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Effect of Stocking Density on Growth, Oxidative Stress and HSP 70 of Pacific White Shrimp *Litopenaeus vannamei*

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Abstract

The objective of this research was to evaluate the growth, oxidative stress and HSP70 of pacific white shrimp under different stocking densities. Juvenile shrimps (average weight 2 g) were stocked under three densities (30，40，50 ind/cage, expressed as S30, S40 and S50 respectively) for 60 days. All treatment groups setting in net cages (size 40 cm × 40 cm × 40 cm) were submerged in a pond to ensure the same rearing conditions. At the end of the experiment, the survival rate decreased with increasing stocking density, ranged 83.3%, 79.2% and 78.7% respectively. The finial average size of shrimp in S30 was higher than that of the group S50. SGR, WG and FCR of shrimps in S30 group were better compared to that of the S40 and S50. Furthermore, antioxidant abilities in the hemolymph, hepatopancreas and muscle of shrimp were higher at low stocking density. HSP70 increased in hepatopancreas with increasing stocking density. The results of this study demonstrated that, when juvenile shrimps were reared under high stocking density, growth, feed utilization, antioxidant capability and stress resistance ability was decreased, indicating that high stocking density would affect growth and welfare of juvenile white shrimp.

Key words: density, oxidative stress, HSP70, *Litopenaeus vannamei*

Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is a worldwide cultured species especially in developing countries (FAO, 2010). Recently, industrial shrimp culture has become widely spread and applied because of the diminishing land and discharge of wastewater. And shrimps tend to be cultured at high stocking density under industrial model. Stocking density is one of the most important factors in shrimp culture, high stocking density can influence growth and survival of shrimp due to the stress response induced by crowding (Mena-Herrera, Gutierrez-Corona, Linan-Cabello, & Sumano-Lopez, 2006).

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The behavioral and physiological responses of crustaceans to crowding have been well studied (Saroglia & Liu, 2012) in order to search for stress biomarkers. Heat shock proteins (HSP) are highly conserved group of proteins that are synthesized as a response to different forms of stress (Robert, 2003). Because of the high sensitivity to changes in the environment, HSP is suggested as possible early biomarker of exposure in ecotoxicological studies.

Aquatic animals are likely to suffer oxidative stress when cultured under high stocking densities. And the endogenous antioxidant system plays a crucial role in protecting against oxidative stress (Junqueira, Barros, & Chan, 2004). This system is composed of enzymes and other (non-enzyme) molecules that scavenge ROS, including superoxidate dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (Halliwell & Gutteridge, 2007; Mohankumar & Ramasamy, 2006). These biomarkers are frequently used both in environmental monitoring and laboratory assays (Pandey, Parvez, Sayeed, Haque, Bin-Hafeez, & Raisuddin, 2003).

Previous studies indicated that high stocking densities would result in negative growth, low survival rate, low production, poor water quality and the emergence of pathogen outbreaks (Lin, Chen, Chen, Yeh, Chen, Huang, Hsieh, & Li, 2015). The immune response and performance of pacific white shrimp stocked at high density was reduced by both crowding stress and water quality deterioration (Aguilar, Racotta, Goytortúa, Wille, Sorgeloos, Civera, & Palacios, 2012). