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Research Article

Sensitivity of Larvae and Adult and the Immunologic Characteristics of *Litopenaeus vannamei* under the Acute Hypoxia

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Litopenaeus vannamei is one of the most commercially important species of shrimp in the world. In this study, we performed acute hypoxia tests with *Litopenaeus vannamei* to estimate 12 h median lethal concentration (LC_{50}) values at different life stages. The results indicated that the 12 h LC_{50} values were significantly different in different life stages of shrimp ($P < 0.05$). The maximum value of 12 h LC_{50} was 2.113 mg L^{-1} for mysis III, and the minimum value was 0.535 mg L^{-1} for adult shrimp with an average total length of 6 cm. The study also determined the hemocyanin concentration (HC) and the total hemocyte counts (THC) in the conditions of hypoxia and reoxygenation. These results showed that the THC decreased and the HC increased under hypoxia, and the THC increased and the HC decreased in the condition of reoxygenation. These results can provide fundamental information for shrimp farming and seedling and also can guide the breeding selection, as well as being very helpful to better understand the hypoxia stress mechanism of shrimp.

1. Introduction

In recent years, because of human activities and the changes of natural environment, the severity, frequency of occurrence, and duration of hypoxia are increasing, resulting in high mortality of valuable living resources [1, 2]. Hypoxia can make coastal benthic ecosystems generate so-called dead zones, and it was reported that more than four hundred ecosystems generated dead zones [3, 4]. Hypoxia may occur as a result of single factor or combined action of several factors such as eutrophication, stratification of the water column, freshwater runoff, weather, and biological processes. However, water eutrophication caused by human activities is the main reason of deterioration and increase in hypoxia zones [5–7]. White shrimp (*Litopenaeus vannamei*) is a very commercially important species in the world. However, hypoxia has seriously affected the growth and development and even resulted in mortality of shrimp [8].

Previous researches had shown that juvenile *Metapenaeus ensis* had 25% mortality at dissolved oxygen (DO) levels between 0.35 and 0.60 ppm, 8.3% mortality at DO levels between 0.60 and 0.85 mg L^{-1}, and 0% mortality at DO levels between 1.0 and 1.36 mg L^{-1} in 24 h lab experiment [9]. The 24 h median lethal concentration (LC_{50}) values for 3- and 10-day-old mysids were 1.51 mg L^{-1} and 1.56 mg L^{-1}, respectively, and 24 h and 48 h LC_{50} for pink shrimp (*Penaeus duorarum*) whose mean total length was 90.8 mm were 1.36 and 1.46 mg L^{-1}, respectively. So these results show that larvae are more sensitive than adult to hypoxia and that is similar to red drum (*Sciaenops ocellatus*) [10]. The majority of mortality owing to hypoxia happened in the first four hours of hypoxia and the test duration of more than 24 h had little effect on medium lethal concentration, and most of the LC_{50} values for fish at 2–4 days fell within 0.1 mg L^{-1} compared with that obtained from 24 h [11].

Marine invertebrates, including mussels (e.g., *Mytilus galloprovincialis*) [12, 13], clams (e.g., *Ruditapes philippinarum*) [14–17], oysters (e.g., *Crassostrea gigas*) [18], and shrimps, are widely studied in immunology and environmental science [19–23]. Because of the lack of the acquired immune system, marine invertebrates, such as shrimp, just rely on innate immune system to resist environmental stress [24, 25]. Humoral immunity includes antimicrobial peptides, clotting cascade, and prophenoloxidase, and the cellular defenses of shrimp depend on hemocytes that have many functions such as production of antimicrobial compounds, apoptosis, nodule formation, encapsulation of pathogens, cell adhesion, wound healing, coagulation, and phagocytosis [26, 27]. Phagocytosis and apoptosis play an important role in shrimp response to virus infection [28]. Hypoxia can evidently decrease the total hemocyte counts (THC), bacteriolytic activity, antibacterial activity, phenoloxidase activity, and phagocytic activity of prawn [29, 30]. Therefore, hypoxia can increase shrimp sensibility to pathogens and decrease defense capability to diseases.

Hemocyanin is an important respiratory protein and a major plasmatic protein in crustaceans and plays an important role in binding and transporting oxygen and CO_2 [31]. Moreover, the cleaved fragments of hemocyanin have antibacterial activity and may improve immunity of shrimp [32]. Under the condition of hypoxia, shrimp can increase the hemocyanin concentration (HC) and the affinity of hemocyanin for oxygen to improve the tolerance to hypoxia [33]. The concentration of copper ion reduces in hepatopancreas; however, the HC and copper ion increase in the hemolymph under hypoxia. These may suggest that shrimp can transport copper ion to compound hemocyanin to enhance the capability of carrying oxygen. Hypoxia also can make the gene expression of hemocyanin increase [34].

Most of previous studies focused on measuring the LC_{50} values in the special life stage and HC and THC in the condition of hypoxia, but there was very little information about the LC_{50} values in the whole life stages in the condition of hypoxia and the changes of HC and THC in the phase of reoxygenation. So, we performed studies to determine the LC_{50} values in whole life stages and investigated the changes of HC and THC in the condition of hypoxia and reoxygenation. These results can better understand the physiological mechanism of shrimp in the condition of hypoxia. Furthermore, it can provide fundamental data for shrimp farming and seedling.

2. Materials and Methods

2.1. Animals. *Litopenaeus vannamei* were obtained from a farm at Wenchang of Hainan province in China. The larvae of shrimp were directly collected from nursery pond, and the adult shrimps were acclimated in tank containing seawater (the salinity, temperature, and pH are displayed in Table 1) for three days before experiment. Half of the seawater in tank was replaced once daily and the shrimps were fed with formulated shrimp diet twice daily during the acclimation period. The shrimps were not fed during the experiment period.

Table 1: Experiment conditions to determine the LC_{50} for dissolved oxygen of white shrimp *Litopenaeus vannamei*.

Life stage	Mean total length (mm)	Temperature (°C)	pH	Salinity (‰)
Zygote	—	29 ± 1	7.96	30
Nauplius	—	30 ± 1	7.98	30
Zoea I	—	30 ± 1	7.96	30
Zoea II	—	29 ± 1	7.98	31
Zoea III	—	29 ± 1	7.95	31
Mysis I	—	30 ± 1	7.96	30
Mysis II	—	30 ± 1	7.91	30
Mysis III	—	30 ± 1	7.91	30
Postlarvae I	—	30 ± 1	7.93	30
Postlarvae II	—	31 ± 1	7.89	30
Postlarvae III	—	30 ± 1	7.92	30
Postlarvae IV	—	30 ± 1	7.88	30
Postlarvae V	—	29 ± 1	7.88	30
Postlarvae VI	—	29 ± 1	7.82	30
Adult	50 ± 2	29 ± 1	7.92	31
Adult	60 ± 4	28 ± 1	7.91	31
Adult	75 ± 4	29 ± 1	7.93	31
Adult	85 ± 5	26 ± 1	7.92	31
Adult	100 ± 5	23 ± 1	7.95	31
Adult	115 ± 3	22 ± 1	7.96	31
Adult	133 ± 5	25 ± 1	7.79	31

2.2. Experimental Design. The first experiment was conducted to determine the LC_{50} values at different life stages of *Litopenaeus vannamei*. The larvae shrimps were cultivated in a 5 L beaker, and six dissolved oxygen levels (0.43, 0.85, 1.70, 2.56, 3.40, and 5.00 mg L^{-1}) were established. The adult shrimps were cultivated in tanks (75 L) and five dissolved oxygen levels (0.3, 0.5, 1.0, 2.0, and 6.0 mg L^{-1}) were established. Each dissolved oxygen level was conducted in triplicate, and 50 shrimps were contained in each replicate. Test conditions were presented in Table 1. After 12 hours, the numbers of deaths and survivals were counted, respectively.

