Effects of temperature on hematological and histopathological changes and survival rate of juvenile *Fenneropenaeus vannamei* experimentally challenged to White Spot Virus

Kakoolaki S. [1*]; Sharifpour I. [1]; Afsharnasab M. [1]; Sepahdari A. [1]; Mehrabi M. R. [1]; Ghaednia B. [2]; Nezamabadi H. [1]

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Abstract

Many shrimp farmers were suffering from White Spot Disease (WSD) onset in last decades. Oscillation of environmental factors could lead mortality in susceptible hosts. Our study was aimed to investigate the effect of different temperatures on juvenile *Fenneropenaeus vannamei* experimentally exposed to White Spot Virus (WSV). Five hundred and forty juveniles were distributed among 3 treatments in triplicates, 22, 25 and 30°C and experimentally WSV were injected in the shrimps. Our results showed mortality started at 36 h post inoculation (hpi) in the treatment at 25°C (T_{25}), meanwhile the mean value of mortality percent at 54 hpi in T_{25} (71.10±17.35) showed the significant difference ($p=.045$) with T_{22} (3.33±3.33) and T_{30} (Not Observed, NO.). Our results suggest that in site selection, in primary stage of farm designing, water temperature at more than 29°C, should be considered as key environmental factor. This finding can lead us that why the White Spot Disease occurred with high mortality in some area when the days of shrimp culture were prolonged until mid autumn.

Keywords: White Spot Syndrom Virus, Temperature, *Fenneropenaeus vannamei,* Challenge

1- Iranian Fisheries Research Organization., P.O.Box: 13185-116, Tehran, Iran

2- Iran Shrimp Research Center, P.O.Box:1374, Bushehr, Iran

*Corresponding author's email:bsh443@gmail.com

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Introduction

Almost farmers were suffering from White Spot Virus invaded to the shrimp, *Pennaeus indicus* farms in Iran during 1992-2006 (Kakoolaki et al., 2010) and then *F*enneropenaeus *vannemei* SPF (Specific Pathogen Free) were transported from Hawaii to Iran to control the disease (Kakoolaki et al., 2011). Shrimp culture has been a familiar industry and well extended throughout the world especially in south-east of Asia since 1990 (Sánchez-Martínez et al., 2007). Estimated losses due to WSV only in Asia have been approximately $ 6 billion (Lightner, 2011). Small size of the ponds, abrupt alteration in physico-chemical criteria specially salinity and temperature and prolonged the days of culture in the shrimp farms result in few epizootic of White Spot Diseases (WSD) in Iran in last decade (Kakoolaki, 2004; Kakoolaki et al., 2011; Peinado-Guevara and Lopez-Meyer, 2006; Soltani et al., 1998). So far, the effective way of temperature on WSV outbreak has not been well known. Rahman et al. (2007; 2006) showed that higher temperature with 33°C could be used to control the mortality of WSV infected shrimp in the field. Due to effects of temperature on metabolisms, growth, survival rate and immunological criteria, it becomes one of the most important environmental factor in shrimp farms (Wyban et al., 1995). Esparza-Leal et al. (2010) showed if days of shrimp culture extend to autumn, the susceptibility to WSV among the exposure shrimps could be increased (Esparza-Leal et al., 2010). The risk of WSV outbreak is reduced when the water temperature goes up and salinity fluctuation is low (Tendencia et al., 2010b). In contrary to the results of Sahoo et al. (2005), the association between WSV outbreak and environmental risk factors such as temperature (Corsin et al., 2002; Peinado-Guevara and Lopez-Meyer, 2006), ammonia (Corsin et al., 2001) and salinity (Kakoolaki et al., 2011) Some scientist studied the effects of temperature on the expression of WSV gene (Granja et al., 2006; Reyes et al., 2007). Our study was aimed to investigate the effect of different temperature on mortality criteria in *Fenneropenaeus vannemai* exposed to WSV.

