 2) (POULOS et al., 2006). Análises filogenéticas baseadas na região RdRp do IMNV classificou este vírus como membro da família Totiviridae, e o único a infectar um hospedeiro diferente de fungos ou

protozoários (POULOS et al., 2006; NIBERT, 2007), embora Haugland et al. (2011) relatem a identificação de um novo vírus desta família, o vírus Piscine myocarditis (*Piscine myocarditis virus* - PMCV), capaz de infectar vertebrados, como peixes adultos do salmão do Atlântico (*Salmo salar* L.).

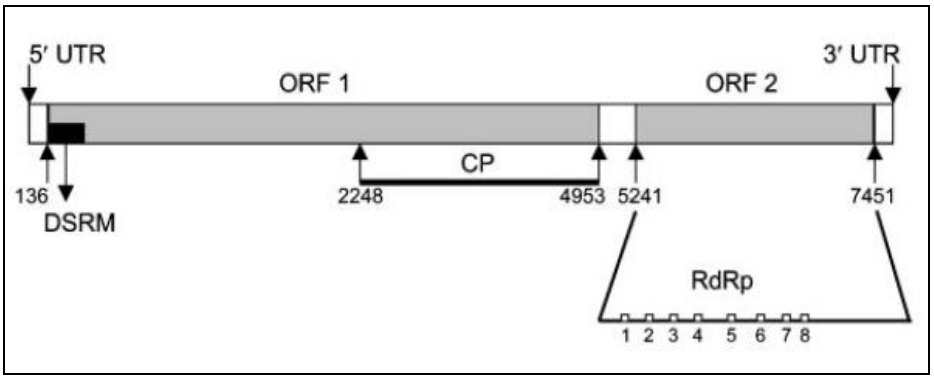

Figura 2. Organização e regiões conservadas do genoma IMNV. Organização do genoma mostrando: as UTRs, o DSRM, as ORFs, a proteína do capsídeo (CP) e a RdRp. Números abaixo das setas indicam as posições dos nucleótideos no genoma. (Fonte: POULOS et al., 2006).

Quanto às estratégias de tradução, segundo Ghabrial e Nibert (2009), membros da família Totiviridae podem utilizar três modelos: (1) a RdRp e a CP são traduzidas como uma proteína de fusão a partir de uma única e contínua ORF, seguida por clivagem proteolítica; (2) a RdRp e a CP são traduzidas como uma fusão, a partir de duas sobreposições, mas fora da ORFs, usando um "ribosomal frame-shift", seguida de clivagem proteolítica e; (3) a RdRp e a CP são traduzidas como proteínas separadas por uma terminação de tradução, seguida de reiniciação interna proveniente de duas ORFs distintas e não sobrepostas. No caso do IMNV, embora a estratégia de tradução adotada seja o terceiro modelo descrito (POULOS et al, 2006), segundo Nibert et al., (2007),

após uma reavaliação da estratégia de tradução deste vírus, há fortes indícios do uso de uma "frame-shift" durante a tradução para gerar uma fusão entre a RdRp e a CP.

Quanto à distribuição geográfica do IMNV, além do nordeste brasileiro, surtos de IMNV foram relatados em fazendas na Indonésia (Ásia) em 2006 (SENAPIN et al., 2007), gerando perdas econômicas superiores a US$ 1 bilhão de dólares em 2010 (LIGHTNER et al., 2012). No Brasil, os surtos de IMNV parecem estar associados a estresses ambientais, tais como variações extremas de salinidade e temperatura, e possivelmente com o uso de alimentos de baixa qualidade (LIGHTNER, 2011). Silva et al. (2010), em estudo realizado no nordeste brasileiro, também apontam longos ciclos e altas densidades de estocagem como fatores capazes de aumentar a ocorrência do IMNV na produção. Em um levantamento epidemiológico realizado por Pinheiro et al. (2007) no estado de Pernambuco em 2004, das 11 fazendas investigadas, nove apresentaram indivíduos infectados.

Com relação à suscetibilidade de camarões peneídeos ao IMNV, infecções experimentais conduzidas por meio de injeção intramuscular de inóculo purificado mostraram que *L. vannamei, Litopenaeus stylirostris, Farfantepenaeus subtilis* e *P. monodon* são susceptíveis ao IMNV, sendo *L. vannamei* a mais susceptível de todas as espécies com base no aparecimento dos sinais clínicos da infecção e mortalidade (TANG et al., 2005; COELHO et al., 2009). Além disso, co-infecções naturais de IMNV com os vírus da Necrose hipodermal e hematopoiética infecciosa (*Infectious hypodermal and haematopoietic necrosis virus* - IHHNV) (COELHO et al., 2009; TEIXEIRA-LOPES et al., 2011) e da Mancha branca (FEIJÓ et al., 2013) em *L. vannamei* têm sido relatadas no nordeste brasileiro.

Segundo a Organização Mundial da Saúde Animal (OIE) (2012) são considerados métodos de detecção *in vitro* para diagnóstico confirmatório a hibridização *in situ* e a reação em cadeia da polimerase (*Polymerase Chain Reaction* - PCR) e suas variações: Transcriptase Reversa acoplada à reação em cadeia da polimerase (*Reverse Transcriptase - Polymerase Chain Reaction* - RT-PCR) e PCR em tempo real (*Real Time Reverse Transcriptase-Polymerase Chain Reaction*). Para o IMNV, estão disponíveis as técnicas de hibridização *in situ* (TANG et al., 2005), RT-PCR (POULOS e LIGHTNER, 2006) e PCR em tempo real (ANDRADE et al., 2007; SILVA et al., 2011; LIU et al., 2013), sendo esta última considerada atualmente como o método mais sensível e reproduzível para detecção e quantificação de vírus de camarão (LIU et al., 2013).

2.4 - Transmissão vertical de vírus em crustáceos.

Vírus são os agentes infecciosos mais comumente "bem-sucedidos" na transmissão vertical por terem a capacidade de persistir dentro das células, muitas vezes ao longo do ciclo de vida do hospedeiro, sem produzir graves perturbações ou interferir na viabilidade, tampouco induzir respostas imunes capazes de eliminá-los (MIMS, 1981).

Entende-se por transmissão vertical, a passagem do vírus de uma geração para outra, seja por intermédio dos gametas ou do embrião. Quando o vírus é transmitido dentro de um óvulo maduro recebe a denominação específica de transmissão vertical transovariana (MARTIN et al., 1987; BROCK e BULLIS, 2001).

Em crustáceos, a transmissão vertical tem sido um dos principais fatores para o aumento da prevalência de muitos vírus em populações selvagens ou domesticadas de

diferentes espécies de peneídeos, tais como: *Penaeus japonicus*, *P. stylirostris*, *L. vannamei* e *P. monodon*) (LO et al., 1997; MUSHIAKE et al., 1998; HSU et al., 1999; MORALES-COVARRUBIAS et al., 1999; TSAI et al., 1999; PENG et al., 2001; COWLEY et al., 2002; MOTTE et al., 2003; WITHYACHUMNARNKUL et al., 2006; LA PEÑA et al., 2007), e carídeos (*Macrobrachium rosenbergii*); (SUDHAKARAN et al., 2007a).

Além do aumento da prevalência, outra implicação da transmissão vertical de vírus é o seu desdobramento em relação à teoria de acomodação viral. Nesta teoria, a integração aleatória de fragmentos do genoma viral no genoma do hospedeiro por meio de transcriptase reversa e integrase do próprio hospedeiro conduziria a transcrição de mRNA *antisense* capaz de suprimir a propagação do vírus decorrente da degradação específica das sequências dos transcritos virais por meio do RNA de interferência (RNAi). Tais inserções acarretariam em infecções ativas, mas de baixo nível em que o hospedeiro não exibiria sinais clínicos da doença, mas permaneceria infectado e infeccioso a outros hospedeiros susceptíveis. Além disso, os indivíduos com estas inserções as transmitiriam a seus descendentes e, durante o processo reprodutivo, a distribuição aleatória dos cromossomos permitiria a rápida mistura destas inserções de proteção, o que resultaria no desenvolvimento de uma prole tolerante ao vírus (FLEGEL, 2009). Entretanto, apesar da identificação de cinco tipos de transcriptase reversa e sete tipos de integrase (TASSANAKAJON et al., 2006), até o momento, nenhum estudo comprovou esta hipótese e a inserção de sequências de DNA derivadas de vírus em células germinativas do hospedeiro.

Para o vírus *penaeid rod-shaped DNA* (PRDV), um estudo sobre sua prevalência em populações selvagens de adultos de *Penaeus japonicus,* em cinco pontos da costa

japonesa, mostrou que 10,1% das fêmeas e 6,7% dos machos capturados eram positivos para o PRDV, com maiores taxas de detecção nas amostras provenientes de estômago, ovário e testículo quando comparadas à hemolinfa. Ainda neste mesmo estudo, uma variação sazonal e sexual indicou um maior número de fêmeas positivas no verão, sugerindo que sua captura torna-se uma fonte de transmissão do PRDV quando estas são usadas como reprodutores (MUSHIAKE et al., 1998).

Embora o IHHNV seja infeccioso para todos os peneídeos, a prevalência deste vírus em reprodutores e a transmissão vertical dependem da espécie infectada. Infecções por IHHNV geram menos impacto na performance reprodutiva de *P. stylirostris* e *P. monodon* do que em *L. vannamei* (MORALES-COVARRUBIAS et al., 1999; MOTTE et al., 2003; WITHYACHUMNARNKUL et al., 2006). Um estudo de Morales-Covarrubias et al. (1999), conduzido em adultos de *P. stylirostris* capturados no Norte do Golfo da Califórnia (México), mostrou que a prevalência de IHHNV não afetava a reprodução, uma vez que esta era de 89% nas fêmeas, de 57% nos machos, de 100% em ovos não fertilizados e de 60% em esperma, com ausência de sinais clínicos da doença em todos os animais capturados.

Resultados similares foram obtidos por Withyachumnarnkul et al. (2006), sobre a influência da transmissão vertical de IHHNV na produção de ovos de *P. monodon* positivos provenientes da Tailândia. Os autores não detectaram redução na produção de ovos e náuplios ou quaisquer efeitos negativos sobre o crescimento da prole quando comparados a animais negativos, sugerindo que este vírus pode ser transmitido verticalmente sem comprometimento da fecundidade ou fertilidade.

A transmissão vertical de IHHNV também foi analisada em *L. vannamei* no Equador em que 85% dos ovários foram positivos, contrastando com apenas 13% do

esperma de machos, sugerindo que a transmissão vertical via materna é a mais importante (MOTTE et al., 2003). Esses autores ainda concluíram que fêmeas negativas mostraram-se 25% mais produtivas do que as positivas, com redução evidente de crescimento heterogêneo em náuplios provenientes de fêmeas livres de IHHNV.

Com relação ao WSSV, reprodutores selvagens de *P. monodon* capturados no sul de Taiwan apresentaram uma alta prevalência com 67,5% de machos e 75% de fêmeas positivos em diagnóstico de nested-PCR. As fêmeas com infecções elevadas (detectadas na 1ª PCR) foram incapazes de desovar, enquanto que aquelas com infecções leves (detectadas na 2ª PCR) desovaram, mas morreram após a desova. Além disso, quando estas fêmeas foram triadas novamente depois de mortas, tornaram-se positivas na 1ª PCR, indicando que o estresse de desova pode induzir à replicação viral (LO et al., 1997). Quanto à detecção de WSSV nos tecidos, esses autores observaram que a infecção por WSSV foi confirmada via ISH nos ovários (células do folículo e oogônias) e espermatóforos, com ausência de óvulos maduros infectados, sugerindo que a transmissão transovariana seja improvável, mas que possa ocorrer com a liberação do vírus no momento da desova.

Em outro estudo com reprodutores selvagens de *P. monodon* capturados, também no sul de Taiwan, ao se analisar a prevalência de WSSV em 45 fêmeas infectadas antes e após a desova, houve um aumento de 67 para 75% de fêmeas infectadas, com evidente acréscimo na severidade de infecção, sugerindo que uma estratégia eficaz seja selecionar reprodutores livres de WSSV após a primeira desova (HSU et al., 1999).

Nas Filipinas, apesar da ausência de diferença entre a prevalência de WSSV entre fêmeas e machos selvagens de *P. monodon*, houve uma variação sazonal significativa, com maior prevalência durante a estação seca ou verão, período no qual há atividade de

desova natural (LA PEÑA et al., 2007). Tal resultado corrobora com os descritos por Lo et al. (1997) e Hsu et al. (1999) no sul de Taiwan, em que maior prevalência de infecção foi obtida após a desova. Além disso, Kou et al. (2001) também relatam que reprodutores infectados por WSSV também podem morrer imediatamente após a desova.

Tsai et al. (1999) e Peng et al. (2001) sugerem que a adoção da estratégia de povoamento com náuplios oriundos de fêmeas com prevalência por WSSV inferior a 50% pós-desova atrelado a condições favoráveis de cultivo (baixa densidade e baixo estresse ambiental), pode resultar em ciclos produtivos com baixa incidência de surtos de doenças na engorda de *P. monodon*.

Para o vírus *gill-associated* (GAV), a transmissão vertical foi demonstrada em reprodutores de *P. monodon* selvagens coletados na costa nordeste de Queensland, Austrália (COWLEY et al., 2002). Neste estudo, tanto espermatóforos e tecido ovariano maduro, como ovos fertilizados e náuplios de fêmeas selvagens fertilizadas naturalmente foram positivos para GAV via nested-PCR, indicando que a propagação do vírus pode ser biparental. No caso dos espermatóforos, níveis de detecção mais elevados (intensidade de banda e amplicons oriundos da 1ª PCR) foram observados em *P. monodon* mantidos em cativeiro por mais de 12 meses, indicando que o estresse do cativeiro em longo prazo pode aumentar a severidade de infecção por GAV (COWLEY et al., 2002).

No caso de camarões carídeos, como o *M. rosenbergii*, a possibilidade de transmissão vertical dos vírus *Macrobrachium rosenbergii nodavirus* (MrNV) e *extra small* (XSV) foi confirmada via desafio oral ou de imersão de reprodutores previamente triados. Nestes experimentos, os reprodutores inoculados sobreviveram ao desafio sem

sinais clínicos de infecção, desovando cinco dias após a inoculação com eclosão de zoeas. Entretanto, após a eclosão, a taxa de sobrevivência das larvas diminuiu gradualmente, atingindo 100% de mortalidade na fase de pós-larvas. A confirmação de infecção por estes vírus foi obtida através de amostras de tecido do ovário, ovos fertilizados e estágios larvais por meio de PCR e nested-PCR (SUDHAKARAN et al., 2007a).

Em populações de *L. vannamei*, a presença de sobreviventes capazes de serem portadores de infecção por IMNV ao longo da vida até se tornarem reprodutores não foi demonstrada cientificamente, mas acredita-se que o vírus possa ser transmitido verticalmente à progênie. Do mesmo modo, informações sobre vetores de infecção ainda não se encontram disponíveis (OIE, 2012).

2.5 - Vetores virais em crustáceos

Do ponto de vista epidemiológico define-se como vetor um animal, geralmente invertebrado, que transmite ativamente um agente infeccioso do indivíduo infectado ao susceptível, podendo fazê-lo de duas maneiras: transmissão mecânica ou biológica (FAO, 1987).

Na transmissão mecânica, os vetores servem como um veículo (um carreador) em que o agente infeccioso é transmitido a partir de um hospedeiro para outro sem passar por uma fase de desenvolvimento ou multiplicação. Neste caso, o agente é transportado na pele ou trato digestório (como a boca) do vetor a partir de um hospedeiro infectado para o susceptível. Assim, o tempo de sobrevivência do agente no vetor é geralmente curto, e como resultado, a transmissão do agente tem que ser

alcançada rapidamente. Já na transmissão biológica, o agente infeccioso se desenvolve dentro do vetor, tornando-se infeccioso ou multiplicando-se e servindo como hospedeiro intermediário ou definitivo, dependendo de quais fases do ciclo de desenvolvimento do agente esteja dentro do vetor. Além disso, os vetores podem ser capazes de passar o agente para sua própria prole via transovariana, o que mantém a permanência do agente infeccioso na população de vetores, através de muitas gerações, sem que a população seja re-infectada, sendo, portanto, uma fonte permanente de risco (FAO, 1987).

Na aquicultura, numerosos organismos aquáticos, incluindo rotíferos, artrópodes (copépodes e microcrustáceo - Artemia), moluscos (bivalves) e anelídeos (poliquetas) têm sido apontados como vetores ou fonte de contaminação viral de WSSV, MrNV, XSV e HPV em crustáceos (LI et al., 2003; VIJAYAN et al., 2005; SUDHAKARAN et al., 2006; ZHANG et al., 2006, 2007, 2008, 2010; SIVAKUMAR et al., 2009; CHANG et al., 2011; CORRE et al., 2012; JIANG, 2012; FENG et al., 2013).

A transmissão e a infectividade de WSSV a partir de rotíferos infectados das espécies *Brachionus urceus* e *B. plicatilis* em *Fenneropenaeus chinensis* e *P. monodon* foram documentados por Zhang et al. (2006) e Corre et al. (2012), respectivamente. No primeiro trabalho, Zhang et al. (2006) demonstraram que rotíferos experimentalmente infectados via rota de adesão vírus-fitoplâncton foram positivos para WSSV via PCR, mas não foram patogênicos para *F. chinensis* devido à baixa susceptibilidade a WSSV antes da fase pós-larval (PL). Já para pós-larvas de *P. monodon*, *B. plicatilis* infectados via imersão foram altamente patogênicos, com 82% de mortalidade nos camarões co-cultivados com rotíferos positivos (CORRE et al., 2012). Ainda neste estudo, ovos em diapausa também foram positivos para WSSV, confirmando as evidências anteriores deste rotífero como hospedeiro e vetor na transmissão de WSSV. Similarmente, Yan et

al. (2004) também detectaram a presença de WSSV em ovos em dipausa de *B. urceus*, submetidos à desinfecção da superfície, coletados em sedimentos de viveiros de *F. chinensis*, indicando que este organismo serve como um reservatório de WSSV em viveiros, o que segundo Corre et al. (2012), explicaria a recorrência de surtos virais em viveiros de camarão que tiveram uma preparação preventiva com secagem, calagem e dessecação do solo.

Além dos rotíferos, artrópodes da subclasse Copepoda, como os copépodes *Acartia clausi*, *Nitocra* sp. e *Apocyclops royi* foram confirmados como vetores de infecção por WSSV via PCR. Copépodes do gênero *Nitocra* sp. foram expostos ao WSSV através da rota de adesão vírus-fitoplâncton, sendo utilizados como material infeccioso para a infecção de pós-larvas de *P. japonicus* através de desafios oral e de injeção intramuscular, para os quais obtiveram 52,5 e 72,5% de mortalidade , respectivamente (ZHANG et al., 2008). Similarmente, o copépode *A. clausi* foi submetido a desafio de imersão e a rota de adesão vírus-fitoplâncton para WSSV, sendo positivos apenas os animais desafiados via vírus-fitoplâncton por nested-PCR, indicando que estes animais, ao ingerirem fitoplâncton carreador de WSSV, acumulam partículas virais em seu intestino e eliminam o agente infeccioso no ambiente (ZHANG et al., 2007). Além disso, o receptor para WSSV foi encontrado na membrana celular do copépode *A. clausi*, evidenciando o papel deste invertebrado como hospedeiro (FENG et al., 2005).

Chang et al. (2011), ao investigar a função do copépode *Apocyclops royi* e do molusco bivalve *Meretrix lusoria* na transmissão de WSSV, mostraram que o genoma de WSSV pode ser detectado via nested-PCR e que as cargas virais, detectadas por PCR em tempo real, aumentaram de acordo com o tempo de curso da infecção após desafios

via vírus-fitoplâncton em *A. royi* e *M. lusoria*. Entretanto, a análise temporal de transcrição da expressão de genes de WSSV via RT-PCR revelou que o vírus conseguia se replicar em *A. royi*, mas não em *M. lusoria*, sugerindo que este último é apenas um vetor mecânico devido à bioacumulação de partículas virais. Um desafio viral via ingestão revelou ainda que WSSV acumulado em *M. lusoria* infectado poderia ser transmitido ao *L. vannamei* e provocar infecção grave (CHANG et al., 2011).

Outro artrópode, o microcrustáceo *Artemia* sp., também tem sido apontado como vetor de WSSV. Em duas investigações, diferentes estágios de *Artemia* sp. (metanáuplios e adultos) foram inoculados através da rota de adesão vírus-fitoplâncton com resultados positivos via PCR, mas sem sinais clínicos de infecção (LI et al., 2003; ZHANG et al., 2010). Li et al. (2003) também mostraram que os resultados obtidos a partir de estudos de infectividade e transmissão vertical sugerem que a *Artemia* sp. pode transmitir o vírus das fases larvais até adultos e de adultos a cistos reprodutivos. No entanto, a constatação de que náuplios eclodidos destes cistos foram negativos, indica que o WSSV é removido durante a incubação e lavagem dos náuplios. Similarmente, Chang et al. (2002) não detectaram WSSV em náuplios eclodidos e lavados derivados de cistos positivos, nem em pós-larvas de *P. monodon* alimentadas por estes náuplios, sugerindo uma contaminação externa dos cistos. Entretanto, a presença de receptores celulares de WSSV na membrana de células de *Artemia* sp., sugere que este microcrustáceo seja um reservatório de WSSV (FENG et al., 2013).

Além do WSSV, os vírus MrNV, XSV e HPV também foram capazes de infectar o microcrustáceo artemia. A patogenicidade de MrNV e XSV para todos os estágios de desenvolvimento da *Artemia* sp. (náuplio, metanáuplio, juvenil, pré-adulto e adulto) foi avaliada por meio de desafios de imersão e oral, com resultados positivos para todas as

fases via RT-PCR, embora nenhum sinal clínico de doença tenha sido observado. Por outro lado, ao se utilizar *Artemia* positiva para a transmissão destes vírus em pós-larvas de *M. rosenbergii*, todos se tornaram positivos com sinais de infecção com 100% de mortalidade ao 9° dia pós-inoculação, indicando que este microcrustáceo funciona como reservatório ou vetor mecânico (SUDHAKARAN et al., 2006). Do mesmo modo, em similares desafios experimentais, diferentes fases da *Artemia franciscana* foram positivas ao HPV por PCR, com transmissão horizontal comprovada quando estes animais infectados foram usados em desafios de ingestão de pós-larvas de *P. monodon*. No entanto, não ficou claro se a *Artemia* é realmente infectada com HPV ou simplesmente age como portadora passiva (SIVAKUMAR et al., 2009).

Embora maior importância tenha sido dada a organismos zooplanctônicos como vetores de WSSV, de acordo com Bondad-Reantaso et al. (2001), vírions de WSSV podem permanecer infecciosos nos tecidos em decomposição ou em detritos por até quatro dias e ser consumidos por invertebrados bentônicos, evidenciando a possibilidade de poliquetas (anelídeos) como vetor mecânico deste vírus.

Em estudo conduzido por Vijayan et al. (2005), poliquetas da espécie *Marphysa gravelyi* foram coletadas em oito estações selecionadas na costa nordeste da Índia e avaliadas para a presença de WSSV via nested-PCR. Destas áreas, as cinco que recebiam diretamente efluentes da carcinicultura apresentaram poliquetas positivas para WSSV com prevalência entre 16,7 e 75%, enquanto que as três restantes, em que não havia atividade de cultura, todas foram negativas. Além disso, ao desafiar poliquetas com a adição de inóculo na água, mais de 60% tornaram-se positivas após sete dias de experimento, sendo capazes de infectar reprodutores de *P. monodon* (até 83% de

infectados), comprovando, assim, seu papel como vetor na transmissão horizontal de WSSV (VIJAYAN et al., 2005).

2.6 - Aplicação de modelos *in vitro* para crustáceos

Modelos *in vitro* baseados na utilização de cultura celular são sistemas experimentais que permitem alto controle das condições de ensaio, reduzindo a variabilidade das respostas que possam ser observadas devido a condições de estresse, nas quais as interações entre patógeno e hospedeiro possam ser analisadas (VILLENA, 2003). Por este motivo, sua aplicação tem permitido a abordagem de alguns elementos cruciais para o manejo integrado de saúde na aquicultura, incluindo a validação e padronização de métodos de diagnóstico, o desenvolvimento de novos produtos terapêuticos e a implementação de metodologias de controle de doenças eficazes (VILLENA, 2003). Adicionalmente, estes modelos também têm sido utilizados como ferramentas alternativas para a experimentação animal (RINKEVICH, 1999) e para a produção de grande quantidade de material viral e sua caracterização (HAQ et al., 2013).

Culturas celulares podem ser distinguidas em três tipos: primária, secundária e de linhagem estabelecida. Os cultivos primários são obtidos diretamente do organismo (animal ou vegetal) em condições assépticas e os fragmentos de tecido submetidos à desagregação enzimática, mecânica ou química para a obtenção de uma suspensão de células livres. As culturas secundárias são resultantes do repique (passagem) de culturas primárias e as de linhagens estabelecidas, são cultivos cujo crescimento celular é prolongando decorrente de característica neoplásica da célula (DE ROBERTIS, 2006).

Em crustáceos, culturas primárias oriundas de diferentes tecidos (órgão linfóide, hepatopâncreas, ovário e hemócito) de peneídeos têm permitido a propagação dos vírus da Mancha branca (KASORNCHANDRA e BOONYARATPALIN, 1998; UMA et al., 2002; MAEDA et al., 2004; JIANG et al., 2006; JOSE et al., 2010; JOSE et al., 2012), da Taura (GEORGE et al., 2011) e da Cabeça amarela *in vitro* (ASSAVALAPSAKUL et al., 2003). No entanto, culturas primárias são de difícil estabelecimento, contêm uma mistura heterogênea de células com vida útil limitada e propensa à contaminação, além de baixa reprodutibilidade. Por outro lado, linhagens de células imortais podem ser continuamente cultivadas, eliminando a necessidade de fonte de células oriundas de animais vivos; apresentam culturas homogêneas com relação ao genótipo e fenótipo e estabilidade em nitrogênio líquido por vários anos e; culturas celulares em larga escala em curto período de tempo (CRANE, 1999; CLAYDON et al., 2010).

Atualmente, apesar dos avanços na decodificação das necessidades nutricionais de células *in vitro* e nas abordagens moleculares em nível genômico para transformação e imortalização de células, linhagens celulares provenientes de invertebrados permanecem sem ser desenvolvidas. Tal fato ocorre, principalmente, devido à falta de informações sobre os mecanismos moleculares que inibem transformações neoplásicas em camarão (JAYESH et al., 2012).

Visando solucionar este problema, inúmeros trabalhos têm empregado linhagens celulares de mosquito (*Aedes albopictus* subclone C6/36) e lepidópteros (*Spodoptera frugiperda* subclone SF9) para a propagação *in vitro* de vírus de camarão.

Sudhakaran et al. (2007b) relatam a replicação dos vírus *Macrobrachium rosenbergii nodavirus* (*Mr*NV) e *extra small virus* (XSV) em cinco passagens sucessivas de C6/36, confirmada através da análise de RT-PCR de amostras do

sobrenadante da cultura após três dias de inoculação e de microscopia (coloração de RNA com laranja de acridina), na qual foram observados o desenvolvimento de vírions após 48 horas e aumento da coloração do RNA no citoplasma. Adicionalmente, estudos de infectividade conduzidos por estes autores em pós-larvas de *Macrobrachium rosenbergii*, por meio de desafios de imersão de inóculo preparado a partir do sobrenadante da cultura de células infectadas, mostram a presença de sinais clínicos de infecção similares aos obtidos em surtos naturais e 100% de mortalidade em 6 dias pós-infecção, com confirmação positiva via RT-PCR dos animais desafiados (SUDHAKARAN et al., 2007b).

No caso de camarões peneídeos, Sriton et al. (2009) descrevem 100% de células C6/36 e SF9 positivas para WSSV e YHV detectadas via análise imunohistoquímica em mais de 100 passagens celulares. Entretanto, apesar da fluorescência positiva, nenhum efeito citopático (CPE) foi observado nas células infectadas em relação às negativas e nenhum vírion ou nucleocapsídeo de WSSV ou YHV fora observado no núcleo de células imunopositivas via microscopia eletrônica, indicando que a persistência de antígenos virais possa estar associada à transmissão ocorrida por divisão celular (subdivisão de genes entre células mãe e filhas) ou a algum processo endocítico inicial durante a inoculação viral (SRITON et al., 2009).

Resultados similares foram obtidos por Gangnonngiw et al. (2010), que, ao inocular YHV em C6/36, obtiveram 100% de células positivas detectadas via análises imunohistoquímica e RT-PCR oriundas de passagens celulares. Entretanto, quando amostras do sobrenadante celular destas culturas foram analisadas via RT-PCR, todas as passagens mostraram-se negativas, sugerindo a não liberação de partículas virais no meio de cultura. Tais resultados foram ratificados através dos estudos de infectividade

em *P. monodon* inoculados intramuscularmente com inóculo produzido a partir da quinta passagem celular (usando apenas a célula) e do sobrenadante da cultura. No primeiro (passagem celular), 57% de mortalidade foi alcançada ao 2° dia pós-inoculação nos camarões desafiados, com confirmação da infecção por histologia e imunohistoquímica, enquanto que para o segundo (sobrenadante), nenhum camarão injetado foi infectado (GANGNONNGIW et al., 2010).

Para o vírus da Taura, células C6/36 foram inoculadas e mostraram-se positivas por imunohistoquímica dentro de 11 passagens sucessivas, com constatação de partículas virais junto a estruturas vesiculares no citoplasma de C6/36 imunopositivas semelhantes àquelas encontradas em células de camarões infectados com TSV (ARUNRUT et al., 2011). Contudo, *L. vannamei* desafiados intramuscularmente com homogeneizado proveniente do sobrenadante da 15° passagem celular, foram negativos para TSV por imunohistoquímica e RT-PCR. Por outro lado, quando estes animais foram submetidos a injeções contendo um milhão de células positivas oriundas da 15° passagem celular, alta percentagem de hemócitos imunopositivos para TSV foi obtido ao 8° dia pós-inoculação (ARUNRUT et al., 2011).

Recentemente, a propagação de outro vírus de camarão, o da Parvovirose hepatopancreática (*Hepatopancreatic parvo-like virus* - HPV), foi demonstrada em um sistema *in vitro* usando C6/36 (MADAN et al., 2013). Os resultados revelaram que C6/36 foram susceptíveis ao HPV e as células infectadas mostraram CPE sob a forma de formação de vacúolo, redução do tamanho das células e degeneração da monocamada após cinco dias de infecção. A infecção por HPV em C6/36 foi confirmada via análises de RT-PCR, imunocitoquímica e western blot, com quantificação da carga viral através de PCR em tempo real em intervalos regulares de 4,

6, 8, 10 e 12 dias pós-inoculação, na qual ficou evidente um aumento da carga viral durante o curso da infecção, com maiores cargas encontradas ao 12° dia quando comparadas ao 4° dia. Em relação aos estudos de infecciosidade, pós-larvas de *P. monodon* foram desafiadas via imersão com inóculo proveniente da 20° passagem de cultura positiva, causando alta mortalidade após seis semanas de infecção (MADAN et al., 2013).

Os resultados destes estudos mostram que sistemas *in vitro* de cultura celular tornam-se uma opção atraente para o crescimento, o isolamento e a obtenção de vírus sem a dependência de fontes originárias de surtos naturais, além de permitir a avaliação de quais células são suscetíveis a determinado patógeno. Tais implicações tornam as culturas celulares uma ferramenta adicional à padronização de material infeccioso a ser usado na identificação de animais susceptíveis, infectados e resistentes em programas de seleção de linhagens resistentes a doenças.

2.7 - Melhoramento genético para resistência a doenças em camarões marinhos

Nos últimos anos, programas de seleção para resistência a doenças têm recebido cada vez mais importância na aquicultura mundial (ØDEGÅRD et al., 2011). Estes programas baseiam-se em testes de desafio viral a patógenos específicos (um patógeno a um intervalo de tempo, usando diferentes subamostras de todas as famílias do núcleo de seleção) em condições ambientais controladas (ØDEGÅRD et al., 2011).

Em camarões, programas de seleção para resistência a doenças têm sido desenvolvidos para doenças com elevadas taxas de mortalidade (entre 70 e 100%) e com crescimento reduzido nos animais sobreviventes, tais como o vírus da Taura e da Mancha branca (COCK et al., 2009). Para o vírus da Taura, desafios virais por ingestão

de tecido infectado em condições controladas foram realizados para a seleção combinada (massal de famílias resistentes), havendo um aumento de 18,4% de sobrevivência nos indivíduos selecionados quando comparado ao grupo controle (ARGUE et al., 2002). White et al. (2002) relatam um aumento na sobrevivência média de 24 para 37% após a exposição de famílias selecionadas de *L. vannamei* ao TSV ao longo de três anos, com incremento na sobrevivência de 65 para 100% entre as famílias de melhor performance durante o mesmo período. Após 15 gerações de seleção de *L. vannamei* para resistência a TSV, observa-se uma alta frequência de famílias por geração exibindo 100% de sobrevivência após a exposição ao TSV (MOSS et al., 2011).

Para a Mancha branca, apesar de terem sido usados desafios semelhantes com ingestão de tecido para a obtenção de famílias resistentes, por seleção combinada, em ambiente controlado e em condições comerciais, o baixo número de sobreviventes decorrente de mortalidades superiores a 98%, impossibilitou a formação de famílias iniciais (GITTERLE et al., 2005). No entanto, estudos recentes mostram a produção de famílias de *L. vannamei* com maiores taxas de sobrevivência (22,7-57%) após poucas gerações de seleção para o WSSV (HUANG et al., 2011; CUÉLLAR-ANJEL et al., 2012). Já para o vírus IHHN, a comprovação de uma linhagem resistente de *P. stylirostris* (Super Shrimp®) foi obtida através da oferta de tecido infectado picado como rota de infecção (TANG, et al., 2000), sugerindo que programas de melhoramento de resistência podem ser realizados de forma eficaz com *L. vannamei*.

No que diz respeito ao modo de exposição viral, Lotz (1997a, b) relatou que a mortalidade para TSV foi maior quando os camarões foram submetidos a desafios por injeção com o vírus em relação aos que foram apenas expostos ao patógeno na água (*waterborne assay*). Por outro lado, pesquisadores do U.S. Marine Shrimp Farming

Program (USMSFP) do Havaí observaram que não houve correlação fenotípica na sobrevivência familiar ao TSV, quando camarões foram expostos por injeção versus ingestão, enfatizando a importância de padronizar protocolos utilizados nos laboratórios de desafios a doenças (MOSS et al., 2005).

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4- Artigo científico

4. 1 - Artigo científico I

Experimental infection of Infectious myonecrosis virus (IMNV) in the Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931)

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Experimental infection of Infectious myonecrosis virus (IMNV) in the Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931)

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Running title: IMNV infection protocol for marine shrimp.

Keywords: virus infection; IMNV resistant shrimp; survival; breeding program.

ABSTRACT

Bioassays are essential in detecting resistant animals based on standardized challenge tests. We developed an experimental infection of Infectious myonecrosis virus (IMNV) through two methods of infection, by ingestion and injection, in *Litopenaeus vannamei*. For ingestion bioassays, shrimp (2-3.9 g body weight) were exposed to four feeding infection treatments associated to stress. No signs of disease were observed and 30% of the animals were infected. In the injection treatment, dilutions from $1:10^1$ (mean viral load of $9.82x10^5$ IMNV copiesμg^{-1} of total RNA) to $1:10^9$ were tried at 28°C and from $1:10^5$ to $1:10^9$, at 26°C. All challenged animals exhibited IMNV signs of infection for dilutions from $1:10^1$ to $1:10^7$ for both temperatures. Between $1:10^5$ and $1:10^7$ dilutions, the number of infected animals was superior to 60%, while at 26°C; these values were between 30 and 50%, with a clear decrease in the proportion of infected shrimp as a consequence of declining water temperature. Median infectious doses (ID_{50}) was determined between $2.44:10^8$ and $1.19:10^7$ at 28°C and between $1.76:10^6$ and $1.28:10^5$ at 26°C for 15 days post-infection, respectively. As the highest infection rates were detected in the dilution of $1:10^7$ at 28°C and $1:10^6$ at 26°C, these were chosen as the most appropriate infective methods.

INTRODUCTION

Penaeid species include the most economically important cultured marine shrimp species worldwide (Moss, Moss, Arce, Lightner & Lotz 2012). In the Americas, the shrimp farming industry accounted for nearly 20% of total global production, and the Pacific white shrimp, *Litopenaeus vannamei*, for more than 95% of the Western Hemisphere's production (Lightner 2011), with a total production of 2,720,929 tons in 2010 (FAO 2012).

Despite high levels of production, viral diseases have been a serious limiting factor in penaeid shrimp industry (Lightner 2011). The increasing rate of emerging viral diseases in aquatic species has been associated with the transition from wild environment to aquaculture systems due to degradation of environmental quality of culture system, high stocking densities and manipulations (Pinheiro, Lima, Souza, Neto, Adrião, Gonçalves & Coimbra 2007; Walker & Winton 2010).

In Brazil, the economic impact of an outbreak of Infectious myonecrosis virus (IMNV) was estimated to have cost US$ 100 million for the period from 2002 to 2006 (Lightner 2011). IMNV-infected shrimp exhibited necrosis of striated muscles (abdomen and cephalothorax), which can become reddened in chronic stage of infection and mortality rates ranged from 40 to 60% (Nunes, Martins & Gesteira 2004).

Crustaceans lack the acquired immunity found in vertebrates and hence their resistance to disease is based on their innate immune system (Luo, Zhang, Shao & Xu 2003), thus limiting alternatives for disease control. Under these circumstances, the

development of genetic resistance stock to infectious diseases becomes an attractive option (Storset, Strand, Wetten, Kjøglum & Ramstad 2007).

Shrimp aquaculture industry has adopted the strategy of developing disease-resistant populations on the basis that high survival rates can be associated with lower viral loads in selected families. In this strategy, when both viral load (a continuous indicator variable) and survival are used as a selection index, the number of observations to achieve an acceptable statistical power is reduced (Moss, Doyle & Lightner 2005).

Breeding programs aiming disease resistance are based on viral challenges for specific pathogens under laboratory conditions (Ødegård, Baranski, Gjerde & Gjedrem 2011a). The development of the experimental infection protocol has to simulate the most likely exposure route in culture conditions, repeatability, stocking densities and uniform dosage of inoculum (White, Schofield, Poulos & Lightner 2002). Thus, inoculation techniques are essential to minimize environmental variance components and maximize genetic variation in challenge tests (Gitterle, Gjerde, Cock, Salazar, Rye, Vidal, Lozano, Erazo & Salte 2006).

Recently, the selection for disease resistance in shrimp has been developed to improve survival for severe diseases such as Taura syndrome virus (TSV) and White spot syndrome virus (WSSV), which causes mortalities close to 100% and decreases growth rates in surviving animals (Cock, Gitterle, Salazar & Rye 2009). In TSV resistance program, selected shrimp exposed to TSV by ingestion of infected tissue had an 18.4% increase in survival after the first generation of selection (Argue, Arce, Lotz & Moss 2002). In the case of WSSV, high mortalities (>98%) prevented the selection of

dams and sires (Cock *et al.* 2009; Gitterle, Salte, Gjerde, Cock, Johansen, Salazar, Lozano & Rye 2005). However, recent studies demonstrated that WSSV selected families produced higher survival rates (from 22.7 to 57%) in few generations, suggesting that resistance breeding programs can be conducted effectively with *L. vannamei* (Huang, Yin, Ai, Huang, Li, Weng & He 2011; Cuéllar-Anjel, White-Noble, Schofield, Chamorro & Lightner 2012).

Because primary cell cultures from crustaceans have been used for growing or viral titration purposes only (Villena 2003) and attempts for producing crustacean hybrid cell lines have not demonstrated success in *in vitro* viral growth (Claydon, Roper & Owens 2010), *in vivo* assays may provide an important tool in breeding selection (Prior, Browdy, Shepard, Laramore & Parnell 2003).

A preliminary experiment of IMNV infection has been reported for *L. vannamei* (White-Noble, Lightner, Tang & Redman 2010). However, the development of a standardized challenge protocol to evaluate IMNV resistance in *L. vannamei* families remains unavailable. Based on this information, this study aimed at developing an experimental infection protocol for IMNV infection in *L. vannamei*, throughout two methods of exposure, ingestion of infected tissue and injection of viral inoculums, quantifying the viral load in all challenged animals and evaluating the viral load and survival rates correlations for the methods of exposure.

MATERIAL AND METHODS

Experimental conditions

Specific Pathogen Free (SPF) *L. vannamei* juveniles (2-3.9 g body weight) from different families were obtained at Genearch Aquacultura Ltda (Brazilian commercial hatchery; Rio Grande do Norte, Brazil) and maintained under a biosecurity facility. All tanks were covered to prevent virus transmission by aerosol and water was disinfected with 5 mgL^{-1} chlorine.

Shrimp were randomly selected and distributed into 100 L fiberglass tanks with continuous aeration (salinity between 29 and 31 gL^{-1}), according to stocking density of each route of infection and acclimatized to experimental conditions for 24 h prior to the experiments. Animals were fed twice a day with a commercial pellet feed (35% crude protein) at a rate of 5% body weight day^{-1}. Water quality parameters (temperature and pH) were monitored twice daily using an YSI 556 MPS meter (Yellow Spring Instruments Co., Yellow springs, OH, USA), with daily exchange of sea water at 50% and samples were collected weekly for total ammonia and nitrite measurements using commercial colorimetric kit (Alcon Labcon, Camboriú, Santa Catarina, Brazil). All water parameters were kept within recommended limits for *L. vannamei* (Van Wyk & Scarpa 1999).

Inoculum Preparation

The IMNV inoculum used in this study was obtained from samples of adult (7-11 g) *L. vannamei* collected during an outbreak in farms located in Northeastern Brazil, which exhibited gross signs of acute-phase IMN disease. The presence of IMNV was confirmed by PCR, and the absence of Taura syndrome virus (TSV), White spot

syndrome virus (WSSV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Necrotizing hepatopancreatic bacterium (NHPB) was detected by PCR and nested-PCR. All PCR and nested-PCR analyses were conducted through IQ2000^{TM} kits, according to the manufacturer's instructions (Farming IntelliGene Tech. Corp., Taiwan) (data not shown).

Tissue (100 g) from abdominal muscle of naturally IMNV-infected shrimp without cuticle was homogenized in 300 ml of chilled TN buffer (0.4 M NaCl and 20 mM Tris-HCl, pH 7.4) to a final concentration of 0.3 g tissue mL^{-1} TN buffer. The amount of tissue for inoculum preparation was based on TSV challenge described by Zarain-Herzberg, Hernandez-Saavedra & Ascencio-Valle (2003). The homogenate (stock inoculum) was diluted to $1:10^1$ (mean viral load of $9.82x10^5$ IMNV copiesμg^{-1} of total RNA) with sterile 2% saline solution and centrifuged at 3,000 rpm for 25 min at 4ºC. Supernatant fluid was centrifuged at 14,000 g for 20 min at 4°C and filtered through a sterile 0.22 μm syringe filter (TPP, Switzerland) followed by serial dilutions ($1:10^2$ to $1:10^9$). All new virus dilutions were prepared from stock inoculum before each assay and dilution dosages were expressed in stock inoculum ml^{-1} of 2% saline solution. All dilutions were stored at -80 °C until further use in injection challenges.

Oral inoculation procedure

The remaining muscle tissue used for stock inoculum preparation was preserved at -80°C and used as infective material for oral challenges by ingestion of infected tissue.

Oral inoculation procedure consisted of four treatments: feeding with infected tissue with salinity stress (FITS); feeding with infected tissue with temperature and salinity stresses (FITES); feeding with infected tissue with alkalinity stress (FITA) and feeding with infected tissue without stress (FIT) (Table 1). Stress tests design were based on information provided by shrimp farmers who experienced IMNV outbreaks between 2002 and 2004 in Brazil and by optimal water quality standards for *L. vannamei* culture (Van Wyk & Scarpa 1999).

Each treatment was carried out in two replicates of 13 shrimp at a density of 1 individual 7 L^{-1} and one control group. After molt stage, shrimp were orally infected with IMNV-infected minced muscle tissue at 4% of the biomass/day for three days. From the fourth day onwards, animals were submitted to stress tests for 48 hours at intervals of one week. This procedure was repeated three times. Negative controls were fed IMNV-free shrimp tissue confirmed using PCR, and submitted to same stress tests conditions.

The experiment lasted 50 days and throughout this period, animals were fed a commercial feed in the intervals of stress tests. Mortalities and gross signs of IMNV disease were monitored daily. Throughout the experiment, all dead animals were collected and stored at -80°C until real-time PCR analysis was performed. After 50 days, surviving shrimp were also stored at -80°C and submitted to real-time PCR analysis. The IMNV infection loads were assessed in dead and surviving shrimp over time.

171 **Table 1** Stress tests of oral inoculation procedure

Treatments	Stress	Optimal standards (Van Wyk & Scarpa 1999)
Feeding with infected tissue with salinity stress (FITS)	5 gL^{-1}	28 – 35 gL^{-1}
Feeding with infected tissue with temperature and salinity stresses (FITES)	20ºC and 5 gL^{-1}	28 - 32°C and 28 – 35 gL^{-1}
Feeding with infected tissue with alkalinity stress (FITA) by adding HCl.	30 mg $CaCO_3L^{-1}$	>100 mg $CaCO_3L^{-1}$
Feeding with infected tissue without stress (FIT)	-	-

172

173 **Intramuscular inoculation procedure**

174 After molting, shrimp were inoculated intramuscularly in the third abdominal
175 segment using a 1-mL insulin syringe with each dilution (100 µL per animal). In the
176 negative control treatment, shrimp were injected with sterile 2% saline solution with the
177 same volume. Shrimp were monitored daily to observe mortality rate and IMNV clinical
178 signs.

179 Nine exponential serial dilutions were tested: from $1:10^1$ to $1:10^9$. Initially,
180 dilutions from $1:10^1$ to $1:10^3$ were challenged with IMNV in duplicate of 13 animals,
181 however massive mortalities were observed on the 13th day. Thus, new virus inoculum
182 dilutions were set at $1:10^5$ to $1:10^9$ and three replicates of 20 animals were used. These
183 experiments were carried at 28ºC at a density of 1 shrimp 5 L^{-1} and one control group.

In order to verify the effect of low water temperature in IMNV infection, a second experiment was conducted at 26ºC using the same virus serial dilutions previously tested at 28°C, from $1:10^5$ to $1:10^9$, with three replicates of 20 animals at a density of 1 shrimp 5 L^{-1} and one control group.

In both either cases, dead and surviving animals were collected during the course of the experiment and stored at -80°C for real-time PCR analysis (except for dilution $1:10^4$ at 28ºC). Moribund or surviving animals from each dilution were preserved in Davidson's AFA fixative for histological analysis (Bell & Lightner 1988) and a pair of their pleopods was collected for real-time PCR diagnosis.

A third experiment was carried out at 26ºC with dilutions from $1:10^6$ to $1:10^8$ with three replicates of 20 animals at a density of 1 shrimp 5 L^{-1} and one control group, aiming to validate the survival probability trend obtained in the second experiment. Thus, no PCR analyses were carried out, only annotation of dead and survivors was recorded.

RNA extraction and RT-PCR

Total RNA was extracted from the abdominal muscle (50 mg) of challenged and control animals using Trizol (Invitrogen, Carlsbad, CA, USA), according to Chomczynski & Sacchi (1987), and stored at –80°C. The presence and quality of extracted RNA were evaluated by means of electrophoresis 1% agarose-formaldehyde gel in accordance with the standard protocol and the RNA concentration was estimated visually by comparison of known RNA sample band intensity (130 $\eta g \mu L^{-1}$) (Sambrook,

Fritsch & Maniatis 1989). The cDNA synthesis was performed by Improm-II™ Reverse Transcription System (Promega, Madison, WI, USA), with 0.5 µg oligo(dT)$_{15}$ and 2 µL of extracted total RNA in a 20 µL reaction volume, following the manufacture's protocol.

Real-time PCR quantitation of IMNV in challenged animals

Real-time PCR detection assay was made according to Silva, Pinheiro & Coimbra (2011) and the primer sequences used were 5'- GGA CCT ATC ATA CAT AGC GTT TGC A-3' (IMNV412F) and 5'-AAC CCA TAT CTA TTG TCG CTG GAT-3'(IMNV545R), as described by Andrade, Srisuvan, Tang & Lightner (2007).

Amplification was carried out with 1X SYBR Green Master Mix (Applied Biosystems, Warrington, UK), 0.2 µM of each primer and 1 µL of cDNA in a final volume of 25 µL. Real-time PCR runs were conducted in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the quantitation of the viral load was performed using the ABI 7500 program (version 2.0.1). Each sample was tested in duplicate. To determine the copy number of the IMNV genome, an IMNV recombinant plasmid was constructed and used as a quantitative standard, as described in Silva *et al.* (2011). Amplification specificity was confirmed by analyzing the dissociation curve (Ririe, Rasmussen & Wittwer 1997).