The second experiment was conducted to determine the changes of HC and THC in the phase of hypoxia and reoxygenation. The average body lengths of shrimp were 125 ± 0.5 mm and the animals were acclimated in tank containing seawater (salinity 31, pH 7.89, and temperature 27 ± 1°C) for three days before use. Three dissolved oxygen levels (1.0, 3.0, and 7.0 mg L^{-1}) were established and each level was conducted in triplicate. Each test tank (75 L) contained 30 shrimps.

The desired dissolved oxygen levels were established by bubbling nitrogen gas and air into the seawater, and the dissolved oxygen levels were monitored with a DO meter (HI 9146, HANNA) every half an hour. The pH and salinity values were measured by pH meter (HI 8424, HANNA) and salinity meter (RSH-28), respectively.

Table 2: Estimated 12 h LC_{50}, 95% confidence intervals (CI), 90% lethal concentration, and 10% lethal concentration of the test white shrimp *Litopenaeus vannamei*.

Life stage	LC_{50}	CI	90% lethal concentration	10% lethal concentration
Zygote	1.288		0.546	3.042
Nauplius	1.335	0.899–1.737	0.868	2.054
Zoea I	1.404	1.257–1.525	1.057	1.866
Zoea II	1.387	1.055–1.611	1.046	1.838
Zoea III	1.722	1.569–1.856	1.230	2.413
Mysis I	1.722	1.569–1.856	1.230	2.413
Mysis II	1.762	1.600–1.910	1.178	2.637
Mysis III	2.113	1.967–2.254	1.537	2.907
Postlarvae I	1.490	1.340–1.632	0.951	2.335
Postlarvae II	1.504	1.353–1.651	0.920	2.460
Postlarvae III	1.349	1.197–1.502	0.709	2.564
Postlarvae IV	1.074	0.954–1.197	0.607	1.900
Postlarvae V	1.135	1.006–1.266	0.620	2.076
Postlarvae VI	1.299	1.153–1.446	0.696	2.424
5 cm	0.577	0.463–0.775	0.377	0.883
6 cm	0.535	0.430–0.731	0.351	0.816
7.5 cm	0.625	0.404–1.477	0.429	0.911
8.5 cm	0.640	0.564–0.735	0.419	0.977
10 cm	0.593	0.537–0.762	0.463	0.759
11.5 cm	0.593	0.537–0.762	0.463	0.759
13.5 cm	0.651	0.582–0.740	0.482	0.878

Note. The results were derived from the mean of repeated experiments and the duration of hypoxia was 12 hours.

2.3. Hemolymph Collection and Total Hemocyte Counts (THC). Hemolymph was collected randomly from each replicate at 0, 3, 6, 12, 18, and 24 h during the period of hypoxia and reoxygenation. Hemolymph was withdrawn individually from the cardiocoelom of shrimp with a 1.0 mL syringe filled with an equal volume of anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, and 0.115 M glucose pH 7.55) and stored, respectively, in 1.5 mL Eppendorf centrifuge tubes. 30 μL of anticoagulant hemolymph was added immediately into the blood counting chamber by micropipette. Then hemocytes were observed under the optical microscope (Olympus) and THC were recorded by a cell counter. Another anticoagulant hemolymph was stored at −20°C for HC assay.

Anticoagulant hemolymph was centrifuged at 800 g for 10 min under 4°C for HC assay. Then 100 μL supernatant fraction was moved individually into 5 mL centrifuge tubes and diluted 1 : 30 with Tris-Ca buffer (50 mM Tris, 10 mM $CaCl_2$, and pH = 8.0). The OD values of the diluted plasma were measured at 335 nm using a UV spectrophotometer (1 cm path length) (PerkinElmer Lambda 25), and hemocyanin concentration (unit: mg mL^{-1}) was calculated using the following formula: $E_{335\,nm}$ (mg mL^{-1}) = 2.3 × $OD_{335\,nm}$ (E stands for HC; 2.3 is the extinction coefficient of hemocyanin for mg mL^{-1}) [35, 36].

2.4. Statistical Analyses. All data were tackled using SPSS 19.0. Suppose that $P < 0.05$ was the significant level. All data in the present study were shown as mean ± SD ($n = 3$). All figures were made by Origin 8.0.

