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# *Artemia franciscana* as a vector for infectious myonecrosis virus (IMNV) to *Litopenaeus vannamei* juvenile

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ABSTRACT

In 2004, the infectious myonecrosis virus (IMNV) was recognized as the main cause of *Litopenaeus vannamei* shrimp culture's drop in Brazil. 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 the present study was to investigate the susceptibility of *Artemia franciscana* to IMNV through an immersion challenge and virus-phytoplankton adhesion route and to elucidate its role as a vector for IMNV transmission to *L. vannamei*. *A. franciscana* adults were infected with IMNV through both routes, as demonstrated by PCR-positive reactions. However, infected *A. franciscana* showed no signs of infection. More than 40% of *L. vannamei* juveniles fed with IMNV-infected *A. franciscana* by virus–phytoplankton adhesion route were positive by real-time PCR, whereas only a 10% infection rate was found among shrimp fed with IMNV-infected brine shrimp using the immersion challenge. Significant differences were found in mean viral load between immersion and virus-phytoplankton adhesion shrimp treatments ($p \leqslant 0.05$). Moreover, the mean viral loads were $1.34 \times 10^2$ and $1.48 \times 10^4$ copies/$\mu g^{-1}$ of total RNA for virus-phytoplankton adhesion and IMNV-infected tissue treatments, respectively, and the difference was not significant ($p \geqslant 0.05$). The results indicated that *A. franciscana* act as a vector for IMNV transmission under the experimental conditions examined. Although no mass mortalities were detected in *L. vannamei* fed with IMNV-infected brine shrimp, these infected shrimp should not be disregarded as a source of IMNV in grow-out units.



## 1. Introduction

Among the organisms used as live feed in aquaculture, brine shrimp (*Artemia* sp.) have been the most frequently used for marine fish and crustacean hatcheries purposes (Sorgeloos et al., 2001; Sui et al., 2013). Although *Artemia* nauplii and adults do not constitute the zooplankton naturally consumed by many cultured aquatic species, the availability of dormant cysts capable of being stored for long periods makes them a versatile live feed for use in aquaculture (Sorgeloos et al., 1998, 2001). However, non-processed live feed may act as a vector for bacterial or viral pathogens (Sivakumar et al., 2009).

A number of studies have investigated *Artemia* susceptibility to different viral pathogens of crustaceans and their role as a reservoir, vector or source to susceptible hosts. Li et al. (2003) and Zhang et al. (2010) experimentally infected different larval stages of *Artemia* sp. with White spot syndrome virus (WSSV) and demonstrated its vertical transmission from instars to adults and from adults to reproductive cysts.

In addition, white tail disease (WTD) and hepatopancreatic parvovirus (HPV) are also capable of infecting *Artemia* sp. The pathogenesis of WTD, caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV), has been established in *Artemia* sp. (nauplii, metanauplii, juveniles, sub-adults and adults) through viral challenges, with 100% positivity in all phases, as confirmed by molecular analysis (Sudhakaran et al., 2006). Similarly, in experimental infections, different phases of *Artemia franciscana* have proved HPV positivity by PCR, with horizontal transmission to *Penaeus monodon* postlarvae (Sivakumar et al., 2009).

For infectious myonecrosis virus (IMNV), there are no specific data on the vectors. However, due to the structure of its

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non-enveloped viral particle, it is likely that IMNV remains infectious in the gastrointestinal tract of organisms that feed on infected individuals (OIE, 2012). Therefore, the propagation of this virus through the ingestion viral particles by other invertebrates is also believed.

IMNV emerged in 2002 in *Litopenaeus vannamei* grow-out farm located in Piauí state (Brazil), which producers first reported a new disease characterized by loss of abdominal muscle transparency in moribund shrimp due to extensive necrotic areas of skeletal muscle and daily mortality beginning at 7 g (Nunes et al., 2004). In 2006, IMNV outbreaks were also reported on shrimp farms in Indonesia (Senapin et al., 2007) and caused economic losses greater than US$ 1 billion since its emergence until 2010 (Lightner et al., 2012), which resulted in the inclusion of IMNV in World Organization for Animal Health (OIE) list of notifiable crustacean diseases (OIE, 2007).