Statistical analysis

227

228 The proportions of infected shrimps and of survival are the response variables
229 considered in this study. Bayesian approach to statistical inference has experienced fast
230 growth over the last decades in several sciences (Berger 2000; McCarthy 2007; Kinas &
231 Andrade 2010). In this work, Bayesian approach was used to estimate the probability
232 (θ) of infection (or surviving) for each treatment of ingestion and inoculation
233 experiments. In this approach, the likelihood for the data is combined with a prior
234 distribution that conveys all other relevant information to calculate an updated posterior
235 distribution of the parameter of interest (θ). The Bayes formulation is:

236 (1) $$p(\theta \mid x) = \frac{\pi(\theta) l(x \mid \theta)}{\int \pi(\theta) l(x \mid \theta) \mathrm{d}\theta}$$

237 where x stand for the data, $l(x \mid \theta)$ is the likelihood, $\pi(\theta)$ is the prior and $p(\theta \mid x)$ is the
238 posterior distribution. The integral of the above equation can be difficult to calculate.
239 However, in some situations the posterior distribution can be easily calculated by using
240 families of conjugated probability distributions or numerical methods (e.g. Markov
241 Chain Monte Carlo – MCMC).

242 If the likelihood function and the prior distribution are conjugated, the posterior
243 distribution will of the same type of the prior distribution. Binomial and beta
244 distributions are conjugated. Hence, if we assume a binomial distribution for the
245 likelihood $Bin(n, \theta)$ where n is the number of tries in which we observe x "successes"
246 (e.g. infected shrimps) and θ is the probability of success (e.g. infection), and a beta

distribution $Beta(\alpha,\beta)$ for the prior, it is easy to show that the posterior will be also a beta distribution with parameters $\alpha' = \alpha + x$ and $\beta' = \beta + n - x$ (Kinas & Andrade 2010). That solution arises because binomial and beta distribution are conjugated. We have used the conjugated solution to calculate the posterior distribution of probability of infection for each of the ingestion and inoculation experiment treatments. We also used the conjugated solution to calculate the posterior for the survival probability for each ingestion treatment. In all the cases we assumed ignorance about θ and used a non-informative prior distribution $Beta(1,1)$. Intervals of credibility (95%) were calculated based on the posterior distribution of θ.

Generalized linear models (GLM) (McCullagh & Nelder 1989; Dobson 2002) were used to model the relationship between the proportion of surviving shrimps (response variable), and the dilutions (explanatory variable) tested in inoculation experiments. The GLM is:

(2) $$g[E(Y)] = X\theta$$

where X is the design matrix that stands for the explanatory variables, θ is the vector of parameters, $E(Y)$ is the expectation of the response variable, and $g[\ ]$ is the link function. If we assume a binomial distribution to model the number of successes in n tries, the expectation is the probability of success and the model is a logistic regression. Logit is often used as link function. We again assume ignorance about θ and used normal prior distributions with large variance $N(\mu = 0, \sigma^2 = 1000)$. Here opted by the MCMC numerical method to calculate the posterior distribution. The posterior

distribution for the probability of survival is then calculated based on the posterior distribution of θ. Intervals of credibility (95%) where calculated for the survival probability. Kruskal-Wallis test was used to compare mean viral load values between dilution groups. All the analyses mentioned in this section were carried out using the software R (R Core Team 2013). The software JAGS (Plummer 2005) was used together with R to run the MCMC algorithm.

RESULTS AND DISCUSSION

Oral inoculation procedure

Throughout the bioassay by ingestion of infected tissue, no gross clinical signs of IMNV infection were found in challenged animals. Viral load ranged from 23.95 to $8.39x10^6$ copiesμg^{-1} of total RNA for positive samples (Table 2) and 75% of challenged animals presented undetectable levels of viral load. Similar results were reported by White-Noble *et al.* (2010), which registered lower prevalence of diagnosable IMNV infections for orally inoculated shrimp.

Table 2 Viral load (copiesμg^{-1} of total RNA) of positive samples by oral inoculation procedure

Treatment	Nº Examined	PCR Positive	Viral load	
			Minimum	Maximum
FITS	26	17	2.11×10^2	8.39×10^6
FITES	26	7	23.95	85.48
FITA	26	1	48.61	48.61
FIT	26	1	7.14×10^2	7.14×10^2

Although inoculation by ingestion of infected tissue simulates the most likely natural mode of transmission in culture conditions (White *et al.* 2002), in the present study it was ineffective due to the low number of infected animals. According to Gitterle *et al.* (2005), it is unlikely that all animals will be equally infected by feeding minced infected muscle, since only voracious animals will access infected tissue.

In order to produce TSV resistant lines, Argue *et al*. (2002) have challenged 65 families of *L. vannamei* by oral infection of minced tissue and found an increase of 18.4% in survival. Similarly, an IHHNV resistant line of *Penaeus stylirostris* was obtained by feeding minced tissue as an infection route (Tang, Durand, White, Redman, Pantoja & Lightner 2000). For WSSV, three selected families of *L. vannamei* were orally infected by minced frozen tissue, resulting in higher survival rates up to 57%, after nine generations of selection (Cuéllar-Anjel *et al.* 2012). In addition, Gitterle, Gjerde, Cock, Salazar, Rye, Vidal, Lozano, Erazo & Salte (2006) and Escobedo-Bonilla, Audoorn, Wille, Alday-Sanz, Sorgeloos, Pensaert & Nauwynck (2006)

reported 100% infected *L. vannamei* in challenge tests that were conducted by a known oral dose of WSSVmL^{-1} using a catheter, suggesting new possibilities for other shrimp viruses.

In this study, although mean viral load in the FITS treatment was lower than those found in natural IMNV infections by Silva *et al.* (2011), the values were statistically different among stress treatments ($p \leq 0.05$). Moreover, the proportion of infected animals was significantly higher for FITS treatment (Fig. 1).

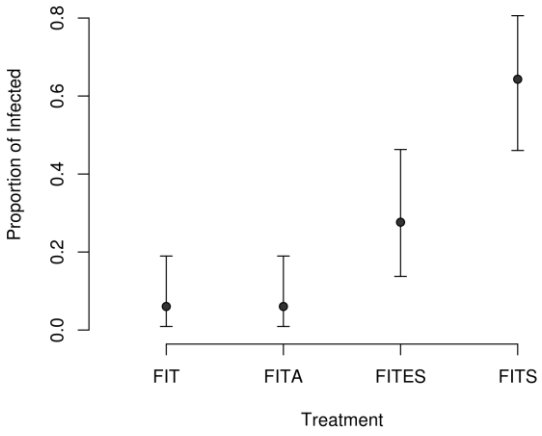

Figure 1 Credibility interval (95%) of the proportion of infected shrimp of oral inoculation procedure as calculated based on the posterior distributions. Circles stand for the median. Treatments: FITS – Feeding with infected tissue with salinity stress; FITES – Feeding with infected tissue with temperature and salinity stresses; FITA – Feeding with infected tissue with alkalinity stress; and FIT – Feeding with infected tissue without stress.

Carbajal-Sánchez, Castro-Longoria & Grijalva-Chon (2008) reported that the severity and progression of WSSV infection were higher in experimentally infected animals submitted to 15 gL^{-1} salinity rather than to 35, 25, 5 and 2 gL^{-1}. Although salinity itself had little influence on metabolism of euryhaline species (Bray, Lawrence & Leung-Trujillo 1990; Le Moullac & Haffner 2000), Pan & Jiang (2002) reported that sudden changes of salinity (30 gL^{-1} to 15 gL^{-1}) reduced antibacterial activity of *L. vannamei* and *Fenneropenaeus chinensis*. Likewise, changes in salinity (from 25 gL^{-1} to 5 gL^{-1}) led to higher susceptibility to *V. alginolyticus* infection in *L. vannamei*, suggesting that change in salinity is capable of causing disease outbreaks (Wang & Chen 2005).

Estimations of survival probabilities of infected shrimp until the end of the experiment (50 days) were high for both FITS and FITES treatments. On the other hand, probability of survival of infected shrimp was low for FITA and FIT groups. In the case of FITA and FIT treatments, most deaths were associated to molting or to stress conditions. However, the number of infected shrimps was low, hence the confidence intervals are wide (Fig. 2). No mortality or detectable viral load was observed in the negative control groups.

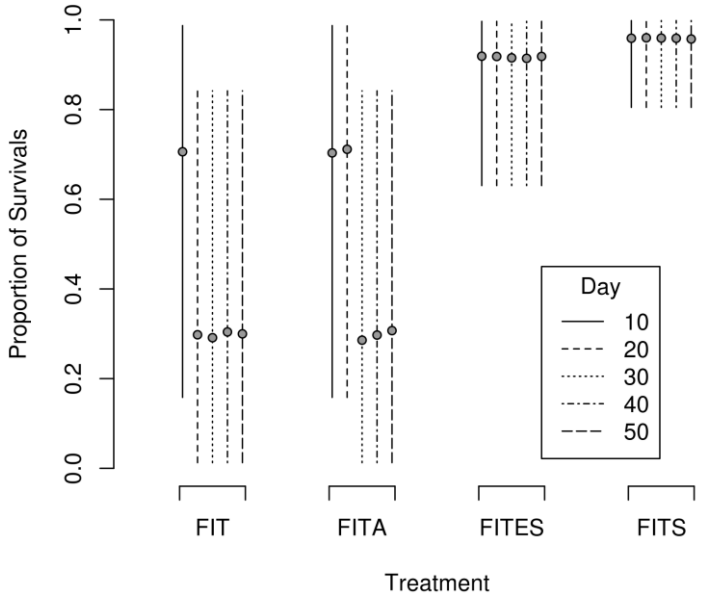

Figure 2 Credibility intervals (95%) of the estimations of survival probabilities of *L. vannamei* after IMNV challenge by oral inoculation treatments: FITS – Feeding with infected tissue with salinity stress; FITES – Feeding with infected tissue with temperature and salinity stresses; FITA – Feeding with infected tissue with alkalinity stress; and FIT – Feeding with infected tissue without stress.

Intramuscular inoculation procedure

Mean viral load of all dilutions was not significantly different at 28ºC for dilutions between $1:10^1$ and $1:10^7$ ($p \geq 0.05$) and values were similar (Table 3) to those reported for the chronic phase in naturally IMNV infected shrimp (between $6.85x10^8$ and $3.09x10^4$ copiesμg^{-1} of total RNA) registered by Silva *et al.* (2011). In contrast, significant differences in the mean viral load between $1:10^1$ and $1:10^7$ group and $1:10^8$ and $1:10^9$ dilutions were found, with the first group exhibiting higher viral loads.

Shrimp kept at 28°C and challenged by dilutions $1{:}10^{1}$ to $1{:}10^{7}$ showed higher mean infection levels (Table 3) than those found for dilutions between $1{:}10^{5}$ to $1{:}10^{7}$ for shrimp maintained at 26°C ($p \leq 0.05$), whereas for $1{:}10^{8}$ and $1{:}10^{9}$ dilutions similar mean viral load were observed for both temperatures.

In this study, the effect of decreasing water temperature from 28 to 26 °C in the course of IMNV infection resulted in lower IMNV infection levels. Opposite results were found for WSSV challenged shrimp kept at 33ºC, which displayed no sign of WSSV infection, whereas those maintained at 27 and 30°C developed signs of disease, showing the effect of water temperature on the progression of infection in shrimp (Rahman, Escobedo-Bonilla, Corteel, Dantas-Lima, Wille, Alday Sanz, Pensaert, Sorgeloos & Nauwynck 2006; Rahman, Corteel, Wille, Alday-Sanz, Pensaert, Sorgeloos & Nauwynck 2007).

371 **Table 3** Mean viral load (copiesµg^{-1} of total RNA) of intramuscular inoculation
372 procedure at 28 and 26ºC

Dilutions at 28°C	Nº Examined	PCR Positive	Viral load		
			Minimum	Mean	Maximum
1:10^{1}	26	26	2.19x10^{2}	6.04x10^{6a}	1.1x10^{8}
1:10^{2}	26	26	1.69x10^{3}	4.82x10^{6a}	7.35x10^{7}
1:10^{3}	26	26	58.3	6.31x10^{6a}	7.65x10^{7}
1:10^{5}	60	52	2.99x10^{2}	2.06x10^{7a}	5.17x10^{8}
1:10^{6}	60	38	71.90	1.03x10^{7a}	4.66x10^{8}
1:10^{7}	60	47	30.20	4.94x10^{6a}	6.79x10^{7}
1:10^{8}	60	23	2.19x10^{2}	4.23x10^{6b}	2.04x10^{8}
1:10^{9}	60	8	1.90x10^{2}	6.12x10^{6b}	3.67x10^{8}
Dilutions at 26°C	Nº Examined	PCR Positive	Viral load		
			Minimum	Mean	Maximum
1:10^{5}	60	28	62.8	6.82x10^{4b}	7.56x10^{5}
1:10^{6}	60	27	80.7	2.09x10^{4b}	3.39x10^{5}
1:10^{7}	60	18	41.7	8.88x10^{4b}	1.25x10^{6}
1:10^{8}	60	9	3.74x10^{3}	2.49x10^{5b,c}	1.25x10^{6}
1:10^{9}	60	1	6.13x10^{5}	6.13x10^{5b,c}	6.13x10^{5}

373

374 All challenged animals for dilutions from 1:10^{1} to 1:10^{7} for both temperatures
375 displayed typical clinical signs of IMNV infection (opacity or muscle necrosis) and

lesions suggestive of IMNV infection were found by histology, as demonstrated by coagulative necrosis of striated muscle fibers accompanied by infiltration of hemocytes (Fig. 3). Furthermore, a cannibalism trend was observed for these dilutions. Few affected individuals were observed under infection for $1:10^8$ or $1:10^9$ dilutions and shrimp used as controls displayed no clinical signs.

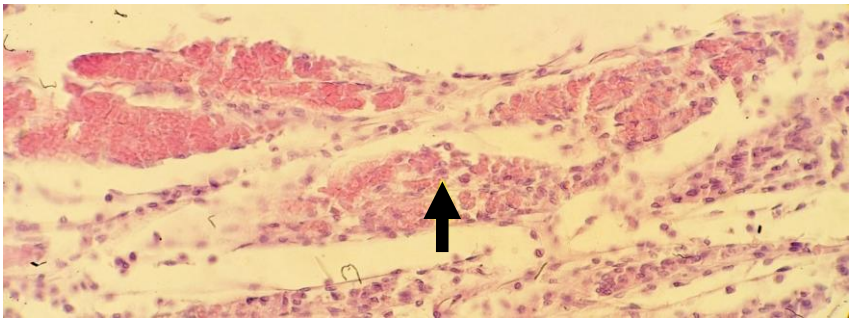

Figure 3 Photomicrograph of striated muscle (400X) from *L. vannamei* analyzed for IMNV lesions by H&E. Coagulative necrosis of striated muscle fibers accompanied by infiltration of hemocytes (arrow) in IMNV infection.

Wu, Namikoshi, Nishizawa, Mushiake, Teruya & Muroga (2001) reported the presence of cannibalism in *Penaeus japonicus* infected experimentally with WSSV by intramuscular route. White *et al.* (2002) suggested that optimal biomass needs to be considered in viral challenges in order to prevent cannibalism, though the eradication of cannibalism is difficult, since *L. vannamei* cannibalizes dead or weak members of the population (Gitterle *et al.* 2005). In this study, we could not detect consistent effect of biomass on infection protocol.

Regarding the number of infected animals, serial dilution and proportion of infected shrimp were inversely proportional for both temperatures. Over 60% of animals challenged for dilutions between $1:10^1$ and $1:10^7$ were infected, while for $1:10^8$ and $1:10^9$ dilutions less than 40% of shrimp were infected at 28°C (Fig. 4). However, shrimp challenged at 26°C showed a proportion of infection superior to 30% for dilutions between $1:10^7$ and $1:10^5$ (Fig. 4). Therefore, a decrease in the proportion of infected shrimp was observed as a consequence of lower water temperature.

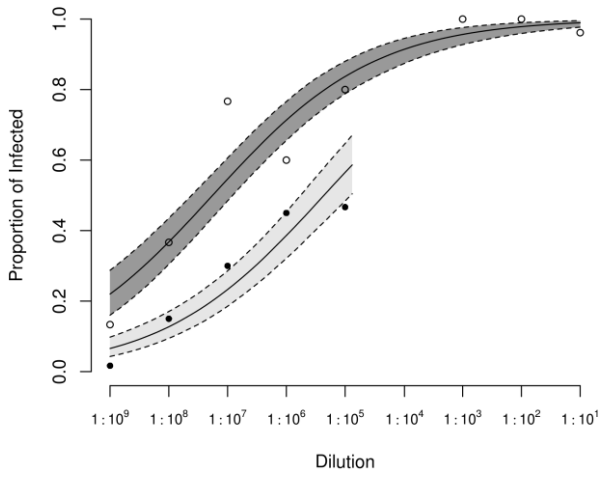

Figure 4 Model fittings and infection probability estimation in the intramuscular inoculation experiment. Light gray area bounded by dashed lines stands for the credibility interval (95%) for the experiment with 26ºC, while dark gray stands for the experiment with 28ºC. Continuous lines are the mean. Solid and empty circles are the observed proportion of infected shrimps in the experiments with 26ºC and 28ºC, respectively.

In viral challenges by intramuscular route, the injected virus spreads rapidly throughout the hemolymph, reaching target organs in a short interval (Carbajal-Sánchez *et al.* 2008). Infectious viral particles are released directly into shrimp body, surpassing natural barriers and reaching susceptible cells (Escobedo-Bonilla *et al.* 2006). On the other hand, in viral challenges by oral route, infectious viral particles can be damaged by lumen of shrimp foregut, leading to their inactivation (Escobedo-Bonilla *et al.* 2006).

In this study, greater proportions of infected animals were achieved by intramuscular challenges, although mean viral load values were similar for all dilutions (from $1:10^1$ to $1:10^7$ for both temperatures) and FITS ingestion treatment.

Survival probabilities were similar among dilutions from $1:10^1$ to $1:10^6$ at 28°C for infected shrimp at the 13^{th} day post-inoculation, though dilutions from $1:10^1$ to $1:10^5$ showed massive mortalities at that time (Fig. 5). In general, high survival probabilities of infected shrimp were achieved five days after exposure to the virus (for both temperatures), but decreased quickly at the 10^{th} day post-inoculation (less than 40% of survivors) especially for $1:10^6$ and $1:10^7$ dilutions at 28ºC, whereas for dilutions from $1:10^6$ to $1:10^8$ at 26°C, the survival probability was superior to 60% at the 10^{th} day of experiment (Fig. 5).

In the third experiment, the results of survival probability at 26°C were similar from dilutions $1:10^6$ to $1:10^8$ up to the 5^{th} day post-inoculation, followed by a decline in the 10^{th} day post-inoculation. A high survival was registered at the end of the experiment (15^{th} day post-inoculation) for $1:10^6$ and $1:10^7$ dilutions (Fig. 6).

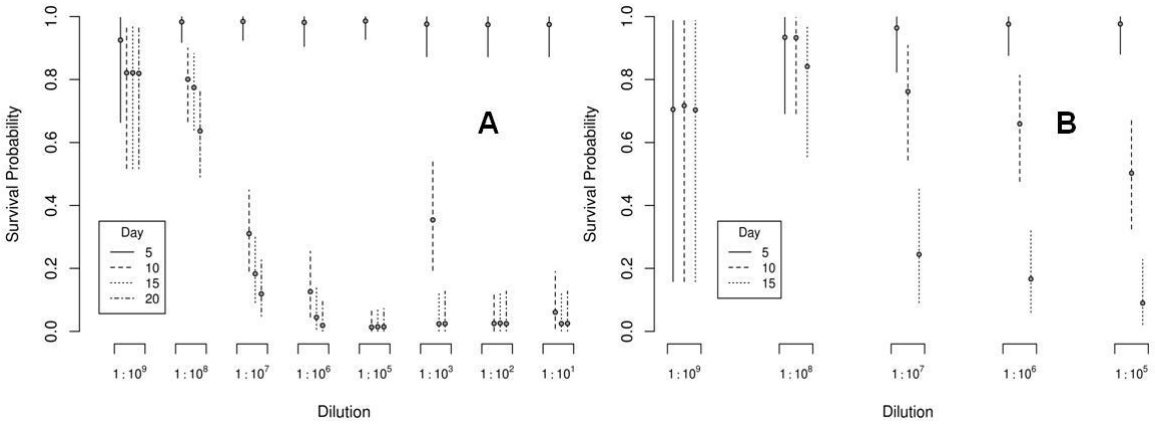

Figure 5 Credibility intervals (95%) of the estimations of survival probabilities of *L. vannamei* infected after intramuscular inoculation procedure at 28°C (A) and 26°C (B).

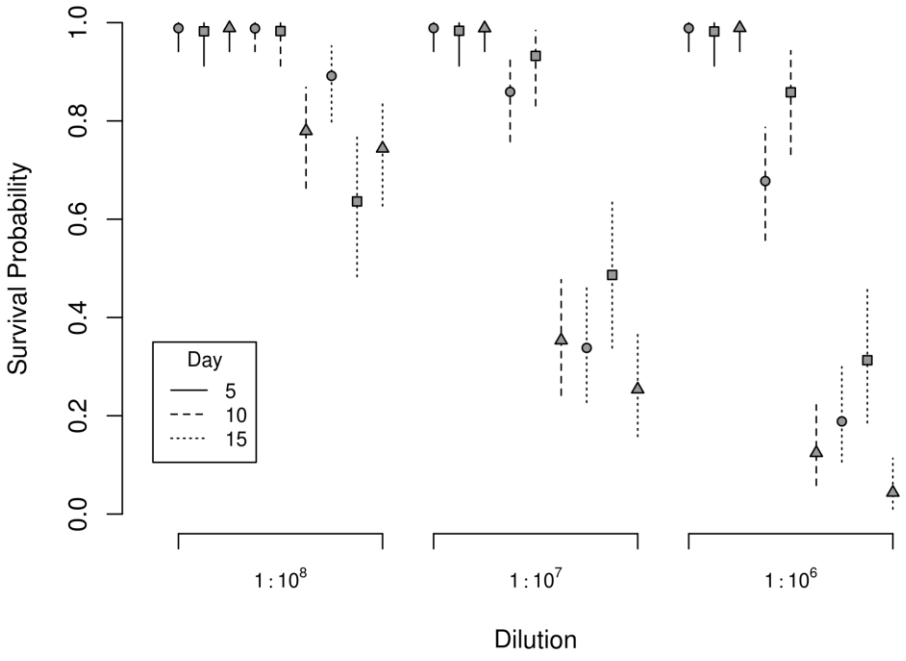

Figure 6 Credibility intervals (95%) of the estimations of survival probabilities of *L. vannamei* infected by intramuscular inoculation. Solid circles correspond to observed survival probability at 28°C, whereas solid square and triangle are survival probabilities at 26°C for the second and third experiments, respectively.

Prior *et al.* (2003) found no differences in the mortality of *L. vannamei* infected intramuscularly with serial exponential dilutions of TSV and WSSV inoculums recorded for 7 days post-infection. In the present study, there was no significant difference in the mortalities for IMNV dilutions from $1:10^1$ to $1:10^9$ recorded five days post-infection ($p \geq 0.05$).

In the injection groups for both temperatures, the first mortalities were recorded at the 6^{th} day post infection and there was one mortality peak between the 7^{th} and 10^{th} days for dilutions of $1:10^5$ to $1:10^7$, suggesting that the time course required by the virus to reach susceptible cells and start an infection process is around 10 days post-inoculation.

In breeding programs, challenge tests for infectious diseases have been carried out by terminating experiments at intermediate mortality or when it is still increasing. A fundamental point for this is that genetic evaluation is based on survival as a binary trait, which is favorable when the aim of selection is resistance rather than reduced susceptibility (Ødegård, Gitterle, Madsen, Meuwissen, Yazdi, Gjerde, Pulgarin & Rye 2011b).

In the present study, median infectious doses (ID_{50}) were determined between $2.44:10^8$ and $1.19:10^7$ dilutions at 28°C and between $1.76:10^6$ and $1.28:10^5$ dilutions at 26°C for 15 days post-infection, respectively. Moreover, if only the proportion of infected individuals (positive animals) is taken into account, the dilution of $1:10^7$ at 28°C and the dilution of $1:10^6$ at 26°C revealed an infectious dose in which more than 45% of the test animals were infected and nearly 80% died. Furthermore, most of the animals that died at these dilutions were infected with high levels of viral load,

indicating that almost every infected shrimp died. Thus, these dilutions were the most appropriate infective methods for driving challenges for IMNV resistance selection in *L. vannamei* families due to its efficiency in the infection rate.

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4.2 - Artigo científico II

Investigação da replicação do vírus da Mionecrose infecciosa em linhas de célula do mosquito *Aedes albopictus* subclone C6/36 e *Spodoptera frugiperda* subclone SF9.

Artigo científico a ser encaminhado a Revista - Journal of Virological Methods.

Todas as normas de redação e citação, deste capítulo, atendem as estabelecidas pela referida revista (em anexo).

Investigação da replicação do vírus da Mionecrose infecciosa em linhas de célula do mosquito *Aedes albopictus* subclone C6/36 e *Spodoptera frugiperda* subclone SF9.

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ABSTRACT

In Brazil, the Infectious myonecrosis virus (IMNV) caused a significant decrease in the production of *L. vannamei* in 2004, stressing the need of developing standardized *in vitro* systems for isolation of virus in order to develop strategies for disease control. Although there was no permanent shrimp cell line, some attempts were conducted for propagation of shrimp viruses in mosquito cell line. In this study, C6/36 and Sf9 cell lines were challenged with IMNV and ten serial passages were analyzed by PCR and indirect immunofluorescence (IFI). The PCR analysis confirmed the IMNV in the five successive passages from C6/36 cells, whereas SF9 cell was positive just for inoculation passage. For IFI analysis, a monoclonal antibody was constructed and used to confirm IMNV infection. All ten serial passages from C6/36 and SF9 cells were negative by IFI, suggesting that there was no viral replication, but that residual virus remained from inoculation. The results of the present study showed that IMNV did not replicate in C6/36 and SF9 cells, thus limiting *in vitro* system for IMNV replication purposes. This is the first attempt of using C6/36 and SF9 cell lines for IMNV propagation.

Keywords: C6/36 mosquito cell line; Lepidopteran cell line; IMNV; RT-PCR; Monoclonal antibody; Indirect immunofluorescence.

1. Introdução

Segundo a Organização das Nações Unidas para Alimentação e Agricultura (FAO), cerca de 57% (2,7 milhões de toneladas) da produção mundial aquícola marinha, em 2010, consistiu de camarões marinhos, com predominância de 71,8% da produção concentrada no camarão branco do Pacífico, o *Litopenaeus vannamei* (FAO, 2012). Nas Américas, a carcinicultura marinha está baseada quase inteiramente na cultura de *L. vannamei* (Lightner, 2011), com uma cadeia produtiva que englobava, em 2004, 14 países (Lightner, 2011) e uma produção de 594.960t em 2011, geradora de mais de 2,5 bilhões em receita (FAO, 2014).

No entanto, durante o último século, a rápida adoção de práticas de cultivo intensiva na indústria de peneídeos marinhos acarretou tanto em um aumento global do movimento de animais vivos e seus produtos, e sua subsequente possibilidade de transferência e estabelecimento de certos patógenos de um hemisfério para o outro, quanto nas várias fontes de estresse antropogênica aos ecossistemas aquáticos, tais como: mudança de habitat; alimentação artificial e elevadas densidades de estocagem, conduzindo ao surgimento e a propagação de doenças (Walker e Winton, 2010).

Doenças de origem viral e bacteriana respondem atualmente por 80% das perdas por doenças na carcinicultura marinha mundial, sendo os vírus quatro vezes mais geradores de impacto negativo na produção do que as bactérias (Flegel, 2012). No Brasil, uma nova doença transmitida por vírus que afeta crustáceo, chamada de doença da Mionecrose infecciosa, foi primeiramente relatada em fazendas de engorda de *L. vannamei* no estado do Piauí (Brasil) em 2002 e na Indonésia em 2006 (Nunes et al., 2004; Senapin et al., 2007), ocasionando prejuízos econômicos que ultrapassam os US$ 100 milhões de dólares para o período de 2002 a 2006 no Brasil (Lightner, 2011) e de US$ 1 bilhão de dólares para o período de 2006 a 2010 na Indonésia (Lightner et al., 2012).

A doença da Mionecrose infecciosa é causada pelo vírus de RNA de dupla fita pertencente à família Totiviridae, denominado de vírus da Mionecrose infecciosa (*Infectious myonecrosis virus* – IMNV) (Poulos et al., 2006). Os sinais clínicos da doença incluem opacidade e necrose muscular dos segmentos abdominais, cauda e urópodos, com observação da redução ou suspensão no consumo alimentar, perda do volume do hepatopâncreas, dificuldade no endurecimento da carapaça e natação errática

nos animais infectados. Os viveiros afetados pelo IMNV podem apresentar mortalidades variando de 40 a 60% (Nunes et al., 2004).

Culturas celulares constituem uma ferramenta básica para o estudo de infecções patogênicas, particularmente para aquelas em que os patógenos são replicantes intracelulares, como os vírus (Sudhakaran et al., 2007). Seu uso permite a análise de interações entre o vírus e o hospedeiro no sentido de identificar os mecanismos envolvidos em um processo de infecção viral (Villena, 2003).

Em crustáceos, embora culturas primárias oriundas de diferentes tecidos (órgão linfóide, hepatopâncreas, ovário e hemócito) de peneídeos tenham sido usadas para a propagação dos vírus da Mancha branca (Kasornchandra e Boonyaratpalin, 1998; Uma et al., 2002; Maeda et al., 2004; Jiang et al., 2006; Jose et al., 2010; Jose et al., 2012), da Taura (George et al., 2011) e da Cabeça amarela (Assavalapsakul et al., 2003), a inconsistência de informação sobre o número de passagens celulares (número de dias) que uma cultura pode ser mantida têm tornado estes protocolos de difícil reprodução (Jayesh et al., 2012).

Atualmente, linhagens celulares provenientes de invertebrados marinhos permanecem sem ser desenvolvidas apesar das abordagens moleculares em nível genômico para transformação e imortalização de células. Tal fato se dá, principalmente, devido à falta de informações sobre os mecanismos moleculares que inibem transformações neoplásicas em camarão (Jayesh et al., 2012). Assim, estudos direcionados ao IMNV e a outros vírus de camarão permanecem limitados pela ausência de linhas de células contínuas de crustáceos.

Visando solucionar este problema, inúmeros trabalhos têm empregado o uso de linhagens celulares de mosquito (*Aedes albopictus* subclone C6/36) e lepidópteros (*Spodoptera frugiperda* subclone SF9) para a propagação *in vitro* de vírus de camarão. Sudhakaran et al. (2007) relatam a replicação dos vírus *Macrobrachium rosenbergii* nodavirus (*Mr*NV) e extra small virus (XSV) em cinco passagens sucessivas de C6/36, confirmada através da análise de RT-PCR de amostras do sobrenadante da cultura. No caso de camarões peneídeos, Sriton et al. (2009) descrevem 100% de células C6/36 e SF9 positivas para o vírus da Mancha branca (*White Spot Syndrome Virus* – WSSV) e da Cabeça amarela (*Yellow Head Disease* - YHV) detectadas via análise imunohistoquímica em mais de 100 passagens celulares. Resultados similares foram

obtidos por Gangnonngiw et al. (2010) que ao inocular YHV em C6/36 obtiveram 100% de células positivas detectadas via análises imunohistoquímica e RT-PCR oriundas de passagens celulares. Para o vírus da Taura (TSV), C6/36 foram inoculadas e mostraram-se positivas por imunohistoquímica dentro de 11 passagens sucessivas, com constatação de partículas virais em C6/36 imunopositivas (Arunrut et al., 2011). Recentemente, a propagação de outro vírus de camarão, o da Parvovirose hepatopancreática (*Hepatopancreatic parvo-like virus* - HPV), foi demonstrada em um sistema *in vitro* usando C6/36 por RT-PCR, imunocitoquímica, western blot e PCR em tempo real, ficando evidente a replicação com o aumento da carga viral durante o curso da infecção (Madan et al., 2013). Assim, o presente estudo tem por objetivo investigar a propagação do IMNV nas linhas de célula C6/36 e SF9 através de análises de PCR e imunofluorescência indireta. Este é o primeiro estudo a avaliar o IMNV em um sistema *in vitro*.

2. Material e métodos

2.1 Preparação do inóculo viral

Adultos de camarão marinho *L. vannamei* (peso médio de 11,6 g) naturalmente infectados foram obtidos durante um surto de IMNV em viveiros de engorda de fazendas localizadas no litoral norte do estado de Pernambuco (Nordeste, Brasil) e usados como material para o preparo do inóculo viral. Após a coleta, estes animais foram imediatamente armazenados em nitrogênio líquido e depois transferidos para um ultrafreezer a -80ºC.

A infecção por IMNV foi confirmada por PCR, usando primers específicos para IMNV (Poulos e Ligthner, 2006), enquanto a presença de outros patógenos de camarões peneídeos (TSV, WSSV, vírus da Necrose hipodermal e hematopoiética infecciosa - IHHNV e Hepatopancreatite necrosante - NHPB) foi descartada por meio dos kits da IQ2000^{TM}, conforme as instruções do fabricante para cada patógeno (Farming IntelliGene Tech. Corp., Taiwan) (dados não mostrados). Este procedimento foi adotado de forma a garantir que apenas o patógeno de interesse (IMNV) estivesse presente no inóculo.

O preparo do inóculo viral foi feito segundo protocolo de infecção experimental descrito por Silva et al. (descrito no 1° artigo desta tese), com algumas modificações. O

tecido muscular de camarão infectado foi descongelado e homogeneizado em tampão TN gelado (20 mM Tris-HCl, pH 7,5, 400 mM NaCl) a uma proporção de 1:3 (w/v). O homogeneizado foi diluído a $1:10^1$ em meio de cultura (Leibovitz L-15 ou Grace, dependendo da cultura celular) e centrifugado a 3000 rpm por 25 minutos a 4ºC e o sobrenadante, removido e centrifugado a 14000 rpm por 20 minutos a 4ºC. Em seguida, filtrou-se o sobrenadante através de um filtro com membrana polietersulfonica (PES) de 0,22 µm (TPP, Switzerland).

Para os estudos de infectividade, o filtrado foi diluído em meio de cultura, sendo esta diluição definida com base na observação de efeitos citotóxicos ocasionados pelo inóculo. Todas as diluições foram armazenadas a –80ºC até a sua utilização para infectar as linhas de célula do mosquito *Aedes albopictus* subclone C6/36 e *Spodoptera frugiperda* subclone SF9.

2.2 Cultura celular e manutenção

Células C6/36 (*Aedes albopictus* subclone C6/36) e SF9 (*Spodoptera frugiperda* subclone SF9) foram obtidas do Departamento de Virologia e Terapia Experimental pertencente ao Centro de Pesquisa Aggeu Magalhães (FIOCRUZ, Brasil). Células C6/36 foram mantidas em meio Leibovitz L-15 (Gibco, EUA) contendo 100 U/ml de ampicilina, 100 µg/mL de estreptomicina e 2,5 µg/ mL de fungizona suplementado com 10% de soro fetal bovino, enquanto que as células SF9 foram cultivadas em meio Grace TNM-FH (Gibco, EUA) suplementado com 100 U/mL de ampicilina, 100 µg/mL de estreptomicina, 2,5 µg/ mL de fungizona, 10 µg/ mL de gentamicina e 10% de soro fetal bovino.

As células foram incubadas a 28°C e à medida que formavam uma monocamada, eram repicadas por meio de deslocamento da monocamada a uma razão de 1:2 ou 1:4, dependendo do número de células.

2.3 Estudos de infectividade

Para os estudos de infectividade, células C6/36 e SF9 foram cultivadas em placas de cultura de seis poços (TPP, Germany) a densidade de $3x10^5$ células /mL e incubadas por 12 horas a 28°C. Após atingir uma confluência de 70%, o meio de cultura foi removido e o inóculo viral diluído adicionado. Para as células C6/36 foram testadas as diluições

de 1:2, 1:5 e 1:10, enquanto que para as SF9, as diluições de 1:10, 1:20, 1:30 e 1:50. Estas diluições foram definidas com base nos efeitos citotóxicos (redução de viabilidade celular e degeneração da monocamada celular) observados para as diluições previamente testadas para estas células (dados não mostrados). Depois da adsorção por 2 horas a 28°C, o sobrenadante foi descartado e novo meio de cultura acrescido de soro fetal bovino a 2% foi adicionado às células, seguido de incubação a 28°C por 15 dias para a observação de efeito citopático (CPE). Diariamente, as células inoculadas foram observadas para efeito citopático, sob um microscópio de contraste de fase invertida. Após 15 dias, o sobrenadante das culturas celulares infectadas foi coletado e 1 mL usado para o estudo de re-infecção em novas células, sendo realizadas alíquotas de amostras do sobrenadante e das células de cada passagem inoculada. As amostras do sobrenadante de cada passagem inoculada foram preservadas a -80°C para análise de PCR e as células, congeladas em meio de congelamento (90% de soro fetal bovino e 10% de DMSO) para a análise de imunofluorescência indireta. Ao total, dez passagens celulares foram efetuadas.

2.4 Confirmação de IMNV por PCR

2.4.1Extração de RNA e RT-PCR

Cem microlitros de cada amostra do sobrenadante de cada passagem inoculada foram usados para a extração do RNA total usando Trizol (Invitrogen, EUA), conforme as instruções do fabricante. Após a extração, a concentração e a qualidade do RNA total foram medidas por análise espectrofotométrica em 260 e 280 ηm por meio de um espectrofotômetro (NanoPhotometer® P-Class, Implen, EUA). Em seguida, o cDNA foi sintetizado através do kit Improm-II^{TM} Reverse Transcription System (Promega, Madison, WI, USA) usando 2µL de RNA total (300 ηg/µL) e 0,5µg de oligo$(dT)_{15}$ em um volume final de 20µL, conforme as instruções do fabricante Promega (USA). O cDNA foi armazenado a -20°C até posterior utilização.

2.4.2 PCR

Análises de PCR foram realizadas para confirmar a infecção por IMNV no sobrenadante das 10 passagens das culturas das células C6/36 e SF9, infectadas experimentalmente.

Cada reação de PCR foi conduzida em um volume final de 25µL contendo 2µL de cDNA, 1U de *Taq* polimerase, 200 µM de cada dNTP, 1,5 mM de $MgCl_2$ e 5 ρmol dos primers específicos para IMNV (IMNV-F 5′-CGA-CGC-TGC-TAA-CCA-TAC-AA-3′ e IMNV-R 5′-ACT-CGC-CTG-TTC-GAT-CAA-GT-3′) e 1X tampão de PCR, conforme descrito por Pinheiro et al. (2007), sendo adicionado a cada reação um controle positivo para o vírus da Mionecrose infecciosa e um controle negativo que substituiu a amostra de cDNA por água ultra-pura. O ciclo térmico empregado foi o mesmo descrito por Pinheiro et al. (2007) e os amplicons foram submetidos à eletroforese em gel de agarose a 2%.

2.5 Produção de anticorpo monoclonal

Para a detecção e determinação de percentagem de células positivas para IMNV nas passagens celulares infectadas via imunofluorescência indireta (IFI) foi produzido um anticorpo monoclonal baseado na sequência codificante da região hidrofílica do capsídeo do IMNV (GenBank no. AY570982.1), previamente descrito por Borsa et al. (2011), com algumas modificações.

O RNA total foi extraído de uma amostra de tecido muscular remanescente utilizado para a preparação do inóculo viral preservado a -80°C, seguido de síntese de cDNA conforme previamente descrito. Para a expressão da região hidrofílica do capsídeo do IMNV, os primers IMMV F - Sítio *Nde*I (5'-CAT ATG GGG CAA TTA CGG TTA CAG G-3') e IMNV R - Sítio *Xho*I (5'- CTC GAG GTA TAC ATA CCA AAT GGC C -3') foram usados para amplificar esta região via PCR usando o cDNA sintetizado como amostra. As condições de amplificação e ciclo térmico foram as mesmas descritas por Pinheiro et al.(2007).

Após a visualização do produto de PCR de 600pb em gel de agarose a 2%, foi usado o kit Illustra GFX PCR DNA and Gel Band Purification (Amersham/GE Healthcare, EUA) para a purificação. O produto purificado foi, então, diretamente ligado ao vetor pDRIVE (Qiagen, EUA) e transformado em células competentes *Escherichia coli* DH5α (New England Biolabs), conforme as instruções do fabricante. As colônias recombinantes (colônias brancas) foram inoculadas em meio Luria-Bertani (LB) (1% triptona, 0,5% extrato de levedura, 1% NaCl) com ampicilina a 50 µg/mL para a obtenção de DNA plasmidial, conforme protoco padrão (Sambrook et al., 1989). A

confirmação do recombinante foi feita através de digestão enzimática com 20U de *Nde*I e 25U *Xho*I (New England Biolabs) e seqüenciamento, usando o seqüenciador automático ABI 3100 (Applied Biosystems, CA, USA).

Após a confirmação dos recombinantes, o fragmento digerido e purificado obtido dos clones recombinantes foi ligado ao vetor de expressão pET-21a(+) (Novagen, EUA), previamente digerido (com 20U de *Nde*I e 25U *Xho*I) e desfosforilado (com 20U de *Antarctic Phosphatase* - New England Biolabs), e transformado em células eletrocompetentes *E. coli* DH10B (Invitrogen, EUA), conforme as recomendações do fabricante.

Após a transformação, cinco colônias obtidas foram propagadas em LB com ampicilina a 50 μg/mL para a obtenção de DNA plasmidial usando o kit QIAprep Spin Miniprep (Qiagen, EUA). A recombinação foi confirmada por digestão e o DNA plasmidial da construção IMNV-pET21a(+) transformado em células competentes de *E. coli* BL21, para a expressão da proteína recombinante.

Seis colônias da transformação em BL21 foram cultivadas em meio LB líquido e a expressão das proteínas foi induzida pela adição de 1mM de isopropiltiogalactosídeo (IPTG) a 37°C a 150 rpm por 4 horas. A cultura celular foi centrifugada (8000 rpm a 4°C por 20 minutos) e o pellet, ressuspendido em 20 mL de PBS 1X estéril gelado, seguido de sonicação com seis pulsos de 30 segundos intercalados com um minuto de repouso (Sonics Vibra Cell - VC x 500, EUA). Em seguida, o lisado celular foi centrifugado a 10.000 rpm por 10 minutos a 4°C e o sobrenadante contendo o extrato protéico total solúvel descartado. Segundo Borsa et al. (2011), a maior concentração da proteína recombinante da sequência do capsídeo do IMNV encontra-se na fração protéica insolúvel. Assim, o pellet foi ressuspendido em tampão desnaturante (DNPI-10) e a purificação da proteína conduzida através do kit Protino® Ni-NTA Agarose sob condições desnaturantes (Macherey-Nagel, Germany). Para renaturação da proteína e recuperação de sua conformação, a proteína purificada foi diluída em tampão ASB (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM β-mercaptoetanol e 0.1% (w/v) ASB-14) acrescido de inibidor de protease a 1,25X e submetida a três ciclos de diálise em PBS 1X (Allonso et al., 2011), seguida de concentração à vácuo (Concentrator 5301, Eppendorf, EUA) para aumento do rendimento da proteína purificada. Todas as etapas de indução e purificação da proteína recombinante foram submetidas à eletroforese em

gel de poliacrilamida SDS-PAGE a 17,5% corados com azul de Comassie a 0,1% para confirmação da presença da proteína. A proteína purificada (~25 kDa) à concentração de 1 mg/mL, foi enviada para a empresa RHEA BIOTECH (Brasil) para a produção do anticorpo monoclonal anti-IMNV.

Para a validação do anticorpo monoclonal anti-IMNV produzido, camarões livres de patógeno específico (*Specific Pathogen Free* -SPF) de *L. vannamei* com 2 g foram desafiados experimentalmente para IMNV via injeção intramuscular de inóculo diluído a $1{:}10^7$. Após a observação de sinais clínicos de infecção (8 dias pós-inoculação), tecidos do músculo abdominal proveniente de animais desafiados e do controle negativo foram removidos assepticamente (imersão por 1 minuto em etanol a 70% estéril, seguido de imersão em PBS 1X estéril acrescido de 100 U/mL de ampicilina e 100 µg/mL de estreptomicina por 1 minuto) e dissociados mecanicamente através de *cell strainer* de 70 µm (Fisher Scientific, EUA). Cem microlitros da suspensão celular foi transferida para o sistema Lab Tek® Chamber Slide (NUNC, EUA), previamente tratado com poli-D-lisina a 0,1 mg/mL, contendo 200 µL de meio de cultura Grace TNM-FH suplementado com 100 U/mL de ampicilina, 100 µg/mL de estreptomicina, 2,5 µg/mL de fungizona, 10 µg/mL de gentamicina, 10% de soro fetal bovino e 10% de extrato de músculo de camarão (George e Dhar, 2010). Após a adesão das células na lâmina, as células foram fixadas em metanol absoluto gelado por 15 minutos para a análise de imunofluorescência indireta.

2.6 Imunofluorescência indireta (IFI)

A análise de imunofluorescência indireta foi efetuada para a detecção da proteína do capsídeo do IMNV nas amostras inoculadas. Para as 10 passagens celulares de C6/36 e SF9 inoculadas congeladas, foi realizado o descongelamento prévio das células a 37°C, seguido de centrifugação a 1200 rpm por 5 minutos e ressuspensão em 1 mL de PBS 1X estéril, com a transferência de 20 µL da suspensão para os poços de lâminas multispot de 12 poços (Thermo Scientific, EUA). Após o assentamento celular, as células foram fixadas em metanol absoluto gelado por 15 minutos, descrito previamente.

Assim, após a fixação, tanto as lâminas usadas para a validação do anticorpo monoclonal, como as produzidas a partir das amostras de passagens congeladas foram acrescidas de 20 µL de anticorpo monoclonal diluído a 1:10 e incubadas a 37°C por 1

hora. Em seguida, as lâminas foram lavadas três vezes em PBS 1X e, uma vez, em água destilada, seguida de secagem a temperatura ambiente. Após secagem, o anticorpo secundário anti-camundongo IgG, produzido em caprino conjugado com isotiocianato de fluoresceína - FITC, foi adicionado a diluição de 1:100 e incubado a 37°C por 1 hora, seguida de lavagens sucessivas em PBS 1X e coloração em azul de Evans a 0,01% por 3 minutos. Em seguida, a lâmina foi montada com adição de glicerol tamponado (glicerol/PBS 1X - 9:1 - v:v) e as células, analisadas sob microscópio confocal (Leica DMI 4000B, EUA).

3. Resultados e discussão

Nenhum efeito citopático (alteração morfológica e degeneração da monocamada celular) foi observado nas dez passagens celulares de C6/36 e SF9 inoculadas com IMNV quando comparadas ao controle.

Amostras oriundas do sobrenadante de cinco passagens celulares de C6/36 desafiadas com IMNV foram positivas por PCR, com redução da intensidade das bandas à medida que o número de passagens aumentava (Figura 1). A partir da 6ª passagem, todos os resultados foram negativos. Já para as células SF9, só a cultura celular inoculada pela primeira vez (passagem 0) mostrou-se positiva (Figura 2). Tal resultado sugere que as células C6/36 e SF9 desafiadas para IMNV foram incapazes de liberar partículas virais (vírions infecciosos) na cultura celular ou que não houve replicação viral.

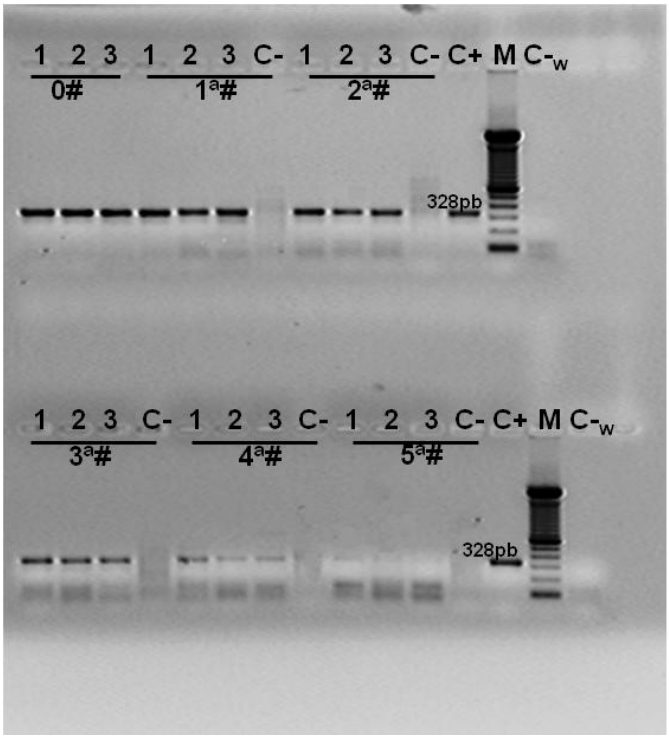

Figura 1. Detecção positiva de IMNV em células C6/36 por PCR. Amostras 1, 2 e 3 correspondem as diluições de 1:2, 1:5 e 1:10 inoculadas em C6/36, enquanto os números abaixo da linha indicam o número de passagens celulares ocorridas, onde: # é passagem celular; C-, controle negativo (célula não infectada); C+, controle positivo; M, marcador de peso molecular de 100pb (Invitrogen, USA) e $C\text{-}_w$, controle negativo (água ultra-pura).