Materials and methods

Five hundred and forty juveniles of *P. vannamei* (7.98±0.54) were gathered from Quarantine research shrimp farm located in Bushehr province in south of Iran and then transferred to the Iran Shrimp Center for experimental and laboratory examinations. Shrimp were then acclimated to the determined conditions as treatments and corresponding controls in triplicates (T_1= 22°C named 22, T_2=25ºC named 25 and T_3=30 ºC named 30 as treatments 1, 2 and 3). The shrimp were then distributed to 18 glass aquariums (50×50×60cm) which 60% of each was filled with well clean aerated seawater (42 ppt). The shrimp were kept, fed and siphoned in the current condition for 40 days before exposure to the virus, according to our previous article (Kakoolaki et al., 2011).

Virus with the titre of $LD_{50}= 1\times10^{5.4}$ and code no. of WSV / irn / 1 / 2011 already had prepared in Motamedi lab in Iran were used in challenging with the treatments but control

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groups were left untreated. The mixture was stored at -80 ºC until use (Motamedi Sedeh et al., 2011) . After acclimation, Shrimps were intramuscularly inoculated with 50 µl of virus solution (Kakoolaki et al., 2011).

After the mortality observation, 0.2ml of Hemolymph were withdrawn from the second leg's basement of ventral segments from 3 moribund shrimp of each triplicates in treatments and controls, using 1 ml syringe along with 26 gauge needle. Each syringe pre-filled with 0.8 ml Alsever solution as anticoagulant (Kakoolaki et al., 2010; Kondo, 2003). According to the method of Afsharnasab et al. (2009) the whole body of these three moribund shrimp were prepared to transfer to histopathology and PCR labs of Iran Shrimp Center. The appendages and gill tissues were cut, placed in Eppendorf micro tubes containing ethanol 70% for PCR technique using IQ2000 WSV commercial kit. Remaining tissues were placed in tubes containing Davidson's fixative. Davidson's fixative were discarded and replacing with ehanol 70% after 48-72 h in the lab and staining by H & E method(Lightner, 1996).

THC was carried out using the Neubauer type's Hemocytometer. A drop of hemolymph-anticuagulant mixture was then placed on the Hemocytometer and counting the cells under a light microscope ×10 as follows:

THC (Cells. Ml^{-1}) =Average count of 5 cells$\times 5\times 10^{4}\times$1/dilution (Kakoolaki et al., 2010).

0.2 ml of withdrawn Hemolymph was pre-filled with 0.1 ml fixative. Differential Hemocyte Count (DHC) was carried out using a slide, a drop of mixture solution was then placed on it and staining with May-Grundwald Giemsa (MGG) method begun after then. The method for fixation and staining of the hemolymph was carried out based on the new methods given in previous article (Kakoolaki et al., 2011)

TPP ($\mu g.dl^{-1}$) was examined using centrifuge (3000 rpm for 10 min. at 4^{o}c) the mixture of distinct anticoagulant and collected hemolymph (n=6) of each triplicate of the treatments. The supernatants were remove and transferred after 24 h with freezing condition to the lab to examine TPP of the treatments using biochemical autoanalyzer (Technicon, RA 1000).

The PCR examinations of the treatments were assessed using IQ2000, WSV commercial kit after exposure the shrimp to the virus.

One Way-ANOVA and Bonferroni multi-comparison tests were carried out to compare the effects of different degrees of temperature on mortality rate, Epigastric hematopoietic spheroid values and THC, TPP and DHC values after challenging the virus against the treatments using SPSS 17. The mean values of criteria are given as Mean ± SE of Mean.

Results

The results of the PCR of the primary stocking of shrimp were negative before the exposure to the virus was begun but based on the Figures 2-6 the hypertrophied cells and the inclusion bodies of the WSV observed through to the target tissues such as gills and epithelial cells of gut, interstitial spaces of hepatopancreas and heart muscle cells. Histopathological finding showed the severe

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infection in T25 in comparison to T22 and T30, respectively. The PCR results of the treatments were positive (based on the IQ2000, WSV commercial kit) after exposure the shrimp to the virus (Fig. 1).