Therefore, in order to prevent the spread of diseases on shrimp farms, animal health management programs have adopted the monitoring of all susceptible species present in culture system, which includes the live food organisms used (Chang et al., 2011). Since *Artemia* constitutes the most widely live food used in penaeid hatchery and maturation, the present study aimed to assess the susceptibility of *A. franciscana* to IMNV and its role as a vector in IMNV horizontal transmission to *L. vannamei*. Moreover, this is the first report to describe a vector for IMNV under experimental conditions.

## 2. Material and methods

### 2.1. Preparation of viral inoculum

One hundred grams of abdominal muscle tissue from naturally IMNV-infected *L. vannamei*, previously analyzed by PCR (Poulos and Lightner, 2006), were homogenized in 300 mL of sterile 2% (w/v) saline solution. The homogenized tissue was diluted in sterile 2% (1:3; v:v) saline solution and filtered successively at 300, 210 and 70 μm. The remaining tissue was centrifuged at 3000 rpm and at 14,000 rpm, both for 20 min at 4 °C, and the supernatant fraction was passed through a 0.22 μm filter (TPP, Switzerland) (Silva et al., In press). The inoculum was divided into 10 mL aliquots and stored at −80 °C until further use in viral challenge.

### 2.2. Production of A. franciscana biomass

*A. franciscana* cysts (HIGH 5 Artemia, INVE Aquaculture, Belgium) were hydrated in freshwater for one hour with continuous aeration, rinsed in running water for one minute on a 100-μm screen and incubated at 3 g/L in seawater (salinity: 30 g/L) for 24 h. The hatched nauplii were separated and transferred to 300-L fiberglass tanks (salinity 30 g/L, temperature of 28 °C and continuous aeration) at a density of 20 nauplii/mL. The brine shrimp were fed as soon as the mouth was open (Instar II) through to the adult phase using the mix described by Naegel (1999) with modifications. The water was exchanged every two days.

For the mix preparation, 50 g of Neston® (Nestlé Brasil Ltda, Brazil), 50 g of FRiPPAK FRESH#1 CAR (INVE Aquaculture, Belgium), 60 mL of Easy SELCO (Artemia International LLC, USA) and 1 g of vitamin C were added in 1 L of distilled water, followed by homogenization for 10 min in a blender and filtering at 70 μm. The daily volume of the mix offered was determined by visual transparency observation.

After 15 days, *A. franciscana* adults (±1 cm) were obtained and stocked in beakers for viral challenge experiments. Five grams of adults were converted into biomass, stored at −80 °C and used as negative control.

### 2.3. Experimental shrimp

A total of 120 Specific Pathogen Free (SPF) *L. vannamei* juveniles (mean body weight of 1.8 g) were obtained from Genearch Aquaculture Ltda (Brazilian commercial hatchery; Rio Grande do Norte, Brazil). The shrimp were kept in experimental units (50 L) at a density of one animal per 5 L (water temperature: 28 °C; salinity: 30 g/L) and fed with commercial pellets (35% crude protein) at 5% of biomass twice a day. A commercial biofilter system (1500 L/h, JEBO 829 canister filter, JEBO, China) was fitted in each three experimental units to obtain a closed recirculation system, totaling four experimental sets.

Temperature and pH were monitored twice a day. Water samples were collected weekly for nitrite, nitrate, ammonium nitrogen and alkalinity analyses using a commercial colorimetric kit (Alcon Labcon, Camboriú, Brazil). The general water quality parameters were kept within the acceptable levels for *L. vannamei* (Van Wyk and Scarpa 1999).

### 2.4. Viral challenge for A. franciscana

Two routes were employed in the viral challenges for *A. franciscana* with IMNV: immersion and virus-phytoplankton adhesion. These challenges were based on experimental infections of *Artemia* sp. with WSSV described by Hameed et al. (2002) and Zhang et al. (2006, 2007, 2008) with modifications. Each challenge was performed with 10 replicates of 500 individuals/L in 2-L beakers with 1 L of seawater (salinity: 30 g/L) at 28 °C under constant aeration. All beakers were covered to prevent cross-contamination.