3. Results

3.1. The Estimated 12 h LC_{50}, 90% Lethal Concentration (LC_{90}), and 10% Lethal Concentration (LC_{10}) for Dissolved Oxygen at Different Life Stages of Shrimp Litopenaeus vannamei. The estimated LC_{50}, LC_{90}, and LC_{10} were shown in Table 2. The results indicated that the life stages had a significant influence on LC_{90}, LC_{50}, and LC_{10} values ($P < 0.01$). The highest LC_{50} value was 2.113 mg L^{-1} in the phase of mysis III, and the lowest LC_{50} value was 0.535 mg L^{-1} in the phase of 6 cm. The results showed that larvae were more sensitive to hypoxia than adult and the changed trend of LC_{50}, LC_{90}, and LC_{10} values was similar at whole life stages of shrimp. The range of LC_{50} in the phase of larvae (1.039 mg L^{-1}) was larger than adult shrimp (0.116 mg L^{-1}).

3.2. Effects of Hypoxia and Reoxygenation on THC and HC in Hemolymph of L. vannamei. Figures 1 and 2 showed that hypoxia had an obvious influence on HC and THC ($P < 0.05$). HC and THC had no significant difference in the control (7 mg L^{-1}) ($P > 0.05$).

Figure 1 indicated that HC was significantly increased under the condition of hypoxia and decreased after reoxygenation, respectively. The HC returned to normal level after reoxygenation for 24 h.

Figure 2 demonstrated that THC obviously decreased in the phase of hypoxia and increased after reoxygenation. The result showed that the more serious and longer the duration of hypoxia, the more the reduction of THC. The THC of treatment group fell to the lowest level after reoxygenation for

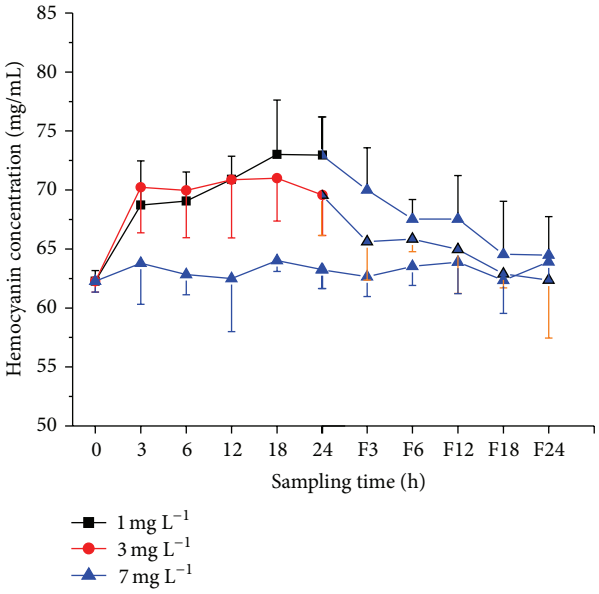

Figure 1: The change trend of HC in the condition of hypoxia and reoxygenation. The F3, F6, F12, F18, and F24 represented, respectively, the treatment time (3, 6, 12, 18, and 24 h) of reoxygenation ($n = 3$).

