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Hypoxia increases susceptibility of Pacific white shrimp to white spot syndrome virus (WSSV)

[*Condição de hipóxia aumenta a susceptibilidade do camarão branco do Pacífico ao vírus da mancha branca (WSSV)*]

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ABSTRACT

The present study aimed to evaluate the mortality, reactive oxygen species production (ROS) and total hemocyte counts (THC) of the marine shrimp *Litopenaeus vannamei* infected with the white spot syndrome virus (WSSV) at three levels of oxygen saturation. For this, 360 shrimp (20±2g) were distributed in 24 tanks (60L), divided in two groups (infected and non-infected), which were subjected to 30, 60 and 100% of dissolved oxygen saturation (in quadruplicate). During 96 hours after infection, daily hemolymph samples were collected for hemato-immunological parameter evaluation (THC and ROS) and dead animals were removed and computed to assess cumulative mortality rates. In the infected group, animals subjected to 100% saturation showed higher ROS production ($P<0.05$) after 48 hours, while THC was significantly reduced ($P<0.05$), regardless of oxygen saturation. The hypoxia resulted in high mortality when compared to 100% saturation condition. In the uninfected group, no significant differences were observed in all evaluated parameters. Thus, the hypoxia condition increased the susceptibility of shrimp to the infection of WSSV, which may be partly related to the low ROS production showed by the animals subjected to 30% oxygen saturation.

Keywords: *Litopenaeus vannamei*, WSSV, dissolved oxygen saturation, immune response, mortality

RESUMO

O presente estudo teve por finalidade avaliar a mortalidade e a contagem total de hemócitos (CTH) e espécies reativas de oxigênio (EROs) de camarão Litopenaeus vannamei *infectados com o vírus da mancha branca (WSSV) e submetidos a três níveis de saturação de oxigênio. Para tanto, 360 camarões (20±2g) foram distribuídos em 24 tanques (60L), divididos em dois grupos, infectados e não infectados e submetidos a 30, 60 e 100% de saturação de oxigênio (em quadruplicata). Após a infecção, diariamente foram coletadas amostras de hemolinfa dos animais para avaliação dos parâmetros hematoimunológicos (CTH e EROs) e foi estimada a mortalidade, por 96 horas. No grupo com infecção, os animais submetidos à saturação de 100% apresentaram um aumento na produção de EROs ($P<0,05$) após 48 horas, ao mesmo tempo em que a CTH demonstrou uma redução ($P<0,05$) independentemente da saturação do oxigênio, e a condição de hipóxia acarretou maiores mortalidades quando comparada à do grupo com 100% de saturação. No grupo sem infecção, não foram observadas diferenças significativas nos parâmetros avaliados nem mortalidade. Dessa forma, pode-se concluir que a hipóxia aumentou a susceptibilidade do camarão à infecção com o vírus da mancha branca, que pode estar, em parte, relacionada com a baixa contagem de hemócitos e produção de EROs observadas nos animais submetidos a essa condição.*

Palavras-chave: Litopenaeus vannamei, *WSSV, saturação de oxigênio, resposta imune, mortalidade*

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INTRODUCTION

Diseases are the result of an unbalanced interaction between pathogen, environment and host. Thus, an animal in a safe environment may carry an infectious agent without manifesting signs of the disease. In the production of aquatic organisms, the occurrence of inadequate water quality parameters may disturb their homeostasis, leading to increased susceptibility to diseases (Le Moullac and Haffner, 2000).

In shrimp, like other invertebrates, protection against diseases solely depends on the innate immune system. There is no immunological memory in these animals, and the non-self recognition depends on pattern recognition proteins (PRPs) that detect pathogen-associated molecular patterns (PAMPs). In general, the innate immune system of invertebrates is related to their hemolymph, composed of a cellular fraction (hemocytes) and a liquid fraction (plasma), where humoral factors are dissolved (Barracco *et al.*, 2008).

Hemocytes are immunocompetent cells responsible for cellular responses in crustaceans. These cells respond to the invasion of microorganisms and parasites, destroying them by phagocytosis or isolating them in hemocyte aggregates, forming nodules and capsules (Barracco *et al.*, 2008). During phagocytosis, the microorganisms are engulfed by hemocytes, and molecules with potent microbicidal activity and cytotoxicity are produced and released. (Bogdan *et al.*, 2000; Campa-Córdova *et al.*, 2002). Reactive oxygen species (ROS) are molecules with unpaired electrons in their outer layers, which oxidize surrounding components, such as membranes and proteins. Thus, thanks to their microbicidal potential and cytotoxicity, they affect the infectious agents (Roch, 1999).