In the immersion challenges, the inoculum was added to the water at 1% of the total volume (10 mL/1000 mL) and *A. franciscana* adults were exposed to it for three hours twice a day for four days. Between one exposure to inoculum and another, the brine shrimp were washed three times and placed to a fresh seawater beaker, in which they remained for the same time (three hours). At the end of the day, *A. franciscana* were fed 10 mL Naegel's mix. After four days of challenge, *A. franciscana* were starved for 24 h to empty the alimentary canal before sampling. Aliquots of 1 g were separated both for PCR analysis (in order to confirm IMNV infection) and for use in the horizontal transmission experiment with *L. vannamei*. All biomass was stored at −80 °C.

As for the virus-phytoplankton adhesion route challenge, two microalgae were used: *Isochrysis galbana* and *Chaetoceros* sp. These microalgae are the most frequently used on penaeid shrimp hatcheries throughout the world (Hemaiswarya et al., 2011). In the challenge, 10 mL of the viral inoculum were previously mixed with 1 L of *I. galbana* and *Chaetoceros* sp. (proportion of 1:1) at $2.6 \times 10^6$ cells/mL for 30 min and then added to each beaker (40 mL) to feed *A. franciscana* for three hours. The brine shrimp were washed three times and transferred to fresh seawater beaker for three hours prior to the second feeding of the virus-phytoplankton mix. As the immersion challenge group, *A. franciscana* were challenged twice a day for four days and fed 10 ml of Naegel's mix at the end of each day. On day 5, they were starved for 24 h, collected, divided into PCR analysis (1 g aliquots) and biomass for *L. vannamei* viral challenge and stored at −80 °C.

### 2.5. Viral challenge for L. vannamei with infected A. franciscana

To determine whether IMNV could be transmitted horizontally between IMNV-infected *A. franciscana* and *L. vannamei* juveniles, four treatments were designed: (1) *L. vannamei* juveniles fed *A. franciscana* infected by virus-phytoplankton adhesion; (2) *L. vannamei* juveniles fed *A. franciscana* infected by immersion; (3) *L. vannamei* juveniles fed minced IMNV-infected *L. vannamei*

tissue; and (4) *L. vannamei* juveniles fed non-infected *A. franciscana* (control group).

Each treatment consisted of three replicates of 10 SPF shrimp (one experimental set) at a density of 1 individual/5 L. All shrimp were fed twice a day at 5% of biomass (10% of biomass/day) for seven days. From the eighth day onwards, shrimp were fed the same volume of commercial pellets and monitored daily for clinical signs of IMNV infection and mortality. The experiment lasted 15 day. Throughout this period, dead shrimp were collected and stored at −80 °C for RT-PCR analysis. Survivors were also sacrificed on the 15th day and stored at −80 °C for molecular analysis.

### 2.6. Molecular analysis

#### 2.6.1. RNA extraction and cDNA synthesis

The extraction of total RNA from challenged *A. franciscana* (both routes) and *L. vannamei* juveniles (all treatments) was performed through digestion of tissue (50 mg) in 1 mL of Trizol (Invitrogen, USA), following the manufacturer's instructions. RNA concentration and quality were analyzed using a spectrophotometer (NanoVue Plus™, GE Healthcare, USA) at 260 and 280 ηm, followed by storage at −80 °C. RT-PCR was performed using Improm-II™ Reverse Transcription System (Promega, Madison, WI, USA) in a final volume of 20 μL containing 300 ηg/μL of total RNA and 0.5 μg of oligo$(dT)_{15}$, according to the manufacturer's instructions. cDNA was stored at −20 °C until used in the conventional and real-time PCR analyses.

#### 2.6.2. Conventional PCR

IMNV infection in challenged *A. franciscana* (both routes) was determined through conventional PCR using the specific primers described by Poulos and Lightner (2006). Two 1-g pools of challenged *A. franciscana* were collected randomly from 10 replicates of each route and used as sample. PCR amplification and cycle conditions were based on an established protocol (Pinheiro et al., 2007). After amplification, the IMNV PCR amplicon (328 bp) was analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide.