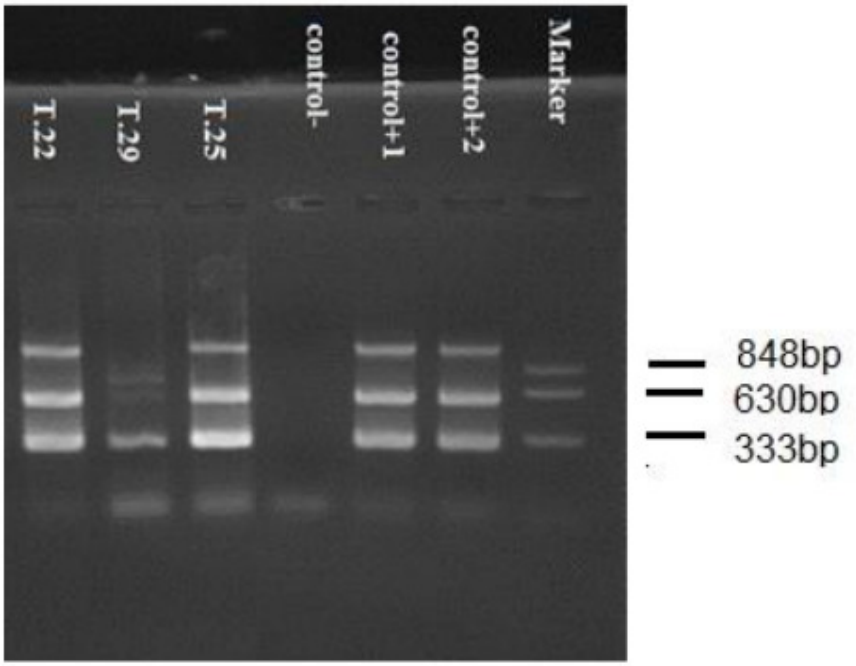

Figure 1: The positive result of PCR for the WSV of the pooled samples from each treatment (IQ2000, WSV commercial kit)

Cumulative mortality and mortality percent were shown in figure 7 and table 1. According to these, the mortality percent at 54 h post inoculation (hpi) in T_{25} (71.10±17.35) had significant difference (*p=.045*) with T_{22} (3.33±3.33) and T_{30} (Not Observed, NO.).

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Table 1: Mortality percent differences between treatments (α=0.05, *n*=3)

		Mean±Std. Error	Sig.
mortality.rate.54h	22	3.33±3.33[a]	.045
	25	71.10±17.35	
	30	.00[a]	
	Total	24.81±12.65	
mortality.rate.72h	22	26.70±.00	.089
	25	28.90±17.35	
	30	11.13±4.82	
	Total	22.24±5.90	
mortality.rate.96h	22	24.43±4.82[a]	.037
	25	.00	
	30	23.30±.00[a]	
	Total	15.91±4.21	
mortality.rate.110h	22	23.33±3.33[a]	.043
	25	.00	
	30	12.23±2.93a	
	Total	11.85±3.60	
mortality.rate.138h	22	16.63±8.81	.051
	25	.00	
	30	31.10±4.45	
	Total	15.91±5.32	
mortality.rate.170h	22	5.56±1.13[a]	.021
	25	.00[a]	
	30	22.23±3.99	
	Total	9.26±3.54	

*Different scripts show the significant differences at the level of .05

Based on the table 2 the quantity of Spheroids more developed ($p< .05$) in T_{25} (7.50±0.18) and T_{22} (6.37± 0.26) in comparison to T_{30} (1.62± 0.53).

[DOR: 20.1001.1.15622916.2014.13.1.8.8]

Table 2: Spheroid formation in different treatments

spheroids	N	Mean±Std. Error	Minimum	Maximum	Sig.
22oc	8	6.37± 0.26[a]	6.00	8.00	
25oc	8	7.50± 0.18[a]	7.00	8.00	.000
30oc	8	1.62± 0.53	.00	4.00	
Total	24	5.16± 0.56	.00	8.00	

*Different scripts show the significant differences at the level of .05

The mortality of T_{25} started in 36 hpi with about 33.30 % of stock and completed after 60 h.