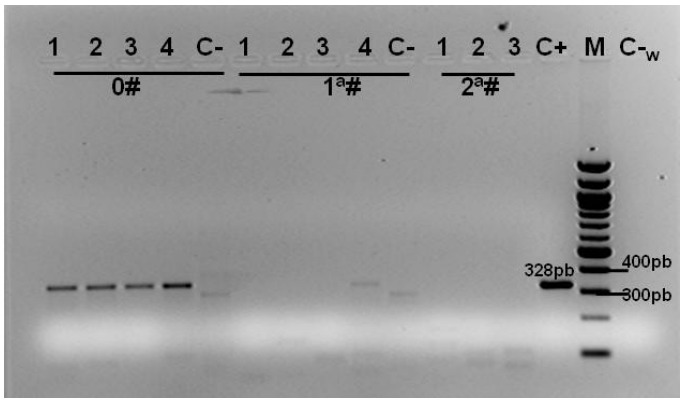

Figura 2. Detecção positiva de IMNV em células SF9 por PCR. Amostras 1, 2, 3 e 4 correspondem as diluições de 1:10, 1:20, 1:30 e 1:50 inoculadas em SF9, enquanto os números abaixo da linha indicam o número de passagens celulares ocorridas, onde: # é passagem celular; C-, controle negativo (célula não infectada); C+, controle positivo;

M, marcador de peso molecular de 100pb (Invitrogen, USA) e C-$_w$, controle negativo (água ultra-pura).

Resultados negativos de PCR do sobrenadante de culturas celulares de C6/36 desafiadas por YHV foram descritos por Gangnonngiw et al. (2010). Embora os autores tenham obtido células imunopositivas para o YHV via análise imunohistoquímica, ao se analisar o sobrenadante destas culturas por meio de RT-PCR, todas se mostraram negativas, sugerindo a não liberação de partículas virais no meio de cultura. Além disso, a patogênese de YHV proveniente do sobrenadante de C6/36 inoculadas foi incapaz de induzir a manifestação da doença em *P. monodon* desfiado experimentalmente via injeção intramuscular, com detecção negativa do vírus nos animais desafiados por histologia e imunohistoquímica, confirmando a não liberação de vírions infecciosos na cultura celular (Gangnonngiw et al., 2010).

Adicionalmente, em outro estudo realizado por Sriton et al. (2009), embora nenhuma análise de RT-PCR tenha sido conduzida para o sobrenadante de células C6/36 e SF9 positivas para WSSV e YHV detectadas via análise imunohistoquímica, a não visualização de vírion de WSSV ou YHV no núcleo de células imunopositivas via microscopia eletrônica indicou que a constância de antígenos virais poderiam estar associadas à transmissão ocorrida por divisão celular (subdivisão de genes entre células mãe e filhas) ou a algum processo endocítico inicial durante a inoculação viral.

Arunrut et al. (2011) ao propagar o vírus da Taura (TSV) em C6/36 constatou que apesar da presença de partículas virais junto a estruturas vesiculares no citoplasma de C6/36 imunopositivas semelhantes àquelas encontradas em células de camarões infectados com TSV, não foi possível infectar novas culturas celulares usando o sobrenadante destas culturas.

Por outro lado, a replicação dos vírus *Macrobrachium rosenbergii nodavirus* (*Mr*NV) e extra small virus (XSV), em cinco passagens sucessivas de C6/36, foi demonstrada através da análise de RT-PCR de amostras do sobrenadante da cultura após três dias de inoculação, com numerosas partículas virais no citoplasma de C6/36 infectadas visualizados via microscopia eletrônica após 48 horas (Sudhakaran et al., 2007). Além disso, pós-larvas de *Macrobrachium rosenbergii* desafiadas via imersão utilizando inóculo preparado a partir do sobrenadante de células infectadas exibiram

sinais clínicos de infecção similares aos obtidos em surtos naturais e 100% de mortalidade em 6 dias pós-infecção, com confirmação positiva via RT-PCR dos animais desafiados (Sudhakaran et al., 2007).

Resultados similares foram obtidos por Madan et al. (2013) ao propagar o vírus da Pavovirose hepatopancreática (HPV) em um sistema *in vitro* usando C6/36, que ao analisar o sobrenadante das passagens infectadas detectou o HPV via PCR e o aumento da carga viral durante o curso da infecção. A patogênese do HPV foi demonstrada em pós-larvas de *P. monodon* desafiadas via imersão com inóculo proveniente da 20° passagem de cultura positiva (Madan et al. (2013).

Com relação às análises de imunofluorescência indireta, o anticorpo monoclonal anti-IMNV produzido neste trabalho foi capaz de reconhecer a proteína nativa viral obtida através de desafio conduzido em *L. vannamei* inoculados experimentalmente (Figura 3), sendo utilizado nas análises de imunofluorescência indireta (IFI) das dez passagens das células C6/36 e SF9 inoculadas com IMNV.

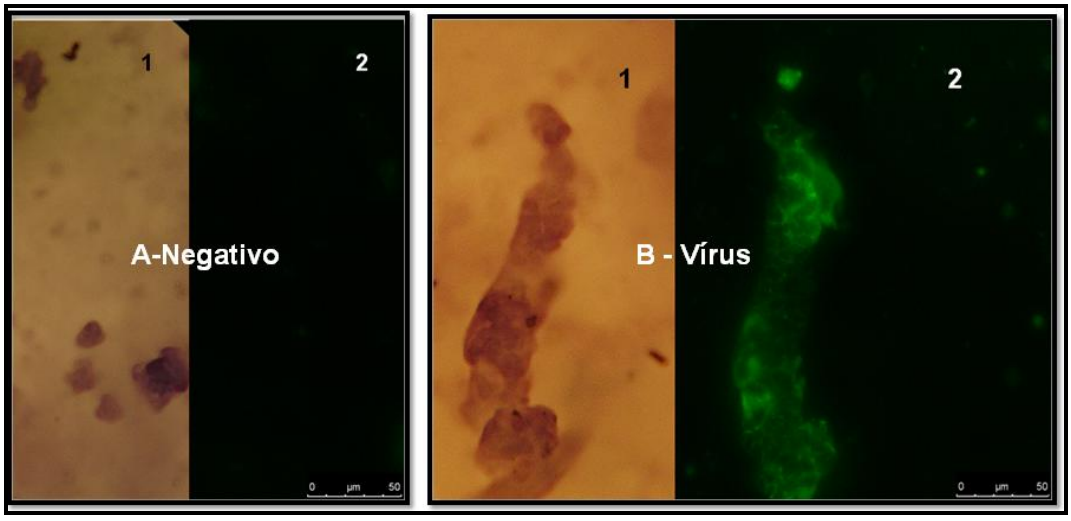

Figura 3. Análise de imunofluorescência indireta (IFI) de *L. vannamei* SPF infectado experimentalmente para IMNV usando o anticorpo monoclonal anti-IMNV, onde: A, tecido oriundo de *L. vannamei* SPF e B, *L. vannamei* SPF infectado experimentalmente para IMNV (1, microscopia óptica e 2, microscopia confocal).

Nenhuma das dez passagens das células C6/36 e SF9 inoculadas com IMNV foram positivas via IFI, indicando que os resultados positivos obtidos por PCR para as 5

passagens de C6/36 e para a inoculação de SF9 (passagem 0) não eram produto de replicação viral, mas vírus residual do inóculo.

De acordo com Sriton et al. (2009) é surpreendente que as transmissões de WSSV e YHD tenham ocorrido tão prontamente nas linhas de célula C6/36 e SF9, uma vez que estes vírus não são capazes de infectar estas células de inseto naturalmente, sendo improvável, portanto, que estas células possuam receptores específicos para estes vírus.

A membrana celular é a principal barreira de proteção dos organismos vivos para evitar a entrada de patógenos e, sendo os vírus, parasitas intracelulares obrigatórios, faz-se necessário encontrar os meios para atravessá-la (Huang et al., 2013). Para a maioria dos vírus, a fase inicial do processo de entrada é a ligação de uma proteína de adsorção viral a um receptor generalizado, seguida da interação com um receptor celular específico do hospedeiro. Estes receptores generalizados, mais comumente conhecidos como fatores de adsorção, concentram as partículas virais sob a célula do hospedeiro e criam condições favoráveis a ligação do receptor (Kalia e Jameel, 2011).

Receptores celulares variam de um vírus para o outro e são em muitos casos célula-específicos. Os vírus de uma mesma família podem ter seletividade para diferentes receptores, enquanto que os vírus de diferentes famílias podem usar a mesma proteína que o seu receptor celular (Kalia e Jameel, 2011).

No caso de vírus não envelopados, a ausência de membrana lipídica impede a adoção de fusão da membrana como mecanismo para entrar nas células. Embora os mecanismos de adsorção e penetração de vírus não envelopados ainda sejam pouco conhecidos, geralmente envolvem uma proteína do capsídeo (capsídeo-dependente) ou proteínas que medeiam a penetração da membrana (Chandran et al., 2002). Para o IMNV, a presença de protrusões na superfície do capsídeo pode estar envolvida aos processos de ligação ao receptor e a penetração da membrana (Tang et al., 2007).

No presente estudo, a ausência de células C6/36 e Sf9 imunopositivas via IFI sugere a falta de um receptor específico destas células para o IMNV, inviabilizando o uso deste sistema *in vitro* para a propagação do vírus como consequência da ausência de replicação viral.

4. Conclusões

No presente estudo, o IMNV não foi capaz de infectar as células de mosquito (C6/36) e de lepidópteros (SF9) devido, provavelmente, a ausência de receptores específicos destas células. Por outro lado, embora estes resultados pareçam desanimadores, eles apontam para a necessidade de outros estudos objetivando a identificação de proteínas presentes nos receptores destas células capazes de inviabilizar a adsorção do IMNV, o que poderá ser favorável ao controle de infecções por este vírus.

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Material and methods

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Results should be clear and concise.

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If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.;

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Acknowledgements

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Nomenclature and units.

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Virus nomenclature.

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M.A.,Maniloff, J., Desselberger, U., and Ball, L.A.(2005) Virus Taxonomy, Classification and Nomenclature of Viruses. Eighth ICTV Report, Academic Press, an imprint of Elsevier. This volume also includes standard abbreviations for species. Once formal taxonomic names have been given in a paper, vernacular terms may be used.

Formal taxonomic nomenclature

In formal taxonomic usage, the first letters of virus order, family, subfamily, genus and species names are capitalized and the terms are printed in italics. Other words in the species names are not capitalized unless they are proper nouns or parts of nouns, for example *West Nile virus*. In formal usage, the name of the taxon should precede the term for the taxonomic unit; for example; "the family *Paramyxoviridae*," "the genus *Morbillivirus*." The following represent examples of full formal taxonomic terminology:

Order Mononegavirales, family Rhabdoviridae, genus Lyssavirus, species Rabies virus.

Family Poxviridae, subfamily Chordopoxvirinae, genus Orthopoxvirus, species Vaccinia virus.

Family Picornaviridae, genus Enterovirus, species Poliovirus.

Family Bunyaviridae, genus Tospovirus, species Tomato spotted wilt virus.

Vernacular Taxonmic Nomenclature

In formal vernacular usage, virus order, family, subfamily, genus and species names are written in lower case Roman script: they are not capitalized, nor are they printed in italics or underlined. In informal usage, the name of the taxon should not include the formal suffix, and the name of the taxon should follow the term for the taxonomic unit; for example "the picornavirus family, the enterovirus genus." One particular source of ambiguity in vernacular nomenclature lies in the common use of the same root terms in formal family, genus or species names. Imprecision stems from not being able to easily identify in vernacular usage which hierarchical level is being cited. For example, the vernacular name "*paramyxovirus*" might refer to the family *Paramyxoviridae*, or one species in the genus *Respirovirus*, such as *Human parainfluenza virus* 1. The solution in vernacular usage is to avoid "jumping" hierarchical levels and to add taxon identification wherever needed. For example, when citing the taxonomic placement of *Human parainfluenza virus* 1, taxon identification should always be added: *Human Parainfluenza virus* 1 is a species in the genus *Respirovirus*, family *Paramyxoviridae*. In this example, as is usually the case, adding the information that this virus is also a member of the subfamily *Paramyxovirinae* and the order *Mononegavirales* is unnecessary.

It should be stressed that italics and capital initial letters need be used only if the species name refers to the taxonomic category. When the name refers to viral objects such as virions present in a preparation or seen in an electron micro

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Abstract:
This should provide a concise description of the purpose of the Protocol and should not exceed 200 words.

Keywords:
Please provide 3 - 6 keywords.

Type of research:

In this section, relevant published studies should be described concisely in list form preceded by Roman lower case numeral characters. The published studies should be appropriately cited.

Time required.

An estimation of the time required to run the protocol should be given per separate step and for the whole protocol.

M*aterials*
The materials used should be described in sufficient detail for the protocol to be replicated. Animals used should include information on breed, breeder, sex, age, weight and the maintenance conditions. Furthermore, this section should be divided into two subsections: (i) Special equipment and (ii) Chemicals and reagents. Any special equipment required should be mentioned, including details of model type/number and (international) supplier. The source or supplier of any special equipment should also be stated, in parentheses, after mentioning the equipment for the first time. A listing (preceded by dashes) of chemicals and reagents used in the protocol, should be provided, if applicable. Special chemicals and drugs with their sources or suppliers should be grouped under a separate subheading ("Chemicals" or "Drugs"). For drugs, generic names should be used; trade names may be given in brackets where the drug is first mentioned. In case of new drugs or chemicals, a full chemical description (formula) should be given. The form of the drug used should be indicated.

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4.3 - Artigo científico III

Transmissão vertical do vírus da Mionecrose infecciosa (IMNV) no camarão marinho *Litopenaeus vannamei*

Artigo científico a ser encaminhado a Revista - Aquaculture.

Todas as normas de redação e citação, deste capítulo, atendem as estabelecidas pela referida revista (em anexo).

Transmissão vertical do vírus da Mionecrose infecciosa (IMNV) no camarão marinho *Litopenaeus vannamei*

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ABSTRACT

In cultured *Litopenaeus vannamei* shrimp, the Infectious myonecrosis virus's (IMNV) infection is characterized by 70% cumulative mortality during grow-out cycle. Although the horizontal transmission of IMNV has been demonstrated, vertical transmission remains unknown, which may be a crucial factor for the prevention of IMNV from one shrimp generation to the next. Hence, we determined the possibility of vertical transmission of IMNV from *L. vannamei* broodstock inoculated by intramuscular procedure or naturally infected with the use of molecular methods (real-time PCR and conventional PCR) in spermatophore, ovary and egg. Moreover, the effect of IMNV infection on sperm viability was also evaluated. Conventional PCR detected IMNV in spermatophores, mature ovary and muscle tissues from experimentally infected SPF broodstock. All samples (spermatophores, mature ovary, muscle and eggs - fertilized or not) from not SPF broodstock naturally and experimentally infected were found to support IMNV replication by real-time PCR. There was no significant difference in viral load of the reproductive organs/tissues between naturally or experimentally infected shrimp groups (for both sexes) ($p \geq 0.05$). However, the mean viral load in the muscle for all animals (males and females) was statistically different from the other tissues, with viral load values for naturally and experimentally muscles dissimilar from each other for both sexes ($p \leq 0.05$). As for spawning, all infected females spawned and there were no significant differences in the viral load of eggs produced by naturally and experimentally infected females for IMNV. In addition, eggs from all females were classified in two morphological types (type B and A_2) based on morphology and post-spawning development. Sperm viability of experimentally infected males was not determined due to lower cell density, whereas for naturally infected males, the percentage of viable sperm showed an average viability of 82%. In the control group, mean viable sperm was 91%. The results suggested that IMNV can be transmitted vertically in *L. vannamei*. However, nauplii did not hatched out, preventing better understanding of the mechanisms by which eggs become infected following spawning (by egg surface or by transovarial transmission). On the other hand, the association of 100% positive samples from ovaries and eggs and low percentage of viable cells from naturally infected males suggests that female appears to be the primary source of IMNV associated with eggs in the vertical transmission.

Keywords: *Litopenaeus vannamei*; IMNV; Real-time PCR; broodstock; sperm cell viability; vertical transmission

1. Introdução

Patógenos de etiologia viral respondem atualmente por 60% das perdas por doenças na carcinicultura marinha mundial (Flegel, 2012). Dentre as estratégias adotadas para a gestão e prevenção de doenças na unidade de produção de camarões marinhos, a redução da prevalência de vírus a partir do controle de reprodutores tem sido a mais eficaz (Brock e Bullis, 2001).

Dos seis agentes infecciosos virais (Necrose hipodermal e hematopoiética infecciosa - IHHNV, Mionecrose infecciosa - IMNV, Síndrome de Taura - TSV, Mancha branca - WSSV, Doença da cauda branca - WTD e da Cabeça amarela - YHD) que infectam crustáceos presentes na lista de notificação obrigatória da Organização Mundial da Saúde Animal (OIE) em 2012 (OIE, 2012a), três (IHHNV, WSSV e WTD) têm a transmissão vertical comprovada associada à alta prevalência em populações selvagens ou domesticadas de diferentes espécies de peneídeos (*Penaeus stylirostris*, *Litopenaeus vannamei* e *Penaeus monodon*) e carídeos (*Macrobrachium rosenbergii*) (Morales-Covarrubias et al., 1999; Motte et al., 2003; Withyachumnarnkul et al., 2006; Lo et al., 1997; Hsu et al., 1999; Tsai et al., 1999; Peng et al., 2001; La Peña et al., 2007; Sudhakaran et al., 2007).

Prevalências superiores a 60% de WSSV em fêmeas foram determinadas em reprodutores selvagens de *P. monodon* capturados em Taiwan, com comprovação de transmissão vertical baseada na detecção positiva via hibridização *in situ* (ISH) do vírus em ovários (células do folículo e oogônias) e espermatóforos, embora óvulos maduros infectados não tenham sido observados, sugerindo liberação do vírus no momento da desova (Lo et al., 1997; Hsu et al., 1999).

Já para o IHHNV, a prevalência e seu impacto no desempenho reprodutivo dependem da espécie envolvida. Em *P. stylirostris* e *P. monodon*, apesar das prevalências médias de 70% em fêmeas tenham sido observadas, nenhuma sintomatologia ou efeito negativo foi notado em termos de número de ovos e náuplios produzidos por desova (Morales-Covarrubias et al., 1999; Withyachumnarnkul et al., 2006). Por outro lado, a alta prevalência de IHHNV em reprodutores domesticados de *L. vannamei* (85% em ovários) pode resultar em uma queda de 25% na produção de náuplios quando fêmeas infectadas são comparadas a negativas, com evidente elevação de crescimento heterogêneo em náuplios originários dessas fêmeas (Motte et al., 2003).

Quanto à doença da cauda branca, a WTD, causada pelos vírus *Macrobrachium rosenbergii* nodavirus (MrNV) e extra small (XSV), a transmissão vertical foi confirmada via desafio viral de reprodutores a pós-larvas, com confirmação de infecção por estes vírus em amostras de tecido do ovário, ovos fertilizados e estágios larvais por meio de PCR e nested-PCR (Sudhakaran et al., 2007).

Com relação ao IMNV, embora estudos sobre a prevalência em reprodutores de *L. vannamei* ainda não se encontrem disponíveis, Pinheiro et al. (2007) em um estudo epidemiológico realizado no estado de Pernambuco (nordeste brasileiro), revelaram que o vírus da IMN esteve presente em 81% das fazendas analisadas quando animais com peso médio de 5 g foram amostrados.

No Brasil, a doença da Mionecrose infecciosa, causada pelo vírus da Mionecrose infecciosa (IMNV), foi apontada no aspecto sanitário, como o principal fator para a perda de 40% da produção do monocultivo de *L. vannamei* na região nordeste em 2003 (Nunes et al., 2011), com estimativas recentes apontando para prejuízos acima de US$ 100 milhões de dólares para o período entre 2002 e 2006 (Lightner, 2011). Como o nome do vírus sugere, esta doença caracteriza-se pela necrose do músculo estriado do abdômen e do cefalotórax, com liquefação dos músculos fibróticos necrosados na fase aguda da infecção, o que confere uma coloração avermelhada às áreas afetadas nos animais infectados (Nunes et al., 2004).

Embora a comprovação de camarões sobreviventes capazes de serem portadores de infecção ao longo da vida até se tornarem reprodutores não tenha sido demonstrada cientificamente, acredita-se que este vírus possa ser transmitido verticalmente à progênie (OIE, 2012b). Assim, o presente estudo tem por objetivo determinar a possibilidade de transmissão vertical do IMNV em *L. vannamei* naturalmente e experimentalmente infectados através de PCR convencional e PCR em tempo real. A avaliação da viabilidade espermática de machos naturalmente e experimentalmente infectados na fase aguda da infecção por IMNV também foi verificada.

2. Material e métodos

2.1 Inóculo viral

A preparação do inóculo viral foi realizada conforme descrito por Silva et al. (descrito no 1° artigo desta tese). Em resumo, tecido do músculo abdominal de *L.*

vannamei (peso médio de 11,6 g) foi coletado durante um surto natural de IMNV e triado para a presença deste vírus por PCR (Poulos e Ligthner, 2006) e ausência de outros patógenos de camarões peneídeos (TSV, WSSV, vírus da Necrose hipodermal e hematopoiética infecciosa - IHHNV e Hepatopancreatite necrosante - NHPB) via kits da IQ2000^{TM} (Farming IntelliGene Tech. Corp., Taiwan). O tecido foi homogeneizado, centrifugado e filtrado em membrana polietersulfonica (PES) de 0,22µm (TPP, Switzerland). A carga viral do homogeneizado foi quantificada via PCR em tempo real (Silva et al., 2011), seguido de diluição serial em solução salina estéril a 2%. A diluição de 1:10^{7} foi armazenada a -80°C até à sua utilização em desafios via injeção intramuscular a 28°C, conforme recomendações de Silva et al. (descrito no 1° artigo desta tese).

2.2 Sistema de recirculação fechado

Todos os animais foram mantidos em um sistema de recirculação fechado (Closed Recirculation Systems - CRS) projetado segundo González-González et al. (2009), com algumas modificações. Neste sistema, a unidade de maturação era composta por três tanques de fibra de vidro preto e circular de 2 m de diâmetro e volume útil de 2200 L, 1 m de altura e um dreno central de 50 mm. Em cada tanque foi instalado um filtro mecânico-biológico de 300 L, dois skimmers de 1500 L, quatro pontos de aeração contínua, um esterilizador UV de 36 W (JEBO, China), quatro airlifts verticais de 70 cm e um *cooler* para manter a temperatura da água a 28°C. Três lâmpadas fluorescentes de 40 W e uma lâmpada incandescente de 100 W foram instaladas 1,2 m acima da superfície da água de cada tanque com um timer digital temporizador para controle de fotoperíodo de 13hL (horas de luz): 11hD (horas sem luz) de acordo com Chamberlain e Lawrence (1981). Todos os tanques foram individualmente cobertos com telas de sombreamento pretas de 70% de bloqueio solar para controle da intensidade luminosa e fotoperíodo. Antes de entrar no sistema, toda água era desinfetada com cloro a 5 mgL^{-1} e durante todo o experimento foi mantida a salinidade de 31 gL^{-1}, temperatura de 28±1°C e pH 8,0±0,4, com taxas de renovação diária de 5%.

Quanto à unidade de desova, três tanques de fibra de vidro preto e circular (1,17 m de diâmetro e 80 cm de altura) e duas caixas circulares de polietileno de média densidade (PEMD) (81 cm de diâmetro e 53 cm de altura) de 300L foram utilizados

para a coleta individual de desovas. Neste sistema a água foi mantida sob aeração leve e contínua, sem circulação, com salinidade de 30 gL^{-1} e temperatura de 28°C, sendo adicionados EDTA a uma concentração final de 10 mgL^{-1} como medida profilática.