#### 2.6.3. SYBR green real-time PCR analyses

IMNV viral load from challenged *L. vannamei* juveniles (abdominal muscle samples) were determined by real-time PCR in accordance with method described by Silva et al. (2011). The reactions were performed in 96-well plates at a final volume of 25 μL and viral load was determined by extrapolating the normalized Ct values from each sample to serial dilution of standard plasmid DNA for IMNV (Silva et al., 2011). Each sample had two replicates and in all 96 well plates were included two negative controls (ultrapure water and positive Taura syndrome virus sample) and a β-actin internal control. The assays were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, USA) with SYBR Green Master Mix (Applied Biosystems, Warrington, UK). All data were analyzed using the StepOne™ software program (version 2.2.2) (Applied Biosystems, CA, USA).

### 2.7. Statistical analyses

The data of IMNV viral load from challenged *L. vannamei* juveniles (all treatments) were analyzed for homogeneity of variance using Cochran's test at a significance level of $p \leqslant 0.05$. Kolmogorov–Smirnov and Shapiro–Wilk tests were used for testing normality ($p \leqslant 0.05$) and a Kruskal–Wallis test was used to compare the viral loads in different treatments. All statistical analyses were performed using the ASSISTAT program, version 7.7 beta (Silva, 2014).

## 3. Results and discussion

All challenged *A. franciscana* were positive for IMNV through both infection routes (immersion and virus-phytoplankton adhesion) (Fig. 1). However, no clinical signs of infection or cumulative mortalities stemming from the challenges were found. Moreover, uninfected brine shrimp biomass used as negative control was IMNV-negative by conventional PCR (data not shown) and no signs of infection were observed in any of the samples.

Previous studies report the positive detection of different shrimp viruses in adult *Artemia*. Li et al. (2003) assessed the susceptibility of nauplii and adult *Artemia* to WSSV during ten days through challenges intake inoculum mixed powdered microalgae and they proved WSSV infection by PCR. In this study, similar results were observed for WSSV vertical transmission tests, which positive adult *Artemia* produced positive reproductive cysts, although the nauplii hatched from these cysts were negative by PCR, suggesting that the removal of WSSV occurred during rinsing of the nauplii. Similarly, Chang et al. (2002) were unable to detect WSSV from positive cysts by PCR analysis after rinsing of hatched nauplii, indicating its external contamination. Moreover, in both studies, no clinical signs of the disease were observed, which according to Li et al. (2003) indicates that further pathogenicity studies of longer duration should be conducted in WSSV-infected *Artemia*.

In another study, four developmental stages of *Artemia* (nauplii, metanauplii, pre-adult and adult) were experimentally challenged by WSSV via immersion and virus-phytoplankton adhesion, however only those exposed through the latter route become positive by two step PCR, indicating low viral loads (Zhang et al., 2010). In the same way, the pathogenicity of WSSV in the *Brachionus urceus* rotifer and *Nitroca* sp. and *Acartia clausi* copepod species has been demonstrated in experimental infections by virus-phytoplankton adhesion route, with positive results using nested-PCR (Zhang et al., 2006, 2007, 2008).

Liu et al. (2007) co-cultured WSSV-infected *Marsupenaeus japonicus* adults with six species of microalgae (*Isochrysis galbana*, *Skeletonema costatum*, *Chlorella* sp., *Heterosigma akashiwo*, *Scrippsiella trochoidea* and *Dunaliella salina*) and they found that all microalgae, except *H. akashiwo*, were reservoirs for WSSV, with positive detection using nested-PCR. Moreover, *Chlorella* sp. and *S. trochoidea* demonstrated the greatest capacity to transport WSSV. However, upon re-infecting *M. japonicus* juveniles with the infected microalgae, only *Chlorella* sp. proved to be a vector of mechanical transmission, suggesting that microalgae may constitute a horizontal transmission route for WSSV (Liu et al., 2007).