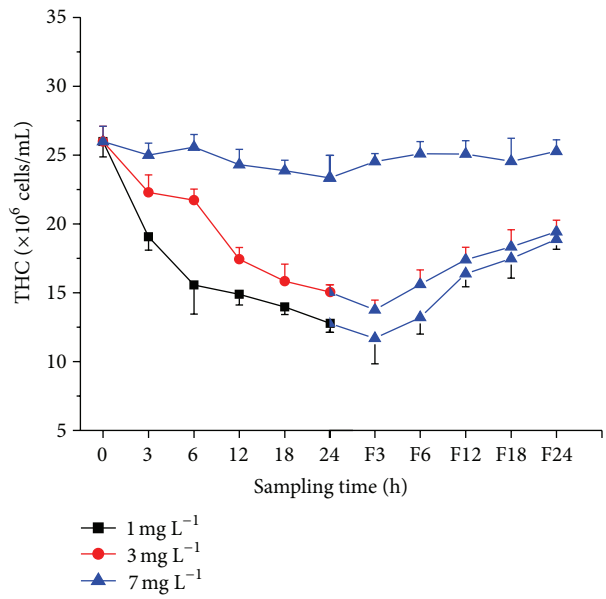

Figure 2: The change of THC in the condition of hypoxia and reoxygenation. The F3, F6, F12, F18, and F24 represented, respectively, the treatment time (3, 6, 12, 18, and 24 h) of reoxygenation ($n = 3$).

3 h, and it did not return to normal level after reoxygenation for 24 h. The THC in the treatment groups had significant difference ($P < 0.05$) compared with control groups.

4. Discussion and Conclusion

The estimated LC_{50} values are different at different life stages of white shrimp, and the larvae stage is more sensitive than adult stage. The range of LC_{50} in the phase of larvae (1.039 mg L^{-1}) is larger than adult shrimp (0.116 mg L^{-1}). The reasons may be that the growth and development of adult shrimp are more perfect and they can more efficiently resist environmental stresses.

Previous researches indicated that the 24 h LC_{50} values for 3- and 10-day-old mysids were 1.51 and 1.56 ppm, respectively; these are similar to the 12 h LC_{50} in this work. The 24 h and 48 h LC_{50} of pink shrimp were 1.36 and 1.46 mg L^{-1}, respectively [10], and the average total length of pink shrimp was about 90.8 mm; the values are bigger than the 12 h LC_{50} in this work. Majority of mortality owing to hypoxia happened in the first four hours of hypoxia and the test duration of more than 24 h had little effect on LC_{50}, and most of the LC_{50} values for fish at 2–4 days fell within 0.1 ppm compared with that obtained from 24 h [11]. This indicates that shrimps may have adapted to hypoxic environment by adjusting their behaviors [37], physiological and biochemical functions [31, 32], respiratory metabolism [38], and even gene expression [39] after a period of treatment.

Mysis III is the most sensitive stage to hypoxia in whole life and the key phase in the process of shrimp culture. The LC_{50} values in the present study are little different from the values of published literature [40]. It may be due to the different sensitivities at different life stages, test conditions, and species of shrimp.

With the increasing experimental time, the change tendency of HC and THC in the present study was similar to published literature [31]. Hemocyanin is the main plasmatic protein and has ability to bind and transport oxygen and metabolically produced CO_2 in crustaceans [41]. In the condition of hypoxia, the increase in HC can improve the ability of shrimp to gain more oxygen to deal with hypoxic stress. After reoxygenation, the decrease in HC indicated that shrimp no longer needed so much hemocyanin to gain oxygen. Published literature reported that the majority of mortality owing to hypoxia happened in the first four hours of exposure and the test duration of more than 24 h had little effect on lethal concentration [10]. In the first three hours of hypoxia, the increasing rate of HC and the decreasing rate of THC are larger than other hypoxia periods; it suggests that shrimp can rapidly activate a complicated system to respond to the hypoxia stress.

Shrimp only has innate immune system including hemocyte and diverse active factors existing in hemocyte or released to hemolymph from the hemocyte, so hemocyte plays an important role in immune defense. The results of the present and previous studies showed that THC, bacteriolytic activity, antibacterial activity, phagocytic activity, and phenoloxidase activity decreased significantly and the sensibility to pathogens increased in the condition of hypoxia [42]. Therefore, we can deduce that hypoxia can reduce the immune defense of shrimp. These results will contribute to the cultivation and seedling of shrimp and also be helpful to better understand the stress mechanism of hypoxia, but there is still much work which needs to be done; for example, we

need to use omic techniques to explore the molecular mechanism of hypoxia stress [43] and need to develop some specific biomarkers to monitor the hypoxia stress; there has been useful information in other invertebrates, such as *Mytilus galloprovincialis* [44–46] and *Venerupis philippinarum* [47].

Disclosure

Yuhu Li is the first coauthor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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