Os animais foram alimentados durante todo o experimento com lulas e mexilhões congelados e dieta comercial própria para reprodutores (Breed S Fresh, INVE Aquaculture, Bélgica), nas proporções de 60, 30 e 10%, respectivamente, quatro vezes ao dia a 20% da biomassa.

Durante todo o experimento, temperatura e pH foram monitoradas duas vezes ao dia, e amostras de água foram coletadas semanalmente para análise de nitrito, nitrato, nitrogênio amoniacal e alcalinidade por meio de kit colorimétrico comercial (Alcon Labcon, Camboriú, Brasil) para verificação destes parâmetros dentro dos limites ideais para peneídeos propostos por Van Wyk e Scarpa (1999).

2.3 Reprodutores SPF e desafio viral

Para a determinação da rota de transmissão vertical do IMNV foram usados reprodutores livres de patógeno específico (Specific Pathogen Free - SPF) de *L. vannamei* provenientes de tanques de maturação da empresa Genearch Aquacultura Ltda (larvicultura comercial brasileira) localizada em Rio do Fogo (RN). Fêmeas (66,27 g) e machos (42,8 g) foram mantidos em um sistema de recirculação fechado (CRS) e distribuídos em tanques de acordo com a presença ou ausência de inoculação para a obtenção de animais experimentalmente infectados.

Desafios virais por injeção intramuscular em um dos reprodutores foram realizados para a identificação da origem do parental envolvido na transmissão vertical. Desta forma, os animais foram distribuídos em três tanques: (1) fêmeas inoculadas com machos negativos (via parental materno); (2) machos inoculados com fêmeas negativas (via parental paterno) e; (3) fêmeas e machos não inoculados (grupo controle).

Para o primeiro tanque, durante o período de intermuda, nove fêmeas unilateralmente abladas de *L. vannamei* foram inoculadas por via intramuscular (300 µL de inóculo diluído a $1:10^7$ por animal) no terceiro segmento abdominal. Após sete dias da inoculação (curso de tempo mínimo necessário para que o IMNV atinja células susceptíveis e inicie um processo de infecção - Silva et al., descrito no 1° artigo desta tese), as fêmeas foram monitoradas diariamente para o desenvolvimento do ovário e as

que apresentavam ovários maduros (Palacios et al., 1999) foram inseminadas artificialmente (Misamore e Browdy, 1997) com espermatóforos de machos negativos (n=20), sendo transferidas para tanques individuais de fibra de vidro (300 L) a 28°C.

Já para a determinação da rota via parental paterno (segundo tanque), 15 machos de *L. vannamei* foram desafiados experimentalmente sob as mesmas condições descritas para as fêmeas. Sete dias após a inoculação, os indivíduos que apresentavam espermatóforos completamente desenvolvidos (Chamberlain et al., 1983) eram selecionados e os espermatóforos retirados, via compressão da região latero-ventral posterior à inserção do quinto par de pereiópodos, inseminados em fêmeas negativas abladas (n=10). Estes desafios contaram ainda com um grupo controle (terceiro tanque) composto por 10 machos e 10 fêmeas, nos quais foram injetados 300 µL de solução salina estéril a 2%.

Entretanto, o longo intervalo de tempo entre a ablação do pedúnculo ocular e primeira desova pós-inoculação (120 dias), atrelado ao surgimento dos primeiros sinais clínicos de infecção por IMNV (10 dias pós-injeção), inviabilizaram a obtenção de desovas. Apenas uma única desova fertilizada com obtenção de náuplios foi obtida proveniente do tanque controle. Assim, todos os animais (desafiados e do grupo controle) foram sacrificados e armazenados a -80°C para confirmação da infecção por IMNV via PCR convencional nos tecidos de músculo, ovário e espermatóforos.

2.4 Reprodutores naturalmente infectados e desafio viral

Visando a obtenção de desovas que pudessem elucidar a rota de transmissão vertical do IMNV, 300 camarões *L. vannamei* (150 fêmeas e 150 machos com peso médio de 28,5 g) foram coletados de viveiros com histórico recorrente de infecção IMNV em uma fazenda de engorda comercial localizada no município de Ceará-Mirim (Rio Grande do Norte, Brasil) com salinidade de 1,5 gL^{-1}.

Estes animais foram transportados para o sistema de recirculação fechado e aclimatados para a salinidade de 31 gL^{-1} através do aumento gradual da salinidade a taxa de 3 gL^{-1} para os três primeiros dias, seguidos de 5 gL^{-1} para os últimos quatro dias. Este processo de aclimatação foi executado em uma semana e resultou na morte de 61 animais (20% de mortalidade).

Após 48 horas a 31 gL^{-1}, as fêmeas restantes foram abladas unilateralmente através de esmagamento do pendúnculo ocular com pinça incandescente e inoculadas via intramuscular (300 µL de inóculo diluído a $1:10^7$ por animal) no terceiro segmento abdominal. Do mesmo modo, 30 machos também foram inoculados experimentalmente.

As fêmeas submetidas à ablação foram observadas ao longo de 10 dias e as que não desenvolveram completamente os ovários, foram descartadas. Desta forma, foram estocados 60 animais por tanque de maturação à proporção sexual de 1:1, sendo estes camarões distribuídos em três tanques conforme as combinações de inoculação: (1) fêmea não inoculada com macho inoculado; (2) fêmea inoculada com macho não inoculado e, (3) fêmea e macho não inoculados. A escolha de fêmeas e machos maduros, bem como as técnicas de extrusão e de inseminação foram conduzidas da mesma forma descrita para os animais SPF. Após as desovas, as fêmeas eram devolvidas ao tanque de origem e os óvulos, fertilizados ou não, eram coletados e armazenados a -80°C para detecção de IMNV via PCR em tempo real.

Objetivando reduzir as condições de estresse que poderiam comprometer a desova e inviabilizar os resultados, nenhuma análise de PCR foi conduzida nestes animais até o fim do experimento. Assim, todos os animais foram sacrificados ao final do experimento (45 dias), identificados de acordo com o tanque e analisados por PCR em tempo real (músculo, ovário e espermatóforo) para a determinação de infecção natural por IMNV e para a definição das reais combinações existentes em cada um dos tanques. Todas as amostras foram armazenadas a -80°C até a sua análise molecular.

2.5 Análises moleculares

2.5.1 Extração de RNA e RT-PCR

A extração do RNA total dos tecidos de todos os camarões (músculo, ovários, espermatóforos e pools de 150 óvulos /fêmea), foi feita conforme o protocolo de Chomezynski e Sacchi (1987), com algumas modificações. Nesta metodologia, o tecido (50 mg) foi macerado individualmente em nitrogênio líquido. Posteriormente, a este macerado foi acrescentado 1 mL de Trizol (Invitrogen) para a digestão. O RNA foi precipitado com álcool isopropílico e ressuspendido em água com DEPC (Dietilpirocarbonato), sendo armazenado a –80°C até a sua utilização. A concentração e a qualidade do RNA extraído foram verificados por meio de espectrofotometria a 260

e 280 ηm via espectrofotômetro (NanoVue Plus™, GE Healthcare, EUA). Em seguida, 300 $\eta g \mu L^{-1}$ de RNA total e 0,5µg de oligo$(dT)_{15}$ foram usados para sinetizar o cDNA através do kit Improm-II™ Reverse Transcription System (Promega, Madison, WI, USA), conforme as instruções do fabricante. O cDNA foi armazenado a -20°C até posterior utilização nas análises de PCR ou de PCR em tempo real.

2.5.2 PCR

As amostras de cDNA provenientes dos tecidos dos reprodutores SPF (músculo, ovários e espermatóforos) foram submetidos a PCR para confirmação de infecção por IMNV usando os primers específicos descritos por Poulos e Lightner (2006). As reações de PCR foram realizadas em um volume final de 25 µL contendo: 2 µL de cDNA, 1U de *Taq* polimerase, 200 µM de cada dNTP, 1,5 mM de $MgCl_2$ e 5 ρmol de cada primer específico. As condições térmicas de amplificação foram as mesmas descritas por Pinheiro et al. (2007) e os produtos de PCR (tamanho esperado de 328 pb) foram submetidos a eletroforese em gel de agarose a 2% corado com brometo de etídio. A cada reação foi adicionado um controle positivo para o vírus da Mionecrose infecciosa e um controle negativo, constituído de água ultra-pura como amostra.

2.5.3 PCR em tempo real

Análises de PCR em tempo real para IMNV foram realizadas conforme descrito por Silva et al. (2011). Nestas análises, cDNA das amostras de pools de 150 ovos (fertilizados ou não)/fêmea, de tecido muscular, de ovários e de espermatóforos de todos os animais oriundos da fazenda de engorda (não SPF) tiveram sua carga viral média de IMNV determinada e a taxa de infecção natural definida.

Em cada placa de 96 poços foi adicionado: uma diluição serial (1:10^2 a 1:10^8) do plasmídeo recombinante-padrão para IMNV; duas réplicas técnicas de cada amostra; dois controles negativos (água ultra-pura e amostra de controle positivo para o vírus da Taura) e um controle interno de β-actina. As condições de reação e análise dos dados foram efetuadas segundo Silva et al. (2011) em um termociclador StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, EUA).

2.6 Avaliação da viabilidade espermática durante a fase aguda da infecção

A fim de determinar o efeito da fase aguda da infecção por IMNV sobre a viabilidade espermática, cinco camarões não SPF de cada tanque que apresentavam espermatóforos completamente desenvolvidos e sinais clínicos da fase aguda (opacidade multifocal no músculo abdominal) foram selecionados e o par de espermatóforos retirados por meio de extrusão manual.

A viabilidade espermática foi efetuada por meio do método de coloração eosina-nigrosina, conforme Jeyalectumie e Subramoniam (1989), Nimrat et al. (2005) e Vuthiphandchai et al. (2007). Após a remoção do par de espermatóforos, a massa espermática do par foi transferida para um tubo contendo 1 mL de solução salina livre de cálcio (21,63 g de NaCl; 1,12 g de KCl; 0,53 g de H_3BO_3; 0,19 g de NaOH; 4,93 g de $MgSO_4.7H_2O$; pH 7,4) e homogeneizada até obter uma suspensão de células. Em seguida, uma alíquota de 50 µL da suspensão celular foi adicionada a 50 µL de uma solução contendo eosina a 0,5% e nigrosina a 10% na proporção de 1:1 (v:v) e misturada sobre uma lâmina, com posterior confecção de esfregaço das células coradas. A amostra foi seca a temperatura ambiente e a viabilidade espermática avaliada sob microscópio óptico. No caso de alta densidade celular (mais de 100 células visualizadas por campo), no mínimo, 100 espermatozoides da lâmina foram avaliados para determinação do percentual de células viáveis, entretanto, se baixa densidade fosse detectada, o percentual era baseado na leitura de toda a lâmina (10x).

2.7 Análises estatísticas

A análise dos dados e a determinação do número de cópias virais das amostras foram feitas através do software StepOne™ (versão 2.2.2) (Applied Biosystems, CA, EUA). Os dados de quantificação viral dos diferentes tecidos foram submetidos ao teste de Bartlett ao nível de 95% de probabilidade ($P \leq 0,05$), para a verificação da homogeneidade. Em seguida, o teste de Kruskal-Wallis foi usado para comparar a carga viral média obtida nos diferentes tecidos (músculo, ovário e espermatóforo) e amostras de desovas coletados. As análises estatísticas foram realizadas com o auxílio do software ASSISTAT versão 7.7 beta (Silva, 2014).

3. Resultados e discussão

Todas as amostras de músculo, ovários e espermatóforos originárias de camarões SPF experimentalmente inoculados foram positivas para a infecção por IMNV via PCR convencional (Figura 1), não sendo detectado o vírus nas amostras de animais não inoculados ou no grupo controle para os mesmos tecidos. Do mesmo modo, sinais clínicos de infecção por IMNV, tais como: opacidade multifocal nos segmentos abdominais e na cauda e lesões necrosadas com coloração avermelhada foram observadas nos animais desfiados. No grupo controle, nenhum sinal de infecção foi notado até o final do experimento (120 dias).

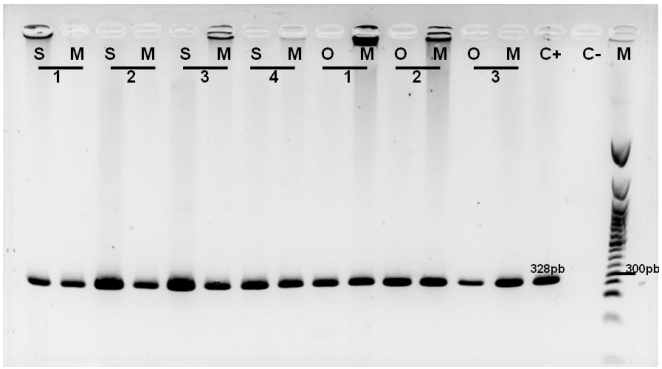

Figura 1. Detecção de IMNV por PCR em diferentes tecidos de reprodutores SPF de *L. vannamei* experimentalmente infectados. As quatro primeiras amostras (1, 2, 3 e 4) correspondem aos machos SPF infectados experimentalmente, enquanto as três últimas (1, 2 e 3), correspondem às fêmeas SPF, onde: S é espermatóforo; M, músculo; O, ovários; C+, controle positivo; M, marcador de peso molecular de 50pb (New England Biolabs, EUA) e C-, controle negativo (água ultra-pura).

Segundo Nunes et al. (2004), a sintomatologia do IMNV pode indicar o grau de severidade da infecção e apresenta-se em três graus: leve, com a presença de pequenos focos de opacidade no músculo abdominal e nos apêndices; moderada, com a expansão da opacidade e perda da transparência muscular e; grave, com a presença de áreas necrosadas e apodrecimento dos segmentos abdominais e leque caudal. No presente estudo, os sinais clínicos observados nos reprodutores SPF indicaram graus de infecção por IMNV de moderado a grave.

Com relação aos animais (não SPF) vindos de viveiros de engorda com histórico de infecção por IMNV nos últimos ciclos, dos 180 animais estocados, 157 foram mantidos até o fim do experimento e destes, 97 não foram submetidos à inoculação, permitindo um levantamento preliminar sobre a percentagem de infectados naturalmente. Assim, quando apenas amostras de músculo foram analisadas via PCR em tempo real, dos 50 machos não inoculados, 60% foram positivos para IMNV, enquanto que das 47 fêmeas não inoculadas, 100% foram positivas. Entretanto, quando as amostras de ovários e espermatóforos foram avaliadas, apenas 44% dos espermatóforos foram positivos versus 100% dos ovários, evidenciando uma maior probabilidade da frequência de fêmeas infectadas em unidades de cultivo.

É importante salientar, ainda, que nenhum adulto coletado e não inoculado mostrou sinal clínico da doença. No segundo experimento, nenhum dos animais (machos e fêmeas) inoculados apresentou sinais clínicos de infecção moderada e grave como observados nos reprodutores SPF, mas um comportamento letárgico após 15 dias de inoculação e discreta opacidade muscular associada à quase total suspensão do consumo alimentar. Por esta razão, transcorridos 20 dias após a inoculação, optou-se por uma renovação de 33% do plantel de animais inoculados, com a substituição de 10 machos no tanque 1 e de 10 fêmeas, no tanque 2. Todos os animais adicionados foram retirados do tanque 3, restando ao final do experimento, 37 animais (20 machos e 17 fêmeas) neste tanque e, no experimento total, 157 animais.

A alta prevalência de infecção por vírus em populações domesticadas de *L. vannamei* tem sido relatada por Motte et al. (2003). Esses autores relataram a prevalência de IHHNV em adultos de *L. vannamei* cultivados em duas gerações em larviculturas no Equador e no Panamá através de nested-PCR de amostras de hemolinfa. No Equador, os camarões de origem colombiana apresentaram prevalência de IHHNV de 50%, sem diferença entre machos e fêmeas, enquanto que nos de origem panamenha, a prevalência foi de 50% para os machos e 63% para as fêmeas. Já no Panamá (de origem panamenha), a prevalência foi estimada em 94% para as fêmeas e 95% para os machos. Ainda neste estudo, ao se analisar a prevalência de todas as fêmeas para todas as origens, antes e depois da primeira desova, um aumento de 12% de fêmeas infectadas foi detectado.

Lo et al. (1997) e Hsu et al. (1999) registraram que maiores severidade e detecção de infecção por WSSV (detecção na 1ª PCR) foram observadas em reprodutores selvagens de *P. monodon* capturados no sul de Taiwan após a desova, indicando que o estresse de desova pode desencadear a replicação viral. Similarmente, La Peña et al. (2007) ao investigar a prevalência de WSSV entre fêmeas e machos selvagens de *P. monodon* nas Filipinas observaram uma variação sazonal significativa, com maior prevalência para ambos os sexos no verão o que coincide com o período de atividade de desova natural. De acordo com Hsu et al. (1999), para aumentar o número de ovos com baixa prevalência de WSSV a recomendação é efetuar uma triagem de reprodutores após a primeira desova.

Tsai et al. (1999) e Peng et al. (2001) sugerem ainda que a adoção da estratégia de povoamento baseada em náuplios provindos de fêmeas com prevalência de WSSV inferior a 50% pós-desova pode resultar em ciclos produtivos de engorda de *P. monodon* com baixa incidência de surtos quando condições favoráveis de cultivo (baixa densidade e baixo estresse ambiental) são praticadas. No presente estudo, todas as análises de PCR em tempo real foram conduzidas ao final do experimento, possibilitando uma triagem de fêmeas pós-primeira desova.

Com relação à detecção de IMNV via PCR em tempo real de todos os animais usados no segundo experimento, para as amostras de músculo, 100% das fêmeas e 75% dos machos foram positivos, ao passo que para os tecidos reprodutivos, 96,1% dos ovários e 57,5% dos espermatóforos foram positivos. Os resultados do número de infectados por tanque no segundo experimento e por tipo de tecido coletado (músculo, ovário e espermatóforo) encontram-se sumarizados na Tabela 1. Com base nestes resultados, as reais combinações de cruzamento obtidas nos três tanques foram: (1) fêmea naturalmente infectada e macho inoculado; (2) fêmea inoculada e macho naturalmente infectado e; (3) fêmea naturalmente infectada e macho negativo (Tabela 1).

Quanto à quantificação da carga viral média de IMNV, não houve diferença significativa entre as amostras de tecido de ovário e espermatóforo analisados para animais (machos e fêmeas) naturalmente ou experimentalmente infectados ($p \geq 0,05$). No entanto, a carga viral média encontrada no músculo para todos os animais (machos e

fêmeas) diferiu estatisticamente dos demais tecidos, sendo os valores de músculo experimentalmente e naturalmente diferentes entre si ($p \leq 0,05$) (Tabela 1).

O IMNV infecta preferencialmente tecidos de origem mesodérmica, embora durante a fase aguda os principais tecidos infectados sejam os músculos estriados (principalmente o músculo esquelético), os tecidos conjuntivos, os hemócitos, e as células do órgão linfóide, podendo este último tornar-se o principal tecido-alvo na fase crônica da infecção (OIE, 2012b). Neste estudo, amostras de músculo tiveram carga viral média significativamente maior que os demais tecidos.

Além disso, em ambos os sexos experimentalmente infectados, os níveis de carga viral média foram similares àqueles obtidos na quantificação de músculo abdominal de animais oriundos de infecções naturais deste vírus (entre $6,85x10^8$ e $3,09x10^4$ cópias μg^{-1} de RNA total) (Silva et al., 2011).

Tabela 1. Número de infectados e carga viral média (cópias μg^{-1} de RNA total) dos reprodutores não SPF para os diferentes tecidos coletados

Tanque	Sexo	Tecido	Infecção	Nº Analisado	PCR Positiva	Carga viral		
						Mínimo	Média	Máximo
1	Macho	Espermatóforo	Experimental	30	24	$1,21x10^3$	$3,61x10^{3a}$	$8,10x10^3$
		Músculo	Experimental	30	30	$1,16x10^3$	$1,96x10^{6b}$	$5,31x10^7$
	Fêmea	Ovário	Natural	30	30	$6,39x10^2$	$2,74x10^{3a}$	$6,91x10^3$
		Músculo	Natural	30	30	$4,07x10^2$	$2,82x10^{3c}$	$6,16x10^3$
2	Macho	Espermatóforo	Natural	30	22	$1,09x10^3$	$4,65x10^{3a}$	$9,82x10^3$
		Músculo	Natural	30	29	$1,06x10^3$	$4,92x10^{3c}$	$7,67x10^3$
	Fêmea	Ovário	Experimental	30	27	$2,29x10^3$	$7,00x10^{3a}$	$1,53x10^4$
		Músculo	Experimental	30	30	$2,87x10^3$	$1,85x10^{6b}$	$4,97x10^7$
3	Macho	Espermatóforo	-	20	0	-	-	-
		Músculo	-	20	1	$2,05x10^3$	$2,05x10^3$	$2,05x10^3$
	Fêmea	Ovário	Natural	17	17	$3,41x10^2$	$2,22x10^{3a}$	$3,67x10^3$
		Músculo	Natural	17	17	$1,13x10^3$	$1,99x10^{3c}$	$3,20x10^3$

[a,b,c] - Médias seguidas de letras iguais não diferem entre si pelo teste de Kruskal-Wallis ($p \leq 0,05$); - Não detectado.

A confirmação da rota de transmissão vertical em camarões tem sido presumida a partir da detecção positiva de vírus via técnica molecular em órgãos reprodutivos (ovários e espermatóforos) e em ovos de adultos maduros infectados.

Resultados positivos via nested-PCR para o vírus *penaeid rod-shaped DNA* (PRDV) em amostras de ovário e testículo procedentes de adultos selvagens de *Penaeus japonicus* durante a estação de maior prevalência (verão) foram apontadas como uma fonte para a transmissão vertical do PRDV (Mushiake et al., 1998).

No caso do IHHNV, a presença do vírus em órgãos reprodutivos tem sido descritas para *P. monodon* (Withyachumnarnkul et al., 2006) e *L. vannamei* (Motte et al., 2003). Reações positivas para IHHNV em ovários e oócitos de *P. monodon* via hibridização *in situ* (ISH) sugerem que este vírus pode ser transmitido verticalmente. Ao mesmo tempo, o fato de que pós-larvas oriundas destes lotes também abrigavam o vírus embasam os resultados obtidos para estes tecidos (Withyachumnarnkul et al., 2006). Em *L. vannamei*, o IHHNV foi detectado em tecidos de ovários, espermatóforos, ovos e estágios pré e pós-larvais (de fêmeas infectadas) triados por PCR, com altíssima frequência em ovários (85%) e rara detecção em espermatóforos (13%), sugerindo uma maior probabilidade de transmissão vertical atrelada ao parental materno.

A comprovação de transmissão vertical também tem sido obtida para WSSV através da presença de células positivas localizadas no espermatóforo e nos ovários (células do folículo e oogônias) em *P. monodon* por meio de análise de microscopia eletrônica e ISH (Lo et al., 1997). Similarmente, Cowley et al. (2002) mostram que a presença do vírus *gill-associated* (GAV) em espermatóforos, ovários maduros, ovos e náuplios via nested-PCR está associada à elevada prevalência de infecções crônicas e a perpetuação deste vírus em larviculturas, quando estes animais são usados como reprodutores, evidenciando a transmissão vertical.

A transmissão vertical dos vírus MrNV e XSV foi reproduzida experimentalmente em *M. rosenbergii*, com a presença destes vírus comprovada via nested-PCR em ovário, ovos fertilizados e larvas derivadas destes lotes infectados (Sudhakaran et al., 2007). No presente estudo, a detecção positiva de IMNV em ovários de fêmeas naturalmente e experimentalmente infectadas e em espermatóforos de machos naturalmente e experimentalmente infectados, demonstra a possibilidade de uma rota de transmissão vertical do IMNV em *L. vannamei*.

Quanto às desovas, todas as fêmeas infectadas (naturalmente ou não) foram capazes de desovar, obtendo-se 13 desovas para fêmeas infectadas naturalmente e quatro, para as experimentalmente, com apenas uma desova fertilizada para ambos os casos. Destas 13 desovas (fêmeas infectadas naturalmente), seis foram de inseminações de machos experimentalmente infectados e, sete, de machos negativos. Para as quatro desovas de fêmeas infectadas experimentalmente, todos os espermatóforos usados na inseminação vieram de machos naturalmente positivos (Tabela 2).

O IMNV foi detectado via PCR em tempo real em 93,4% das amostras de desovas coletadas, apresentando carga viral média por pool coletado variando de $5,84x10^2$ a $5,37x10^3$ cópias de IMNVμg^{-1} de RNA total, não havendo diferença entre a carga viral média obtida para as diferentes combinações de inseminação ($p \geq 0,05$) (Tabela 2).