According to Zhang et al. (2010), virus-phytoplankton adhesion is an efficient transmission route from WSSV to zooplankton. In the present study, both transmission routes (immersion and virus-phytoplankton adhesion) proved to be efficient in infecting *A. franciscana* adult with IMNV, as demonstrated by the positive

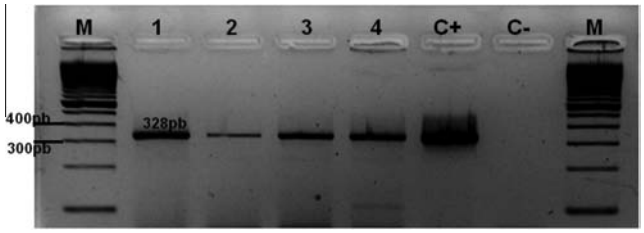


**Fig. 1.** Conventional PCR amplification for detection of IMNV in *A. franciscana* experimentally infected through immersion (samples 1 and 2) and virus-phytoplankton adhesion challenges (samples 3 and 4); C+ (positive control); C− (pure water; Invitrogen, Carlsbad, CA, USA); M (100 bp molecular ladder; invitrogen, USA).

**Table 1**
Number of infected individuals and mean viral load (copies/μg of total RNA) in four treatments of *L. vannamei* challenged through ingestion of infected *A. franciscana*.

| Treatment | No. analyzed | Positive PCR | Viral load | | |
|---|---|---|---|---|---|
| | | | Minimum | Mean | Maximum |
| 1 – *L. vannamei* juveniles fed *A. franciscana* infected by virus-phytoplankton adhesion | 30 | 14 | $8.97 \times 10^1$ | $1.34 \times 10^{2a}$ | $2.19 \times 10^2$ |
| 2 – *L. vannamei* juveniles fed *A. franciscana* infected by immersion | 30 | 3 | $1.75 \times 10^2$ | $9.31 \times 10^{2b}$ | $1.99 \times 10^3$ |
| 3 – *L. vannamei* juveniles fed minced IMNV-infected *L. vannamei* tissue | 30 | 12 | $6.07 \times 10^2$ | $1.48 \times 10^{4a}$ | $1.59 \times 10^5$ |
| 4 – *L. vannamei* juveniles fed non-infected *A. franciscana* (control group) | 30 | 0 | – | – | – |

[a,b] Means followed by the same letter (a and b) did not differ statistically ($p \leqslant 0.05$; Kruskal–Wallis test); – not detected in real-time PCR.

**Table 2**
Cumulative mortality rates in different treatments of *L. vannamei* challenged with ingestion of infected *A. franciscana*.

| Treatment | No. of individuals | Cumulative mortality rate (%) | | | | Percentage of infected individuals |
|---|---|---|---|---|---|---|
| | | 1st day | 5th day | 10th day | 15th day | |
| 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 |

results of conventional PCR (first PCR), which imply a mean viral load greater than $10^5$ copies/μg of total RNA (Silva et al., 2011).

Besides the virus-phytoplankton adhesion route, other viral routes have been used in conducting oral challenges for assessing the *Artemia* susceptibility to shrimp viruses. Hameed et al. (2002) tested two methods (immersion and oral challenge by viral suspension mixed with rice bran) for assessing the pathogenicity of WSSV on different stages of *Artemia* (nauplii, metanauplii, juvenile, pre-adult and adult) and detected the presence of this virus in all development stages following both challenges. However, using similar challenges, Sarathi et al. (2008) were unable to infect the same five development stages of *Artemia* with Monodon baculovirus, as demonstrated by the negative results of nested-PCR.

For *M. rosenbergii* nodavirus (MrNV) and extra small virus (XSV), the use of viral suspension in rice meal as oral challenge was efficient in the propagation of both viruses in all stages of brine shrimp development, as demonstrated by positive nested-PCR results, indicating that *Artemia* sp. acts as a reservoir for these viruses (Sudhakaran et al., 2006).