The immune parameters in shrimp are influenced by environmental factors such as temperature, salinity (Wang and Chen, 2006) and ammonia levels (Jiang *et al.*, 2004). According to Bachére (2000), the immune parameters of *L. vannamei* are very sensitive to many factors and can be changed for no apparent reason.

Dissolved oxygen levels are directly related to good water quality. The effect of dissolved oxygen saturation values below 100% on physiology has been described by many researchers in various crustacean species. Jiang *et al*. (2005) evaluated the effect of hypoxia on *L. vannamei,* and affirmed that in 48 hours, at a dissolved oxygen concentration of 3.5mg/L, phenoloxidase activity increased, while the total hemocyte count decreased. According to Zhang *et al*. (2006), the physical and chemical parameters of water, the stage of development and luminosity can change the sensitivity of shrimp to lack of oxygen. Increased mortality and reduced osmoregulatory capacity were observed in *Litopenaeus stylirostris* under hypoxia during the moult cycle (Mugnier and Soyez, 2005). Le Moullac *et al*. (1998) and Cheng *et al*. (2002) showed that hypoxia increases the susceptibility of shrimp to bacterial diseases.

Several authors have conducted studies on the interaction between white spot syndrome virus infection and water quality parameters. Vidal *et al*. (2001) determined the survival rates of *L. vannamei* infected at different temperatures, obtaining 92.5% of survival at temperatures ranging from 37°C to 40°C, while Rahman *et al*. (2006) indicated that the most effective temperature to reduce viral infectivity is 33°C. Jiravanichpaisal *et al*. (2004) evaluated the effect of temperature on crayfish *Pacifastacus leniusculus* and found that lower temperatures reduced viral infectivity. Also, Liu *et al*. (2006) observed that mortality rates increase with decreased salinity in *Fenneropenaeus chinensis.*

In view of the above, the present study aimed to assess mortality rates, total hemocyte count (THC) and reactive oxygen species (ROS) in *Litopenaeus vannamei* shrimp infected with the white spot syndrome virus (WSSV) at three oxygen saturation levels.

MATERIAL AND METHODS

Three hundred and sixty (360) shrimp (20±2g) of the *Litopenaeus vannamei* species, from a specific pathogen free (SPF) strain of compulsorily notifiable diseases by the World Organization for Animal Health (OIE), bought from Aquatec LTDA (Canguaratema, Rio Grande do Norte) and cultivated in the Marine Laboratory of Universidade Federal de Santa Catarina (UFSC) were used in the experiment.

These animals were transported to the Laboratory of Bioassays of Instituto Federal Catarinense Campus Araquari (IFCCA), distributed in 24 tanks (60L, remaining in acclimatization for 48 hours, in filtered and UV sterilized water at a temperature of 23°C, 35‰ salinity, under constant aeration and without feeding.

Then, the shrimp were divided into three groups, which were subjected to 30 (2.07mg/L), 60 (4.14mg/L) and 100% (6.9mg/L) of dissolved oxygen saturation in water, in a completely randomized design (in quadruplicate). The number of animals housed per tank was 15, observing a maximum of four liters of water per animal. The water used had the same acclimatization conditions.

The aeration control system was composed of two gas supply networks. One network supplied air, using a blower of 0.25 hp to the 24 tanks through a set of hoses, each one with its own air flow control. A second network distributed nitrogen for the treatment tanks with 30 and 60% dissolved oxygen saturation. The desired saturation level was obtained by the animal consumption (Le Moullac *et al*., 1998; Mugnier and Soyez, 2005) allied with bubbling nitrogen gas into the water column until the desired level was reached (Jiang *et al*., 2005). The maintenance of target oxygen saturations was performed by controlling air flow (4x/day) and keeping an ethylene vinyl acetate (EVA) layer on water surface. The layer restricted gas exchanges with the atmosphere due to decrease in air water interface.

After acclimatization, the animals remained for another 24 hours under the three experimental dissolved oxygen saturations. Then, they were divided into two groups for the experiment: 12 tanks for the infected treatment and 12 tanks for the uninfected treatment, under the same oxygen saturation conditions. The shrimp in the infected treatment were challenged with the WSSV, receiving a dose of 50µL of experimental inoculum (needle 13x4.5), in the abdominal muscle between the first and second segments. The animals in the uninfected treatment received shrimp virus-free as inoculum (as described by Guertler *et al*., 2013).

The infective material was obtained from samples collected at shrimp farms in the northern and southern regions of the state of Santa Catarina, during the 2004/2005 outbreak and stored at -20°C. The samples were mechanically crushed and homogenized in buffer (330mM NaCl, 10mM Tris, pH 7.4) (1:10 w/v), being centrifuged at 2000 x *g* for 20 minutes at 4°C. The supernatant was centrifuged at 9000 x *g* for 10 minutes at 4°C, filtered (0.22µm) and preserved in liquid nitrogen (as described by Guertler *et al*., 2013).

The pre-inoculum produced was injected (50µl) in healthy SPF animals (free of WSSV, IMNV and IHHNV). At the first signs of the disease, the animals were sacrificed to obtain the experimental inoculum using the methodology described above. The presence of the WSSV in the experimental inoculum was confirmed by PCR (nested-PCR, Concepto Azul® kit).

During the experiment, every 24 hours the hemolymph was collected for the assessment of total hemocyte count (THC) and reactive oxygen species (ROS), with the elimination of the sampled animals, also, the dead animals were removed and computed to assess cumulative mortality rates, and the presence of WSSV was confirmed by PCR.

The collection of hemolymph was performed with a chilled syringe containing MAS anticoagulant (336mM NaCl, 115mM glucose, 27 mM sodium citrate, 9 mM EDTA, pH 7.2) at the final ratio of 1:3 (hemolymph:MAS). The collection was performed in the region between the last cephalothorax and the first abdominal segments with 1 mL syringe coupled to a 13x4.5 needle. One aliquot of 30µL was added in anticoagulant solution with 4% formaldehyde (formaldehyde/MAS), 1:3 ratio, for cell count, and the remainder was used in ROS evaluation.

Cell count was performed using a Neubauer chamber. The ROS production was quantified by NBT reduction assay, according to Guertler *et al*. (2010), using laminarin (2mg.mL^{-1}) to elicit superoxide anions (O_2^-) production by hemocytes.

The data were tested for homoscedasticity (Levene's test) and then ANOVA factorial was performed, with α=5%. Since differences were

found between the means, Tukey test for comparison of means was used at 5% probability.

RESULTS

No mortality and no abnormal behaviors were observed in the animals from the uninfected treatments. On the other hand, 72 hours later, in the group of infected animals, some shrimp became lethargic or moved erratically, regardless of the oxygen saturation levels. Cumulative mortality was higher in the treatments were shrimp were subjected to lower levels of dissolved oxygen saturation (Figure 1). The animals that died did not develop white spots on the exoskeleton, which is a characteristic of the White Spot Syndrome.

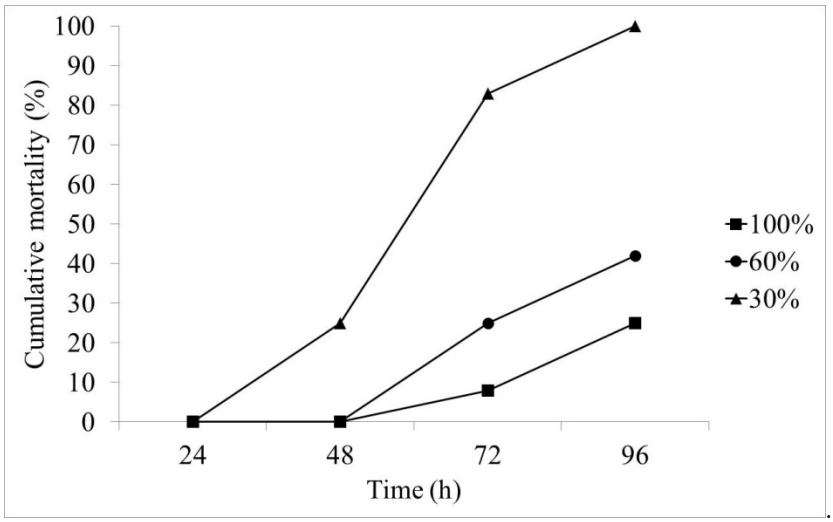

Figure 1. Percentage of cumulative mortality of *L. vannamei* during the experiment under three dissolved oxygen saturations and inoculated with the WSSV

Regarding the total hemocyte count in uninfected animals, there was no significant difference between all levels of dissolved oxygen saturation at any of the assessed periods (unreported data). The infected animals, in turn, showed a significant decrease in THC ($P<0.05$) from 48 hours on (Table 1).

Table 1. The total hemocyte count (THC) of the *L. vannamei* subjected to three oxygen saturation levels and challenged with the WSSV (different letters indicate significant difference by the Tukey test, $P<0.05$).