Embora a PCR em tempo real seja considerada atualmente como método padrão-ouro para o diagnóstico de IMNV (OIE, 2012b), os resultados negativos para pools diferentes da mesma amostra coletada podem estar associados à proximidade do limite de detecção da metodologia. No presente estudo, a metodologia de quantificação foi baseada na técnica descrita por Silva et al. (2011), em que a PCR em tempo real usando SYBR Green foi capaz de detectar até 100 cópias do genoma viral, sugerindo que as amostras negativas de desova no presente trabalho estavam abaixo do limite de detecção.

A detecção positiva de IMNV em ovos (fertilizados ou não) oriundos de fêmeas infectadas (naturalmente e experimentalmente) atrelada à presença deste vírus nos tecidos dos ovários comprova a rota de transmissão vertical deste vírus. Entretanto, os resultados não deixam claro se a propagação do vírus se dá via transmissão transovariana (infecção pelo vírus dentro do ovo) ou na superfície do ovo.

Tabela 2. Combinação de inseminações, número de desovas e carga viral média (cópias de IMNVμg^{-1} de RNA total) dos reprodutores não SPF

Inseminação	Amostras de desovas	Nº Analisado (pools de 150 ovos/fêmea)	PCR Positiva	Carga viral média
♀$_{+ nat}$ + ♂$_{+ exp}$	1	5	4	2,17x10³
	2	2	2	1,68x10³
	3	2	2	3,42x10³
	4	5	5	4,47x10³
	5	4	4	2,23x10³
	6	1	1	1,41x10³
♀$_{+ exp}$ + ♂$_{+ nat}$	1	5	5	2,42x10³
	2	5	4	5,37x10³
	3	5	5	3,24x10³
	4	5	5	2,36x10³
♀$_{+ nat}$ + ♂$_{-}$	1	5	5	3,53x10³
	2	1	1	5,84x10²
	3	4	3	3,97x10³
	4	5	4	4,53x10³
	5	5	5	1,74x10³
	6	1	1	3,29x10³
	7	1	1	1,71x10³

♀$_{+ nat}$ - Fêmea naturalmente infectada por IMNV; ♀$_{+ exp}$ - Fêmea experimentalmente infectada por IMNV; ♂$_{+ exp}$ - Macho experimentalmente infectado por IMNV; ♂$_{+ nat}$ - Macho naturalmente infectado por IMNV e ♂$_{-}$ - Macho não infectado.

Ainda em relação às desovas, ao se analisar os ovos obtidos de todas as fêmeas em relação à morfologia e desenvolvimento pós-desova, dois tipos morfológicos foram identificados: ovos não fertilizados e ovos fertilizados com desenvolvimento anormal. Mais de 97% dos ovos obtidos não foram fertilizados e apresentaram formações citoplasmáticas irregulares, enquanto que cerca de 2% mostraram-se fertilizados,

apresentando náuplio internamente com desenvolvimento retardado e anormal e, não bilateralmente simétrico, seguida da não eclosão (Figura 2).

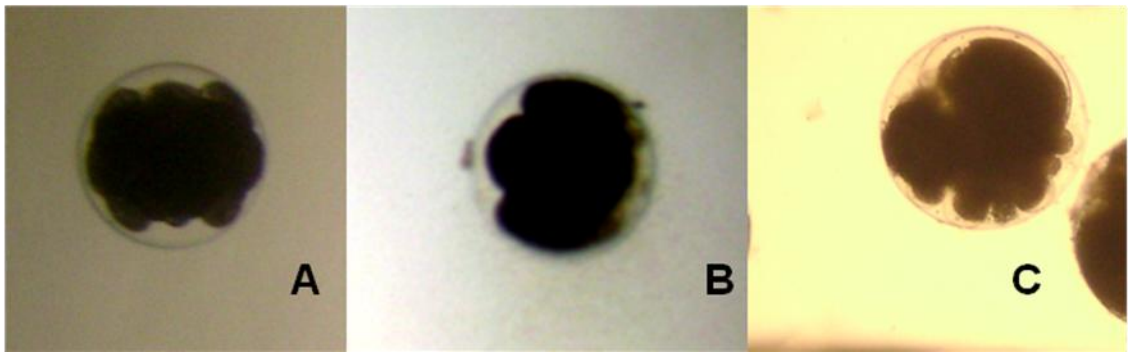

Figura 2. Desenvolvimento de diferentes tipos de ovos de *L. vannamei* observados após 10 horas de desova, onde: A é ovo fertilizado com desenvolvimento normal evidenciado pela simetria bilateral do náuplio interno obtido a partir de reprodutores SPF durante o primeiro experimento; B, ovo fertilizado com desenvolvimento anormal caracterizado por clivagem retardada e assimétrica procedente de reprodutores não SPF e; C, ovo não fertilizado com ausência de clivagem e formações citoplasmáticas irregulares, também provenientes de reprodutores não SPF.

De acordo com Primavera e Posadas (1981), em um estudo conduzido para a identificação da qualidade de ovos em reprodutores de *P. monodon* foram identificados cinco tipos morfológicos de ovos baseado em seu desenvolvimento pós-desova: (A) ovos fertilizados, sendo (A_1) com desenvolvimento normal e (A_2), anormal ou retardado em relação à clivagem; e ovos não fertilizados, classificados por formações citoplasmáticas irregulares (B), por nenhuma alteração na aparência (C) e por grande presença bacteriana (D). Ainda segundo estes autores, aproximadamente 30 minutos após a desova, os ovos já podem ser classificados em àqueles que apresentam clivagem (fertilizados) e os que não apresentam (não fertilizados), com a obtenção de eclosão de náuplios em cerca de 12 a 15 horas pós-desova para o tipo A_1, enquanto que para o tipo A_2, devido ao seu desenvolvimento irregular, baixas taxas de eclosão com a presença de náuplios débeis ou a ausência de náuplios podem ser observadas. Já para os demais tipos (B, C e D), além da ausência de clivagem, após três horas pós-desova, alguns ovos podem apresentar fissuras na célula ou membrana plasmática (Primavera e Posadas, 1981).

Já em outro estudo realizado por AQUACOP (1977) sobre a observação da maturação e reprodução de *P. merguiensis*, *P. aztecus*, *P. japonicus*, *P. monodon*, *L. vannamei* e *P. stylirostris*, quatro tipos morfológicos de ovos em relação ao desenvolvimento pós-desova foram identificados (1, 2, 3 e 4). Destes quatro, os tipos 1, 3 e 4 equivalem-se aos B, A_2 e A_1 descritos por Primavera e Posados (1981), sendo o do tipo 3 (ou A_2) também caracterizado por: grandes células clivadas irregularmente; morte de embrião no ovo e obtenção de náuplios completamente deformados ou que quando normais, apresentam setas dos apêndices alteradas, fazendo-os ser eliminados durante um processo de triagem baseado em seu comportamento de fototaxia positiva (AQUACOP, 1977). Desta forma, com base na classificação proposta por estes autores, os dois tipos morfológicos identificados no presente estudo podem ser classificados como tipo B ou 1 (mais de 97% dos ovos obtidos) e, como A2 ou 3 (menos de 2%). Embora não se saiba exatamente a razão para a obtenção dos ovos do tipo A_2 e B (ou 3 e 1) em *L. vannamei*, segundo AQUACOP (1977), uma possibilidade seria a fraca adesão dos espermatóforos causada por uma má manipulação ou por um mau posicionamento do espermatóforo o que impediria o envolvimento do óvulo com o esperma.

Além desta proposição, outra possibilidade seria a baixa viabilidade espermática presente nos espermatóforos de machos infectados. Para elucidar esta hipótese, uma avaliação da viabilidade espermática durante a fase aguda da infecção de IMNV em machos naturalmente e experimentalmente infectados foi efetuada no presente estudo. O espermatóforo de cinco machos negativos (não infectados) provenientes do tanque 3 foi usado como controle.

Em machos experimentalmente infectados, dos cinco camarões coletados no segundo experimento, apenas uma amostra exibiu mais de 100 células na lâmina por campo, com 75% de células viáveis. As demais exibiram uma baixíssima densidade celular (menos de 72 células visualizadas em toda a lâmina), inviabilizando a determinação do percentual de células espermáticas viáveis para este grupo. Já nos machos naturalmente infectados das cinco amostras analisadas, duas apresentam uma percentagem de espermatozoides viáveis de 87 e 77% (respectivamente), enquanto que as outras três, apresentaram as mesmas características observadas no grupo experimentalmente infectado com baixa densidade celular. Para o grupo controle, todas as amostras apresentaram alta densidade celular (mais de 100 células visualizadas por

campo), com percentual de células viáveis superior a 83% determinada por campo. O número de células viáveis e mortas por macho infectado (naturalmente e experimentalmente) e não infectado (controle) encontra-se sumarizado na Tabela 3.

Tabela 3. Percentual de espermatozoides viáveis de espermatóforos de reprodutores *L. vannamei* não SPF

Tanque	Amostra	N° de células analisadas		% de Células viáveis
		Vivas	Mortas	
1 - Macho experimentalmente infectado por IMNV	1	5	0	-
	2	53	18	-
	3	75	25	75,0
	4	26	12	-
	5	16	30	-
2 - Macho naturalmente infectado por IMNV	1	87	13	87,0
	2	77	23	77,0
	3	5	1	-
	4	17	8	-
	5	36	4	-
3 - Macho não infectado	1	101	20	83,5
	2	100	0	100,0
	3	103	11	90,4
	4	92	9	91,1
	5	104	11	90,4

- Não determinado decorrente do número total de células analisadas ser inferior a 100 por campo.

Ceballos-Vázquez et al. (2003) ao analisar a influência da idade e do peso na qualidade e quantidade espermática de machos de *L. vannamei* advindos de viveiros comerciais usando a contagem de espermatozoides e o percentual de células mortas

como indicadores, relataram que machos com 10 meses e peso médio de 30,3 g apresentam uma percentagem de espermatozoides viáveis de 89,3%. Aqui, o número de células viáveis no controle (não infectados) encontra-se de acordo com os resultados obtidos por estes autores, uma vez que machos com peso médio de 28,5 g apresentaram percentual de células viáveis superior a 83%.

Em relação às células infectadas, embora poucos estudos tratem do papel do espermatozoide na transmissão vertical em espécies aquáticas, segundo Mulcahy e Pascho (1984) em espécies com fecundação externa, a propagação de vírus advinda da transmissão vertical por espermatozoides seria provável e vantajosa. Isto se dá pela adsorção direta do vírus na superfície do espermatozoide, transformando-o em um veículo de entrada mais eficiente no ovo, e pela não diluição de partículas virais durante o processo de desova.

Neste estudo, a presença de poucos machos infectados naturalmente que apresentavam viabilidade espermática próxima às condições encontradas em reprodutores oriundos de viveiros comerciais, sugere uma menor participação parental paterna na propagação do IMNV na fase aguda da infecção.

4. Conclusões

O presente trabalho determinou a possibilidade de uma rota de transmissão vertical do IMNV em *L. vannamei* através da análise molecular via PCR em tempo real de reprodutores desafiados por injeção intramuscular e naturalmente infectados e, seus respectivos ovos. Entretanto, a grande presença de ovos não fertilizados ou com desenvolvimento anômalo inviabilizou a obtenção de náuplios e subsequente triagem que elucidaria a localização do vírus em relação ao interior (transmissão transovariana) ou exterior do ovo. Por outro lado, a detecção 100% positiva de ovários e óvulos associada à baixa percentagem de células espermáticas viáveis de machos naturalmente infectados, sugere uma maior probabilidade de transmissão vertical deste vírus atrelada ao parental materno. Assim, para minimizar a propagação de IMNV em unidades de cultivo, apenas fêmeas negativas após a primeira desova para este e outros patógenos devem ser utilizadas para fins de acasalamento, não devendo, entretanto, negligenciar a baixa participação dos machos na difusão do vírus.

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751 *Penaeus monodon*. Dis. Aquat. Org. 69, 129–136.

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4.3.1 - Normas da Revista - Aquaculture.

Introduction

Types of paper

Original Research Papers should report the results of original research. The material should not have been previously published elsewhere. Articles are expected to contribute new information (e.g. novel methods of analysis with added new insights and impacts) to the knowledge base in the field, not just to confirm previously published work.

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Contact details for submission

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Nutrition:

D.M. Gatlin

The Nutrition Section welcomes high quality research papers presenting novel data as well as original reviews on various aspects of aquatic animal nutrition relevant to aquaculture. Manuscripts addressing the following areas of investigation are encouraged:

1) determination of dietary and metabolic requirements for various nutrients by representative aquatic species. Studies may include environmental/stress effects on animal's physiological responses and requirements at different developmental stages;
2) evaluation of novel or established feedstuffs as well as feed processing and manufacturing procedures with digestibility and growth trials. Such studies should provide comprehensive specifications of the process or evaluated ingredients including nutrients, potential anti-nutrients, and contaminants;

3) comparison of nutrient bioavailability from various ingredients or product forms as well as metabolic kinetics of nutrients, food borne anti-nutrients or toxins;

4) identification of key components in natural diets that influence attractability, palatability, metabolism, growth reproduction and/or immunity of cultured organisms;
5) optimization of diet formulations and feeding practices;
6) characterization of the actions of hormones, cytokines and/or components in intracellular signaling pathway(s) that influence nutrient and/or energy utilization.
7) evaluation of diet supplementation strategies to influence animal performance, metabolism, health and/or flesh quality.

Manuscripts concerning other areas of nutrition using novel or advanced methods are also welcome. Please note that in regard to various diet additives such as probiotics, prebiotics, herbal extracts, etc., a very large number of papers have already been published. Therefore, Aquaculture will not continue to accept manuscripts that present initial and preliminary investigations of such additives. Manuscripts addressing these and other feed additives will be accepted for review only if they are of the highest scientific quality and they represent a significant advance in our knowledge of the mechanisms involved in their metabolism. Manuscripts may also be considered if they present clinical efficacy data generated in large-scale trials and economic cost-benefit analysis of these applications.

Aquaculture Production Science: B.Costa-Pierce

AQUACULTURE PRODUCTION SCIENCE (PS) is one of 5 sections of the international journal AQUACULTURE dedicated to research on improvements and innovations in aquatic food production.

This section supports worldwide dissemination of the results of innovative, globally important, scientific research on production methods for aquatic foods from fish, crustaceans, mollusks, amphibians, and all types of aquatic plants. Contributions are encouraged in the following areas: 1) Improvement of production systems that results in greater efficiencies of resource usage and sustainability of aquaculture; 2) Effective applications of technologies and methods of aquaculture production for improved stocking regimes; 3) The use of new species and species assemblages; and, 4) Investigations to minimize aquaculture wastes and improve water quality, including technologies for nutrient recycling in aquaculture ecosystems, and potential synergy of aquaculture and other food production systems using methods such as polyculture and integrated aquaculture. Aspects of seafood processing and technology will not be considered in this section although aquaculture techniques that may influence the nutritional value of aquatic food products may be considered in the Nutrition Section.

Physiology and Endocrinology: Fish: A. P. (Tony) Farrell Invertebrate: J. Benzie

The Physiology Section welcomes high quality papers that present both novel research data and original reviews, on all aspects of the physiology of cultured aquatic animals and plants. Their content must be relevant to solving aquaculture problems.

Submitted manuscripts must have a valid hypothesis or objective, clearly state the relevance to aquaculture, have proper experimental design with appropriate controls and utilize appropriate statistical analysis. Mention of trade names is limited to the main text.

Relevant physiological topics include, but are not limited to: - Reproductive physiology, including: Endocrine and environmental controls development; Induced ovulation and spermiation; Gamete quality, storage and cryopreservation; control of sex

differentiation; Physiology and endocrinology of gynogenetic, triploid and transgenic organisms; - Molecular genetic assessment of physiological processes; - Larval physiology and ontogeny in relation to aquaculture, including metamorphosis, smolting (salmonids) and molting (crustacea); - Nutritional physiology including endocrine and environmental regulation of growth; - Performance under variable culture conditions, including temperature optima and tolerances; Altered water quality and environmental variables; Stress and disease physiology; Rearing density; - Immunology (physiological studies of probiotics must present statistically valid conclusions); - Respiratory, muscle and exercise physiology of cultured organisms; - Osmoregulatory physiology and control; - Physiology of harvest and handling techniques, including: Anesthesia and transport; Product and flesh quality; Pigmentation.

Genetics:

G. Hulata

The Genetics Section welcomes high-quality research papers presenting novel data, as well as critical reviews, on various aspects of selective breeding, genetics and genomics. Submitted manuscripts must have a valid hypothesis or objective, clearly state the relevance to aquaculture, have proper experimental design with appropriate sample size and controls and utilize appropriate statistical analysis.

Relevant genetics topics include, but are not limited to:

Breeding programs using classic selection procedures, markers or combining marker assisted selection with classic selection

Applications of crossbreeding and interspecific hybridization

Evaluation of commercially important phenotypes among cultured strains, populations or stocks

Applications of biotechnology and genetic manipulation methods

Development of linkage maps, identification of QTL or association of commercially important traits with specific gene(s). Where appropriate, linkage maps should include

co-dominant markers, such as microsatellite DNA and SNP markers, to enable application to other populations and facilitate comparative mapping.

Aquaculture will NOT accept manuscripts dealing with the application of well-described techniques to yet another species, unless the application solves a specific biological problem important to aquaculture production; or manuscripts dealing with gene cloning, characterizing of microsatellites, species identification using molecular markers, EST papers with small collections, or mapping papers with a small number of markers, unless the papers also deal with solving a biological problem that is relevant to aquaculture production.

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Sustainability and Society: D.C. Little

The Sustainability and Society section of the journal Aquaculture invites articles at the interface of natural and social sciences that address the broader roles of aquaculture in global food security and trade.

Aims and scope of the Sustainability and Society section are the: global dissemination of interdisciplinary knowledge regarding the management of aquatic resources and resulting impacts on people. Interconnections with other sectors of food production; resource management and implications for societal impact. Going beyond a narrow techno-centric focus, towards more holistic analyses of aquaculture within well-defined contexts. Enquiry based on understanding trajectories of change amid the global challenges of climate change and food security. Mixed methods and approaches that incorporate and integrate both social and natural sciences. Relevance for the diverse range of policy makers, practitioners and other stakeholders involved. Articles that take a value chain approach, rather than being wholly production orientated, are encouraged.

Disease

B. Austin

The Disease sections welcomes critical reviews and high quality articles containing novel data on all aspects concerning diseases of farmed aquatic species. The aims of the section are: description of new and emerging diseases including characterization of the causal agent(s), development in the understanding of fish pathogens for example including new methods of growth where this has been a problem for fastidious organisms, pathogenicity and epizootiology, developments in the diagnosis of disease going beyond the use of standard well used methods, and methods of disease control, notably new developments in vaccines, immunostimulants, dietary supplements, medicinal plant products, probiotics, prebiotics and genetically-disease resistant stock. Relevance to aquaculture must be demonstrated. Articles, which adapt well known methods without further refinement of those methods, are unlikely to be accepted.

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Example 3: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

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4.4 - Artigo científico IV

Artemia como vetor da Mionecrose infecciosa (IMNV) em juvenis de *Litopenaeus vannamei*?

Artigo científico a ser encaminhado a Revista - Journal of Invertebrate Pathology.

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Artemia franciscana como vetor da Mionecrose infecciosa (IMNV) em juvenis de *Litopenaeus vannamei?*

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ABSTRACT

In Brazil, the Infectious myonecrosis virus (IMNV) was recognized as the main cause of *L. vannamei* shrimp culture's drop in 2004. In health animal control programs, in order to reduce virus prevalence in production units it is necessary to screen live feed used. Among live diets used in aquaculture, the brine shrimp *Artemia* sp. is essential in crustacean larviculture and maturation. The aim of this study was to investigate the susceptibility of *Artemia* sp. to IMNV by immersion challenge and virus-phytoplankton adhesion route and to elucidate its role as a vector for IMNV transmission to *L. vannamei*. Adults *Artemia* became IMNV-infected by both routes, with PCR-positive reactions. However, infected *Artemia* showed no signs of infection. More than 40% of *L. vannamei* juveniles fed with IMNV-infected *Artemia* by virus-phytoplankton adhesion route were positive by real-time PCR, whereas just 10% of infection was observed in shrimp fed with IMNV-infected *Artemia* by immersion challenge. There were significant differences in mean viral load between immersion and virus-phytoplankton adhesion shrimp treatments ($p \leq 0.05$). The mean viral loads were 1.34×10^2 and 1.48×10^4 copiesμg^{-1} of total RNA for virus-phytoplankton adhesion and IMNV-infected tissue treatments, respectively, and the difference was not significant ($p \geq 0.05$). The results indicated that *Artemia* act as a vector for IMNV transmission under the experimental conditions examined. Moreover, despite no mass mortalities have been detected in *L. vannamei* fed with IMNV-infected *Artemia*, these infected shrimp should not be disregarded as a source of IMNV in grow-out units.

Keywords: Live feed; IMNV; Vector; *Litopenaeus vannamei*; Real-time PCR; *Artemia*.

1. Introdução

Nas últimas duas décadas, dentre os organismos usados como alimento vivo na larvicultura de espécies aquáticas, o microcrustáceo *Artemia* sp. tornou-se o item chave para a rápida expansão de larviculturas de camarões e peixes marinhos mundialmente (Sui et al., 2013). Este microcrustáceo é o alimento básico nos estágios larvais dos camarões marinhos das espécies tigre (*Penaeus monodon*) e cinza (*Litopenaeus vannamei)* (Gervais Jr.e Zeigler, 2013).

Embora náuplios e adultos de *Artemia* não constituam o zooplâncton naturalmente consumido de muitas espécies cultivadas, a disponibilidade de cistos capazes de serem armazenados por longos períodos a torna uma fonte de alimento versátil aplicada à aquicultura (Sorgeloos et al., 1998; Sorgeloos et al., 2001). Apesar das inegáveis vantagens, o uso de alimento vivo, como a *Artemia*, ou de outros não processados podem representar uma ameaça a carcinicultura decorrente da possibilidade de atuarem como vetores potenciais de patógenos de origem bacteriana e viral (Sivakumar et al., 2009).

Neste sentido, vários trabalhos têm investigado a susceptibilidade da *Artemia* a diferentes patógenos virais de crustáceos e seu papel como reservatório, vetor ou fonte de contaminação em animais susceptíveis. Para o vírus da Mancha branca (WSSV), Li et al. (2003) e Zhang et al. (2010) relatam que diferentes estágios larvais de *Artemia* sp. foram infectados experimentalmente por este vírus, com a comprovação de transmissão vertical deste patógeno neste microcrustáceo desde as fases larvais até adultos e de adultos a cistos reprodutivos.

Além do WSSV, a doença da cauda branca (WTD) e o vírus da Parvovirose hepatopancreática (HPV) também foram capazes de infectar *Artemia* sp. A patogenicidade da WTD, causada pelos vírus *Macrobrachium rosenbergii nodavirus* (MrNV) e *extra small* (XSV), foi demonstrada para todos os estágios de desenvolvimento da *Artemia* sp. (náuplio, metanáuplio, juvenil, pré-adulto e adulto) via desafios virais, com detecção 100% positiva de todas as fases via RT-PCR (Sudhakaran et al., 2006). Do mesmo modo, por meio de infecções experimentais, diferentes fases da *A. franciscana* foram positivas ao HPV por PCR, com transmissão horizontal comprovada em pós-larvas de *P. monodon* (Sivakumar et al., 2009).

Com relação ao vírus da Mionecrose infecciosa (IMNV) não existem dados específicos sobre vetores, embora devido a sua estrutura de partícula viral não envelopada, seja provável que permaneça infeccioso no trato gastrintestinal de animais que venham a se alimentar de camarões infectados advindos de fazenda de engorda durante um surto (OIE, 2012). Assim, a propagação deste vírus através da ingestão de partículas virais por outros invertebrados também é possível.

O IMNV surgiu em uma fazenda de engorda de camarão marinho *L. vannamei* localizada no estado do Piauí (Brasil) em 2002, em que os produtores relatavam a presença de uma nova doença caracterizada por camarões moribundos que exibiam uma perda da transparência do músculo abdominal devido a extensas áreas necrosadas esbranquiçadas do músculo esquelético e mortalidade diária a partir de 7 g (Nunes et al., 2004). Em 2006, além do nordeste brasileiro, surtos de IMNV foram relatados em fazendas de engorda da Indonésia (Ásia) (Senapin et al., 2007), gerando perdas econômicas superiores a US$ 1 bilhão de dólares desde seu surgimento até 2010 (Lightner et al., 2012), o que culminou com sua inserção na lista de doenças de crustáceos de notificação obrigatória da Organização Mundial da Saúde Animal (OIE) a partir de 2007 (OIE, 2007).