Positive results of infection by HPV have also been achieved with the use of similar viral suspension mix in oral challenges in all development phases of *A. franciscana*, except nauplii (Sivakumar et al., 2009). However, further studies are needed to determine whether this species is actually infected by HPV or acts as a passive carrier, although Feng et al. (2013) reported the presence of WSSV receptors in the cell membrane of brine shrimp, suggesting that this microcrustacean is a reservoir.

In the analysis of *A. franciscana* as a vector for IMNV horizontal transmission to *L. vannamei* juveniles, all treatments showed positive results by real-time PCR. However, just *L. vannamei* juveniles fed *A. franciscana* infected by virus-phytoplankton adhesion and *L. vannamei* juveniles fed minced IMNV-infected *L. vannamei* tissue treatments had infection rates higher than 40%, with no statistically significant difference in mean viral load between the two treatments ($p \geqslant 0.05$) (Table 1).

In contrast, mean viral load in these treatments differed significantly from that found in *L. vannamei* juveniles fed *A. franciscana* infected by immersion treatment, ($p \leqslant 0.05$), in which only 10% of the challenged shrimp become infected (Table 1). Probably, the zero titres (non-infected animals) found in *L. vannamei* juveniles fed *A. franciscana* infected by immersion treatment biased the data distribution, thus making it significantly different to the other treatments.

Our data demonstrated the higher efficiency of virus-phytoplankton adhesion in the propagation of IMNV and, as expected, no viral load was detected in *L. vannamei* juveniles fed non-infected *A. franciscana* (control group).

According to Zhang et al. (2010), viral particles adhered to microalgae surface are ingested and bioaccumulate in *Artemia*, which is a filter feeder organism. The particles are then transmitted to shrimp that feed on this microcrustacean. Although the authors did not observe massive mortality rates during the 15-day experiment, all shrimp challenged with *A. franciscana* previously infected using virus-phytoplankton adhesion route were tested positive for WSSV in nested-PCR.

In general, infection routes involving the ingestion of viral particles by *Artemia*, such as a viral suspension in rice bran, have demonstrated the role of this organism in the horizontal transmission of MrNV, XSV and HPV in the postlarval stage of *M. rosenbergii* and *P. monodon* (Sudhakaran et al., 2006; Sivakumar et al., 2009).

Among all the experimental treatments tested in the present study, only the *L. vannamei* juveniles fed minced IMNV-infected *L. vannamei* tissue treatment led to mean viral loads similar to those found in shrimp naturally infected by IMNV ($3.09 \times 10^4$ to $6.85 \times 10^8$ copies/μg of total RNA) (Silva et al., 2011). Moreover, this was the only treatment in which clinical signs of infection were found in the challenged animals, such as multifocal opacity in the muscles of the abdominal segment and uropods.

These results contrasts to those of Silva et al. (In press) that challenged shrimp groups by feeding them, during three days, with infected minced muscle tissue at 4% body weight. They reported that no gross signs of the disease were present, neither mean viral loads corresponded to natural IMNV infections.

In all treatments, cumulative mortality was kept within the expected range under cultured conditions (no virus), as no massive deaths were observed (Table 2). This finding is in agreement with data reported in previous studies involving the infection of *L. vannamei* postlarvae with WSSV through infected *Artemia* in immersion and virus-phytoplankton adhesion bioassays, in which no massive deaths or differences in mortality rates were found among the different treatments tested (Zhang et al., 2010).

## 4. Conclusions

The present findings demonstrate the susceptibility of *A. franciscana* to IMNV infection by two transmission routes (immersion and virus-phytoplankton adhesion) and the role of this microcrustacean as reservoir or mechanical vector in IMNV

horizontal transmission to *L. vannamei* under experimental conditions tested. Furthermore, although no mass mortalities were detected in *L. vannamei* fed IMNV-infected *A. franciscana* during the 15-day experiment, these infected brine shrimp should not be disregarded as a source of IMNV on shrimp farms units due to increase in this pathogen prevalence.

Another point is that these results represent a crucial issue for selective breeding programs to IMNV resistance, as artificial infection was achieved only by intramuscular injections (White-Noble et al., 2010; Silva et al., In press). Thus, our findings highlighted that *A. franciscana* could be an effective alternative to these laborious process of infection.

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