Total Hemocyte Count (number of cells x 10^6.mL^{-1})					
Oxygen saturation (%)	Time (h)				
	0	24	48	72	96
100	21.38±2.40a	25.69±7.71a	4.68±1.32b	5.48±1.95b	2.46±0.82b
60	20.00±3.16a	30.11±5.01a	8.38±1.20b	7.97±1.33b	2.15±0.90b
30	20.98±3.23a	21.38±5.15a	9.28±2.83b	5.32±1.41b	2.71±0.55b

The production of reactive oxygen species showed a similar behavior in all assessed treatments, with increase in 24 hours and decrease 72 hours after inoculation. In general, the results of the uninfected treatments showed large variation, though not significant (unreported data). In the infected animals, the treatment with 100% dissolved oxygen saturation showed a significant increase ($P<0.05$) in the ROS production in 48h, when compared to the treatment with 30% dissolved oxygen saturation (Table 2).

Table 2. Production expressed by absorbance of *in vitro* NBT reduction assay of superoxide anions in marine shrimps of the *L. vannamei* species subjected to three oxygen saturation levels and challenged with the WSSV (different letters indicate significant difference by the Tukey test, P<0.05)

Reactive oxygen species production (absorbance 630nm)					
Oxygen saturation (%)	Time (h)				
	0	24	48	72	96
100	0.332±0.155abc	0.567±0.127cd	0.629±0.194d	0.372±0.092abcd	0.349±0.078abc
60	0.274±0.082ab	0.496±0.118bcd	0.447±0.065abcd	0.376±0.075abcd	0.295±0.082ab
30	0.217±0.043a	0.450±0.023abcd	0.315±0.036abc	0.297±0.074ab	0.343±0.086abcd

DISCUSSION

The infected animals had lethargy and died, though without the occurrence of white spots, typical of the White Spot Syndrome as indicated by Mathew *et al*. (2007). Cumulative mortality of animals during the experiment showed significant difference regarding the dissolved oxygen saturation level in the infection treatments, indicating an influence of dissolved oxygen on shrimp survival in response to the challenge with WSSV. Similarly, Ruiz-Velazco *et al*. (2010) assessed the dynamic influence of the white spot syndrome virus in shrimp farms in Mexico, and reported that the increased aeration in smaller ponds reduces the impact of the white spot syndrome.

The oxygen saturation levels applied in the uninfected treatments did not show a significant influence on the THC. On the other hand, Jiang *et al*. (2005) noticed a decrease in the THC of shrimp of the *Penaeus stylirostris* species when subjected to low oxygen saturation levels (3.5 and 2mg/L) after 12 hours. Le Moullac *et al*. (1998) also performed an experiment with *P. stylirostris* under severe hypoxia (1mg/L) for 24 hours, obtaining lower THC (7.6%) values, and also observed that hypoxia reduced the hyaline hemocytes (HH) and small granular hemocytes (SGH).

The decrease in hemocyte count observed in the infected treatments is common in many crustacean species infected by the white spot syndrome virus (Sarathi *et al*., 2007;Yeh *et al*., 2009; Guertler *et al*., 2013). Wang *et al*. (2002) affirm that this decrease in hemocyte count is mainly due to the decrease in small and large granular hemocytes (SGH and LGH) because they are the main WSSV targets.

The different oxygen saturation levels also did not impact the production of reactive oxygen species in the uninfected treatments. Zenteno-Savín *et al*. (2006) assessed the ROS production in *L. vannamei* under hypoxia for 24 hours, and did not find a significant difference compared to normoxia. According to Le Moullac *et al.* (1998) and Le Moullac and Haffner (2000), the respiratory burst is not affected by hypoxia. Fridovich (2004) affirms that the percentage of oxygen consumption for reactive species is very low, around 0.1%. However, 48 hours after the infection, the animals with 100% concentrations of dissolved oxygen in water showed a greater ROS production compared to the treatment with 30% saturation. Sarathi *et al*. (2007) also observed an increase in the respiratory burst, in 48 hours, in shrimp infected and maintained in normoxia. Li *et al.* (2006) and Parrilla-Taylor and Zenteno-Savín (2011) affirm that hypoxia increases the antioxidant defense of the animals. Parrilla-Taylor *et al.* (2013) also found that infection with WSSV stimulates the antioxidant defense. Thus, it can be suggested that the increase in antioxidant defenses caused by viral infection and hypoxia led to the low values observed in the treatment with 30% saturation. It is possible that the influence of hypoxia on the mortality of the animals is related to the defense response and not directly to the lack of oxygen.

CONCLUSION

The different oxygen saturation levels did not influence the immune parameters evaluated in uninfected *L. vannamei* shrimp. The highest mortality rates observed in infections by the white spot syndrome virus occurred under hypoxia condition, which can be related to the low ROS production observed in animals subjected to this condition.

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