Entre as ações destinadas à prevenção da propagação de doenças em fazendas de camarão, os programas de saúde animal têm estabelecido como medida de controle que todas as espécies susceptíveis presentes no sistema de cultivo devem ser consideradas como fonte de transmissão de patógenos e, portanto, triadas, o que inclui também os organismos usados como alimento vivo (Chang et al., 2011).

Deste modo, sendo a *Artemia* um alimento vivo amplamente difundido nas unidades de larvicultura e maturação (na forma de biomassa) de peneídeos, o presente estudo tem por objetivo avaliar a susceptibilidade da *Artemia* sp. ao IMNV através de desafios virais e seu papel como vetor na transmissão horizontal deste vírus em *L. vannamei*.

2. Material e métodos

2.1 Preparação do inóculo viral

O inóculo viral foi preparado conforme descrito por Silva et al. (descrito no 1° artigo desta tese), com algumas modificações. Cem gramas de tecido do músculo abdominal de *L. vannamei* naturalmente infectado e previamente triado como positivo

para este vírus por PCR (Poulos e Ligthner, 2006) e negativo para outros patógenos de camarões peneídeos (TSV, WSSV, vírus da Necrose hipodermal e hematopoiética infecciosa - IHHNV e Hepatopancreatite necrosante - NHPB) via kits da IQ2000^{TM} (Farming IntelliGene Tech. Corp., Taiwan), foram homogeneizados em 300 mL de solução salina estéril a 2% (w/v).

O tecido homogeneizado foi diluído em solução salina estéril a 2% (1:3; v:v) e sucessivamente filtrado a 300, 210 e 70 µm para obtenção do inóculo. O inóculo foi dividido em alíquotas de 10 mL de volume e armazenado a -80°C até sua posterior utilização no desafio viral.

2.2 Produção de biomassa de Artemia sp.

Cistos de *Artemia* sp. (HIGH 5 Artemia, INVE Aquaculture, Bélgica) foram hidratados em água doce por uma hora sob forte aeração, lavados em água corrente por um minuto através de tela de 100 µm e incubados a 3 g/L em água à salinidade de 30 gL^{-1} por 24 horas. Após este período, os náuplios foram separados dos cistos não eclodidos ou vazios e transferidos para caixas de fibra de vidro de 300 L (salinidade de 30 gL^{-1}, temperatura de 28°C e aeração constante) a densidade de 20 animais / mL, sendo alimentados a partir da abertura da boca (2ª fase larval - Instar II) até a fase adulta com uma "mistura" descrita por Naegel (1999), com algumas modificações. A cada dois dias, 100% do volume de água eram renovados.

Para o preparo desta "mistura", a cada 1 L de água destilada foram adicionados 50 g de Neston® (Nestlé Brasil Ltda, Brasil), 50 g de FRiPPAK FRESH#1 CAR (INVE Aquaculture, Bélgica); 60 mL de Easy SELCO (Artemia International LLC, EUA) e 1 g de vitamina C, seguido de homogeneização por 10 minutos no liquidificador e filtragem a 70 µm. O volume diário da oferta da "mistura" foi determinado por observação da transparência visual da água.

Após 15 dias, adultos (±1 cm) de *Artemia* sp. foram obtidos e estocados até seu uso nos experimentos de desafio viral. Cinco gramas de adultos foram convertidos em biomassa congelada a -80°C para posterior emprego como controle negativo dos bioensaios virais.

2.3 Obtenção de juvenis de L. vannamei SPF

Um total de 120 indivíduos livres de patógeno específico (*Specific Pathogen Free* - SPF) de *L. vannamei* com peso médio de 1,8 g, provenientes da empresa Genearch Aquacultura Ltda (larvicultura comercial brasileira) localizada em Rio do Fogo (RN), foram usados neste experimento.

Estes animais foram mantidos em unidades experimentais de 50L de volume útil, a densidade de 1 camarão / 5 L, a 28°C e 30 gL^{-1} de salinidade, sendo alimentados com ração comercial (35% de proteína bruta) a 5% da biomassa duas vezes ao dia. A cada três unidades experimentais foi adicionado um filtro biológico de 1500 L/h (Filtro Canister JEBO 829, JEBO, China) para a instalação de um sistema de recirculação fechado, perfazendo um total de quatro conjuntos experimentais.

Diariamente (duas vezes ao dia) eram monitorados a temperatura e o pH e, semanalmente, amostras de água foram coletadas para análise de nitrito, nitrato, nitrogênio amoniacal e alcalinidade por meio de kit colorimétrico comercial (Alcon Labcon, Camboriú, Brasil) para verificação destes parâmetros dentro dos limites ideais para peneídeos propostos por Van Wyk e Scarpa (1999).

2.4 Desafio viral de Artemia spp.

Para os desafios virais de *Artemia* com IMNV, duas rotas foram avaliadas: imersão e adesão vírus-fitoplâncton. Estes desafios baseiam-se em infecções experimentais com artemia para WSSV conforme descrito por Hameed et al. (2002) e Zhang et al. (2006; 2007; 2008), com algumas modificações.

Cada desafio foi composto por 10 réplicas de 500 indivíduos / L em béckers de 2 L com 1 L de água a uma salinidade de 30 gL^{-1} e 28°C, sob aeração constante. Todos os béckers foram cobertos para evitar a contaminação cruzada.

Nos desafios via imersão, o inóculo foi adicionado à água a 1% do volume total (10 mL / 1000 mL) e mantido em contato com os adultos de *Artemia* por três horas, duas vezes ao dia, por quatro dias. Entre uma adição de inóculo e outra, os animais eram lavados três vezes e transferidos para um novo bécker com água limpa, no qual permaneciam por igual tempo (três horas). Ao final do dia, os adultos de *Artemia* eram alimentados com 10 mL da "mistura". Após os quatro dias de desafio, os animais foram mantidos sem alimentação por 24 horas a fim de esvaziar o canal alimentar, sendo

efetuadas alíquotas de 1 g para posterior análise de PCR para confirmação de infecção por IMNV e oferta no experimento de transmissão horizontal em *L. vannamei*. Toda a biomassa foi armazenada a -80°C.

Já para os desafios via rota de adesão vírus-fitoplâncton, duas espécies de microalgas foram utilizadas: *Isochrysis galbana* e *Chaetoceros* sp.. O uso destas microalgas justifica-se por serem as mais utilizadas mundialmente em larviculturas de *L. vannamei* (Hemaiswarya et al., 2011). Neste desafio, 10 mL do inóculo viral foi previamente misturado em um volume final de 1 L contendo *Isochrysis galbana* e *Chaetoceros* sp. a $2{,}6x10^6$ células / mL, na proporção de 1:1 por 30 minutos e, depois adicionado a cada becker (40 mL) para alimentação da *Artemia* por três horas. Em seguida, os animais foram lavados três vezes e mantidos em um bécker com água limpa por três horas antes da segunda oferta de vírus-fitoplâncton. Assim, os adultos de *Artemia* foram desafiados duas vezes ao dia, por quatro dias, sendo alimentados com 10 ml da "mistura" ao final do dia. Da mesma forma descrita anteriormente, ao final do 4° dia de experimento, os animais foram mantidos sem alimentação por 24 horas para coleta e determinação da presença do IMNV. Alíquotas de 1 g foram efetuadas para o uso em desafios com *L. vannamei*, seguida de armazenamento a -80°C.

2.4 Desafio viral de L. vannamei com Artemia infectada

A determinação da transmissão horizontal de IMNV em *L. vannamei* via desafio viral por ingestão de *Artemia* infectada foi avaliada em quatro tratamentos: (1) juvenis de *L. vannamei* alimentados com *Artemia* infectada via adesão vírus-fitoplâncton; (2) juvenis de *L. vannamei* alimentados com *Artemia* infectada via imersão; (3) juvenis de *L. vannamei* alimentados com tecido de camarão infectado e; (4) juvenis de *L. vannamei* alimentados com *Artemia* não infectada (grupo controle).

Cada tratamento constou de três repetições de 10 camarões SPF (um conjunto experimental) e todos os animais foram alimentados para os seus respectivos tratamentos a 5% da biomassa, duas vezes ao dia, durante sete dias, totalizando 10% da biomassa / dia. Ao 8° dia, os camarões foram alimentados com ração comercial à mesma biomassa, sendo monitorados diariamente para a observação de sinais clínicos de infecção por IMNV e mortalidade.

O experimento durou 15 dias e ao longo deste período os animais mortos foram coletados e estocados a –80°C para a análise de PCR em tempo real. Os sobreviventes foram sacrificados ao 15° dia e também armazenados a –80°C para posterior análise molecular.

2.5 Análises moleculares

2.5.1 Extração de RNA e RT-PCR

A extração do RNA total de *Artemia* desafiada (para as duas rotas) e músculo abdominal de camarão inoculado (para todos os tratamentos) foi realizada através da digestão do tecido (50 mg) em 1 mL de Trizol (Invitrogen, EUA), conforme as instruções do fabricante. Após a extração, o RNA foi avaliado qualitativamente e quantitativamente via espectrofotometria a 260 e 280 ηm via espectrofotômetro (NanoVue Plus^{TM}, GE Healthcare, EUA) e armazenado a -80°C. Em seguida, a RT-PCR foi efetuada através do kit Improm-II^{TM} Reverse Transcription System (Promega, Madison, WI, USA) em um volume final de 20 µL, usando 300 ηgµL^{-1} de RNA total e 0,5 µg de oligo(dT)$_{15}$, conforme as instruções do fabricante. O cDNA foi armazenado a -20°C até posterior utilização nas análises de PCR convencional ou PCR em tempo real.

2.5.2 PCR convencional

A infecção por IMNV em *Artemia* desafiada (para as duas rotas) foi determinada através de PCR convencional usando os primers específicos descritos por Poulos e Lightner (2006) usando como amostra, dois pools de 1 g de *Artemia* coletada aleatoriamente nas 10 réplicas de cada rota. As condições de reação e os ciclos térmicos de PCR foram realizadas da mesma forma descrita por Pinheiro et al. (2007). Os produtos de PCR (tamanho esperado de 328 pb) foram submetidos a eletroforese em gel de agarose a 2% corado com brometo de etídio e o tamanho do fragmento estimado por um marcador de peso molecular de 100 pb (Invitrogen, EUA).

2.5.3 PCR em tempo real

Todas as amostras de músculo abdominal de camarão inoculado (para todos os tratamentos) tiveram sua carga viral média de IMNV determinada através de análises de PCR em tempo real conforme descrito por Silva et al. (2011).

As reações foram feitas em placas de 96 poços em um volume final de 25 µL e a carga viral quantificada através da extrapolação dos valores de Ct de cada amostra na diluição serial do plasmídeo recombinante-padrão para IMNV, segundo Silva et al. (2011). Cada amostra foi analisada em duplicata e a cada placa, foram adicionados dois controles negativos (água ultra-pura e amostra de controle positivo para o vírus da Taura) e um controle interno de β-actina. As amplificações foram efetuadas em um termociclador StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, EUA) e os dados analisados por meio do software StepOne™ (versão 2.2.2) (Applied Biosystems, CA, EUA).

2.6 Análises estatísticas

Os dados provenientes da quantificação viral de amostras de músculo de camarão (para todos os tratamentos) foram submetidos ao teste de Cochran ao nível de 95% de probabilidade ($p \leq 0,05$) para verificação da homogeneidade e aos testes de Kolmogorov-Smirnov e de Shapiro-Wilk, para normalidade ($p \leq 0,05$). Em seguida, os dados obtidos com os diferentes tratamentos foram transformados em log da base 10 e submetidos ao teste não paramétrico de Kruskal-Wallis ($p \leq 0,05$) para comparar a carga viral média obtida nos diferentes tratamentos testados. Todas as análises estatísticas foram realizadas com o software ASSISTAT versão 7.7 beta (Silva, 2014).

3. Resultados e discussão

Todas as amostras de *Artemia* desafiadas com IMNV foram positivas via PCR convencional para ambas as rotas de infecção testadas (imersão e adesão vírus-fitoplâncton) (Figura 1), não sendo observados quaisquer sinais clínicos de infecção ou mortalidades acumulativas decorrentes dos desafios. Além disso, amostras de adulto de *Artemia* pertencentes ao mesmo lote usado nestes desafios e que não foram infectadas, mas convertidas em biomassa (armazenada a -80°C), para posterior emprego como controle negativo nos bioensaios virais com os camarões, foram negativas via PCR convencional. Do mesmo modo, nenhum sinal de infecção foi notado.

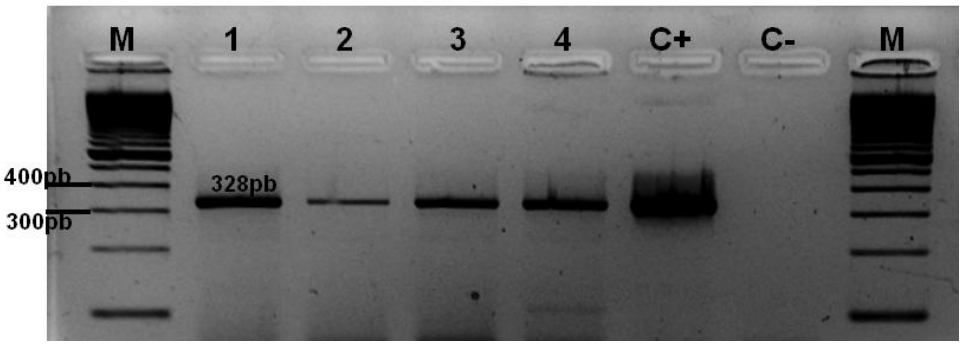

Figura 1. Detecção de IMNV em *Artemia* sp. infectada experimentalmente através de desafios de imersão e de adesão vírus-fitoplâncton. As duas primeiras amostras (1 e 2) correspondem aos adultos de *Artemia* sp. infectada via imersão, enquanto que as duas últimas (3 e 4) correspondem às infectadas via adesão vírus-fitoplâncton, onde: M é marcador de peso molecular de 100 pb (Invitrogen, EUA); C+, controle positivo e C-, controle negativo (água ultra-pura).

Detecção positiva de diferentes vírus em adultos de *Artemia* tem sido relatada por outros autores. Li et al. (2003) ao investigar a susceptibilidade de náuplios e adultos de *Artemia* ao WSSV durante 10 dias por meio de desafios de ingestão de inóculo misturado a microalga em pó comprovaram a infecção por WSSV via PCR. Ainda neste estudo, resultados similares de infecção positiva foram observados em testes para determinação da transmissão vertical deste vírus em *Artemia*, com a presença de adultos positivos gerando cistos reprodutivos também positivos, embora os náuplios eclodidos destes cistos fossem negativos, indicando a remoção do vírus durante a lavagem dos náuplios. Similarmente, Chang et al. (2002) não detectaram WSSV por PCR em náuplios eclodidos, após lavagem, derivados de cistos positivos, sugerindo uma contaminação externa dos cistos. Em ambos os estudos, nenhum sinal clínico da doença foi observado, o que segundo Li et al. (2003) indica que estudos de patogenicidade com maior duração devem ser efetuados para observação de sintomatologia em *Artemia* infectada com WSSV.

Em outro estudo, quatro estágios (náuplio, metanáuplio, pré-adulto e adulto) de desenvolvimento de *Artemia* foram experimentalmente desafiados para WSSV através de desafios de imersão e adesão vírus-fitoplâncton, mas apenas os animais inoculados com a segunda rota (vírus-fitoplâncton) foram infectados, com detecção positiva apenas na 2ª PCR, indicando baixas cargas virais (Zhang et al., 2010). Do mesmo modo, a

patogenicidade de WSSV no rotífero *Brachionus urceus* e em copépodes das espécies *Nitroca* sp. e *Acartia clausi* tem sido demonstrada em infecções experimentais através da rota adesão vírus-fitoplâncton, com resultados positivos obtidos por nested-PCR (Zhang et al, 2006; 2007; 2008).

Liu et al. (2007), ao co-cultivar adultos de *Marsupenaeus japonicus* infectados por WSSV com seis espécies de microalgas (*Isochrysis galbana, Skeletonema costatum, Chlorella* sp., *Heterosigma akashiwo, Scrippsiella trochoidea* e *Dunaliella salina*), determinaram que todas as microalgas foram reservatórios de WSSV com detecção positiva via nested-PCR, exceto para a espécie *H. akashiwo,* e que entre estas, *Chlorella* sp. e *S. trochoidea* foram as com maior capacidade de transporte de WSSV. Entretanto, ao re-infectar os juvenis de *M. japonicus* com as microalgas infectadas, apenas a *Chlorella* sp. foi vetor mecânico de transmissão, sugerindo que as microalgas possam constituir uma via de transmissão horizontal para WSSV (Liu et al., 2007). Assim, segundo Zhang et al. (2010), a rota adesão vírus-fitoplâncton é uma via de transmissão eficiente de WSSV ao zooplâncton. No presente estudo, ambas as rotas de transmissão (imersão e adesão vírus-fitoplâncton) mostraram-se eficientes ao infectar adultos de *Artemia* com IMNV, apresentando detecção positiva via PCR convencional (1ª PCR), o que sugere carga viral média superior a 10^5 cópiasμg^{-1} de RNA total (Silva et al., 2011).

Entretanto, não só a rota adesão vírus-fitoplâncton tem sido usada na condução de desafios orais para avaliação de susceptibilidade de *Artemia* a vírus. Hameed et al. (2002) ao testar a patogenicidade de WSSV em náuplios, metanáuplios, juvenis, pré-adultos e adultos de *Artemia* usando dois métodos de exposição ao patógeno: introdução de 0,1% de inóculo em volume na água (imersão) e adição de suspensão viral em farelo de arroz, seguido de oferta aos animais (ingestão), foi incapaz de detectar a presença deste vírus em todas as fases de desenvolvimento testadas para ambos os métodos. Do mesmo modo, Sarathi et al. (2008) em desafios similares, não conseguiram infectar estes mesmos cinco estágios de desenvolvimento de *Artemia* com Monodon baculovirus (MBV), apresentando resultados de detecção negativa por nested-PCR.

No caso dos vírus *Macrobrachium rosenbergii nodavirus* (MrNV) e *extra small* (XSV), o uso de suspensão viral em farelo de arroz para desafio oral foi eficiente na propagação destes dois vírus em todos os estágios de desenvolvimento da *Artemia*, com

resultados positivos via nested-PCR, indicando que a *Artemia* atua como um reservatório para estes vírus (Sudhakaran et al., 2006).

Resultados positivos de infecção por HPV também foram alcançados com o emprego de suspensão viral associada ao farelo de arroz em desafios orais em todas as fases da *Artemia franciscana*, exceto náuplios (Sivakumar et al., 2009). No entanto, outros estudos devem ser conduzidos para determinar se *A. franciscana* é realmente infectada com HPV ou simplesmente age como portadora passiva (Sivakumar et al., 2009), embora Feng et al. (2013) relatem a presença de receptores celulares de WSSV na membrana de células de *Artemia*, sugerindo que este microcrustáceo seja um reservatório deste vírus.

Quanto à avaliação da *Artemia* como vetor na transmissão horizontal de IMNV em juvenis de *L. vannamei*, todos apresentaram animais positivos via PCR em tempo real. Entretanto, apenas os tratamentos com camarão alimentado com *Artemia* infectada via adesão vírus fitoplâncton (tratamento 1) e com tecido de camarão infectado (tratamento 3) apresentaram percentagem de infectados superior a 40%, não havendo diferença estatística entre a carga viral média obtida para estes tratamentos ($p \geq 0,05$) (Tabela 1). Contudo, a carga viral média destes tratamentos diferiu estatisticamente das encontradas nos camarões alimentados com *Artemia* infectada via imersão (tratamento 2) ($p \leq 0,05$), para os quais apenas 10% dos animais desfiados, foram infectados (Tabela 1), evidenciando a eficiência da rota adesão vírus-fitoplâncton na propagação de IMNV. Nenhuma carga viral foi detectada nos juvenis de *L. vannamei* alimentados com *Artemia* não infectada (grupo controle) e; todos os dados de quantificação viral e número de infectados por tratamento encontram-se sumarizados na Tabela 1.

De acordo com Zhang et al. (2010), usando o método de adesão vírus-fitoplâncton, as partículas virais inicialmente aderidas à superfície da microalga são ingeridas e bioacumuladas pela *Artemia* que é um organismo filtrador, e em seguida, transmitidas a camarões dentro do trato digestório deste microcrustáceo. Em seu experimento, embora mortalidades massivas não tenham sido detectadas durante todo o experimento (15 dias), todos os camarões desafiados por ingestão de *Artemia* infectada previamente pela rota adesão vírus-fitoplâncton, foram positivos para WSSV via nested-PCR.

De maneira geral, rotas que favoreçam a ingestão de partículas virais pela *Artemia*, como o uso de uma suspensão viral em farelo de arroz (por exemplo), têm se mostrado

hábeis ao infectar *Artemia* e provarem seu papel como vetor na transmissão horizontal de MrNV, XSV e HPV em pós-larvas de *Macrobrachium rosenbergii* e *Penaeus monodon* (Sudhakaran et al., 2006; Sivakumar et al., 2009).

Dentre todos os tratamentos experimentais testados, apenas aquele com ingestão de tecido de camarão infectado, mostrou-se com valores de carga viral média semelhante àquelas encontradas em músculo de animais naturalmente infectados com IMNV (entre $6{,}85x10^{8}$ e $3{,}09x10^{4}$ cópiasμg^{-1} de RNA total) (Silva et al., 2011). Além disso, este tratamento foi o único em que foram observados sinais clínicos de infecção por IMNV nos animais desafiados, como opacidade multifocal nos músculos do segmento abdominal e nos urópodos (leque caudal).

Tabela 1. Número de infectados e carga viral média (cópiasμg^{-1} de RNA total) para os quatro diferentes tratamentos de *L. vannamei* desafiado via ingestão de *Artemia* infectada

Tratamento	N° Analisado	PCR Positiva	Carga viral		
			Mínimo	Média	Máximo
1- *L. vannamei* alimentado com *Artemia* infectada via adesão vírus-fitoplâncton	30	14	$8{,}97x10^{1}$	$1{,}34x10^{2a}$	$2{,}19x10^{2}$
2- *L. vannamei* alimentado com *Artemia* infectada via imersão	30	3	$1{,}75x10^{2}$	$9{,}31x10^{2b}$	$1{,}99x10^{3}$
3 - *L. vannamei* alimentado com tecido de camarão infectado	30	12	$6{,}07x10^{2}$	$1{,}48x10^{4a}$	$1{,}59x10^{5}$
4 - *L. vannamei* alimentado com *Artemia* não infectada (grupo controle)	30	0	-	-	-

[a,b] - Letras diferentes indicam diferença estatística significante pelo teste de Kruskal-Wallis ($p \leq 0{,}05$); - Não detectado via PCR em tempo real.

Em relação à mortalidade cumulativa para todos os tratamentos testados, os valores estiveram dentro dos esperados para condições normais de cultivo (sem vírus), não sendo observadas mortalidades massivas (Tabela 2). Estes resultados encontram-se de acordo com os obtidos por outros autores ao infectar pós-larvas de *L. vannamei* com WSSV via *Artemia* infectada por bioensaios de imersão e adesão vírus-fitoplâncton, em que não foram observadas mortalidades massivas ou diferença de mortalidade entre os tratamentos testados (Zhang et al., 2010).

Tabela 2. Percentagem de mortalidade acumulativa para os diferentes tratamentos de *L. vannamei* desafiado via ingestão de *Artemia* infectada

Tratamento	N° de indivíduos estocados	Percentagem de mortalidade cumulativa (%)				Percentagem de infectados (%)
		1° Dia	5° Dia	10° Dia	15° Dia	
1	30	0	0	0	100	46,67
2	30	0	0	0	100	10,00
3	30	0	0	0,03	99,97	40,00
4	30	0	0	0	100	0,00

4. Conclusões

O presente trabalho comprovou a susceptibilidade de *Artemia* a infecção por IMNV via duas rotas de transmissão (imersão e adesão vírus-fitoplâncton) e seu papel como reservatório ou vetor mecânico na transmissão horizontal deste vírus em *L. vannamei*, sob condições experimentais. Além disso, embora mortalidades massivas não tenham sido detectadas durante 15 dias nos camarões desafiados com *Artemia* infectada, não se deve subestimar a presença desses camarões como fonte viral nos sistemas de cultivo quando alimentados com alimento vivo infectado ou contaminado, podendo aumentar a prevalência de patógenos nas unidades de produção.

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