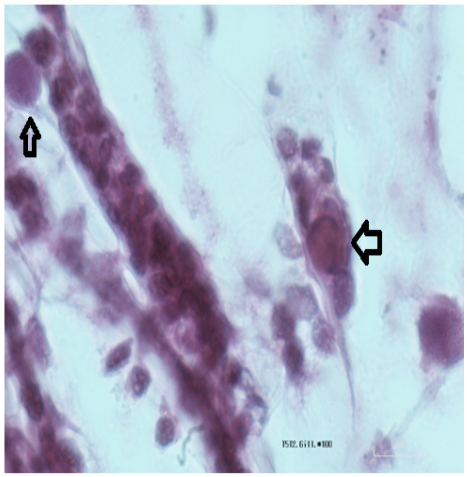

Figure 2: Infected epithelial cells in gill, H & E, Scale bar: 10, ×100

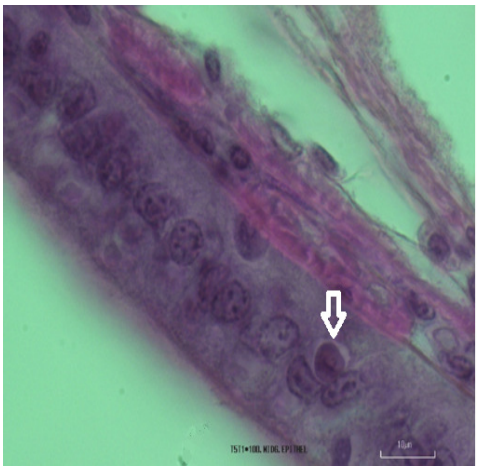

Figure 3: Infected epithelial cell in mid gut, H & E, Scale bar: 10, ×100

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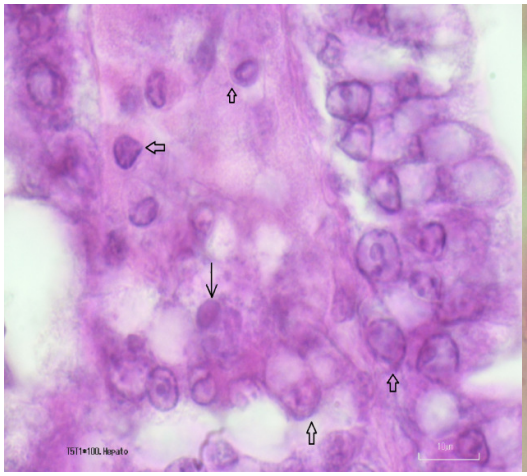

Figure 4: interstitial spaces of hepatopancreas imply to severe infected cell in T_{25}, H & E, Scale bar: 10, ×100

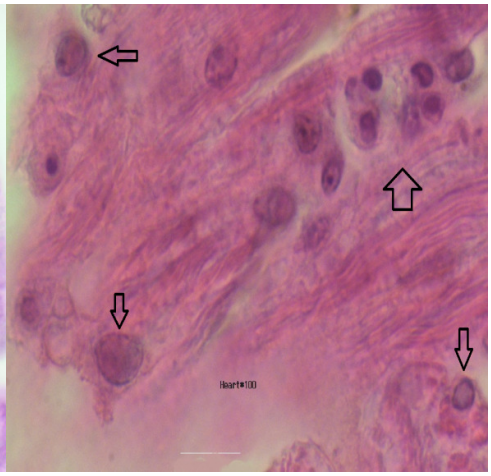

Figure 5: Arrow shows severe Infection in heart muscle cells of shrimp in T_{25}, H & E, Scale bar: 10, ×100

