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Water temperature influences viral load and detection of White Spot Syndrome Virus (WSSV) in *Litopenaeus vannamei* and wild crustaceans

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ABSTRACT

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

1. Introduction

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

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

The amplification of viral loads and onset of disease can be induced by environmental or physiological stress or ambient temperatures (Lotz et al., 2005; Sánchez-Martínez et al., 2007). Optimum temperature for growth and survival of shrimp varies according to the life stage and the species. For *Litopenaeus vannamei*, for example, optimum temperature ranges from 27 °C to 30 °C (Wyban et al, 1995). Highest

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survival of juvenile *L. vannamei* is obtained between 20 °C and 30 °C (Ponce-Palafox et al., 1997). Vidal et al. (2001) discovered that warm-water (32 °C) conditions provided a workable way to control mortalities of *L. vannamei* from WSSV. Mortality of WSSV-infected shrimp or crayfish was reduced or even totally stopped at a higher (32–33 °C) or lower (<15 °C) water temperature in comparison to the optimum temperature range (Du et al., 2006; Guan et al., 2003; Jiravanichpaisal et al., 2004). At optimum temperature (26–27 °C), differences in virulence between WSSV strains have been reported (Rahman et al., 2006; Wang et al., 1999). Guan et al. (2003) reported that viral concentration was lower at 15 °C than at 23–28 °C. The suggested mechanisms to explain this findings include reduced replication (Du et al., 2006), apoptosis (Granja et al., 2003, 2006) and altered gene expression of WSSV (Reyes et al., 2007). WSSV replication was also inhibited at 4 °C and at 32 °C in primary culture of hematopoietic tissue of crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2006).

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

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

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

2. Materials and methods

2.1. Sample collection

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

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

2.2. Inoculum preparation and injection procedure

Approximately 15 g of WSSV-infected *L. vannamei* tissue was diluted 1:5 (w/v) with sterile PBS buffer (pH 7.8) and homogenized. The homogenate was clarified with two centrifugation cycles, 10,000 × *g* for 15 min and 15,000 × *g* for 20 min. The homogenate was filtered in a 0.45 μm filter and used for injecting experimental animals. The concentration of WSSV stock was assessed by real-time PCR. Shrimp were injected intramuscularly into the third dorsal segment with 50 μL of either PBS buffer or WSSV inoculum (6.2×10^3 copies/μL) using a sterile 1 mL syringe fitted with a 21G needle.

2.3. Experimental design

2.3.1. Effect of water temperature on viral replication

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

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

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

2.4. DNA extraction and WSSV qPCR analysis

DNA was extracted from hemocytes samples using a silica matrix (GeneClean Spin Glass Milk—MP Biomedicals, Inc) with slight modifications of the manufacturer's protocol. Hemolymph (300 μL) was withdrawn from the ventral sinus, with a 1 mL syringe and a 21G needle containing anticoagulant solution (EDTA 20 mM, KCl 10 mM, NaCl 450 mM, HEPES 10 mM), proposed by Vargas-