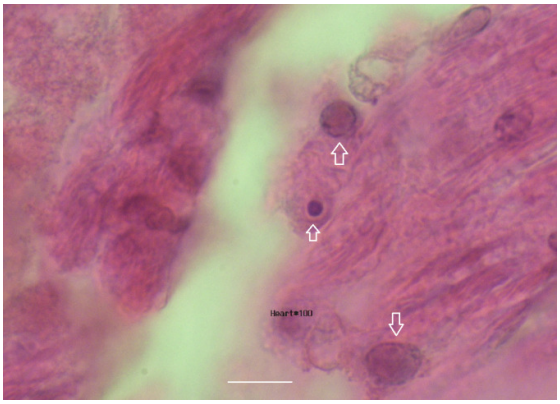

Figure 6: Shows mild Infection in heart muscle cells of shrimp in T_{30}, H & E, Scale bar: 10, ×100

The maximum mortality percent (71.10) was occurred in T_{25} at the primary hours (36 h) after inoculation but and mortality in T_{22} and T_{30} begun at 58 h. The mortality percents of T25 became 100% at 60 h with 28.90% but in T_{22} and T30 were completed at 152 and 170 hpi, respectively. The mean value of total protein, total hemocte count (THC) , total differentiate count containing large granular cell (LGC), semi granular cell (SGC) and hemocyte count (HC) were given in table 3. The mean value of total protein in T_{30} shrimp hemolymph (157.82± 22.59$^{)}$ was significantly higher (p= .012) than that of in T_{22} (76.60± 3.57) and T_{25} (106.72± 17.80) but no significant difference between T_{22} and T_{25} was

[DOR: 20.1001.1.15622916.2014.13.1.8.8]

observed ($p > .05$). The mean value of THC in T_{30} (16.06± 2.48) was significantly higher than those of T_{22} (8.96± 1.81)and T_{25} (7.52± 1.18). Of course, there is a significant difference between the two last (p<.05). *No mortality were observed in whole control groups (fig. 7).

Table 3: Hemolymph parameters in different treatments

		N	Mean ±Std. Error	Minimum	Maximum	Sig.
TOTAL PROTEIN ug/dl	22oc	6	76.60± 3.57 [ab]	66.60	90.90	.012
	25oc	6	106.72± 17.80 [ab]	60.00	175.00	
	30oc	6	157.82± 22.59 [b]	81.80	220.00	
	Total	18	113.71± 12.18	60.00	220.00	
THC×10^5	22oc	6	8.96± 1.81[a]	2.10	15.00	.014
	25oc	6	7.52± 1.18[ab]	3.40	12.00	
	30oc	6	16.06± 2.48[ac]	9.60	22.60	
	Total	18	10.85± 1.37	2.10	22.60	
LGC×10^5	22oc	6	30.33± 1.68[a]	26.00	35.00	.046
	25oc	6	28.83± 1.01[ab]	26.00	33.00	
	30oc	6	34.16± 1.44[ac]	29.00	38.00	
	Total	18	31.11± 0.93	26.00	38.00	
SGC×10^5	22oc	6	46.83± 1.49	43.00	52.00	.002
	25oc	6	56.50± 2.17[a]	48.00	63.00	
	30oc	6	54.50± 0.92[a]	52.00	57.00	
	Total	18	52.61± 1.33	43.00	63.00	
HC×10^5	22oc	6	21.50± 1.38	17.00	27.00	.000**
	25oc	6	14.66± 1.74[a]	10.00	22.00	
	30oc	6	10.33± 0.21[a]	10.00	11.00	
	Total	18	15.50± 1.31	10.00	27.00	

*Different scripts show the significant differences at the level of .05

**Significant difference was observed at the level of .01

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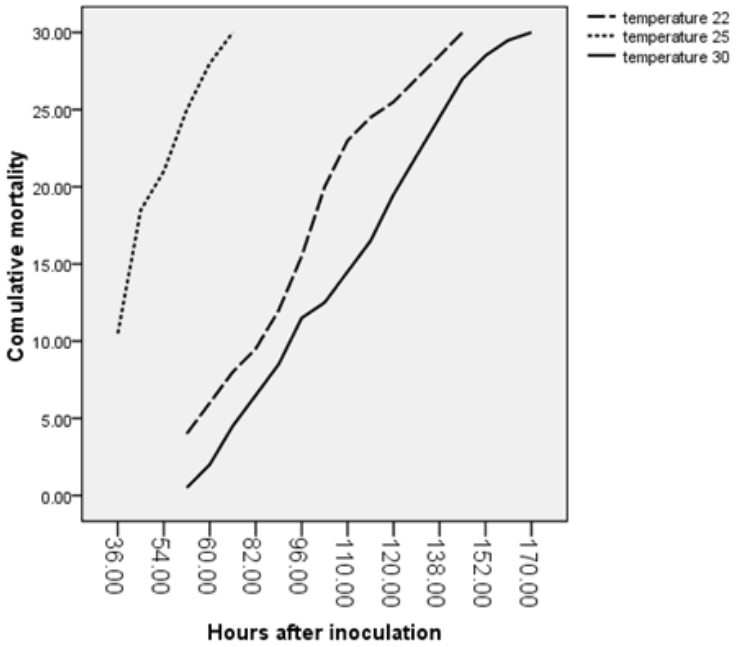

Figure 7: Cumulative mortality in different treatments after determined hpi.

Discussion

Oscillations of environmental factors could result in high mortality rate in shrimp farms. Due to the effect of these factors e.g. temperature increase susceptibility of the host as well the viral replication process(Moser et al., 2012). According to the figure 1, it seems that the load of the virus in T_{30} was lower than that of the T_{22} and T_{25}. In addition, the mortality of T_{30} showed (Fig. 1 , Table 1) a slight trend in contrary to T_{25}. This result is close to finding of Granja et al. (2006) that showed the load of White Spot Virus (WSV) is reduced when the shrimp maintain in higher temperature. Other scientists (Du et al., 2006; Guan et al., 2003) showed that increasing the temperature to around 30 and more could reduce the replication of WSV even stop it in *Marsupenaeus japonicas* and *procambarus clarkia,* respectively. Similar to the results of Granja et al. (Granja et al., 2006) and Vidal et al. (2001) that showed the optimum temperature for loading the virus in *P. vannamei* is 25°C, our finding confirmed that the higher mortality in *P. vannamei* with water salinity of 42 ppt is 25°C and the temperature of 30°C (Figs 1, 7) is less predisposing degree for loading virus and. This findings are contradicted to the result of Moser et al. (2012) that showed the mortality rate is low and the replication of WSV is high in the shrimps kept in 29-30°C. But in similar to our finding, they showed the WSD onset occurred 36 hpi in shrimps kept in temperature at 25°C and contrarily, they found lower temperature allows no replication of the virus. The daily water-temperature fluctuations with hyperthermia (>32 C) could partially reduce WSV replication in some ponds (Rahman et al., 2007; Vidal et al., 2001) but this partial protection decreased when water temperature

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declined from 28.0–33.4^{O}C to 24.9^{O}C while WSD onset was observed in almost cages (Esparza-Leal et al., 2010). Similarly, Tendencia, et al. (2010a) showed the higher prevalence of WSD in the sea was higher in November until January and confirmed WSD onset in earthen ponds is associated with lower degrees of temperature (Tendencia et al., 2010b). Results showed that hyperthermia reduces the expression of WSV genes on shrimp sub-cuticular epithelial cells (Reyes et al., 2007).

Following our results, the mean value of total protein and THC are higher in temperature 30^{O}C than temperatures 22 ^{O}C and 25 ^{O}C. Based on the table 3 the percent value of LGC in T30 is significantly higher (P=.046) than the other treatments and followed by T_{22} and T_{25}. Sudden mortality 36 hpi in T_{25} could be due to the stressfulness of this degree for the shrimp and or optimum degree for replication of the virus. This finding can lead us that why the White Spot Disease occurred in Khuzestan in south of Iran when the days of shrimp culture were prolonged until mid autumn. White Spot Disease onset occurred in all area in south of Iran exception Hormozegan province where the pond water temperature was usually more than the 29-30^{O}C during the summer. But in Khuzestan province the fluctuation of pond-water temperature within 24 h sometimes reach 5-7 degrees (Kakoolaki, 2004). Our results suggest that in site selection in primary stage of farm designing, water temperature more than 29^{O}C, such as Hormozegan province in south of Iran, should be considered.

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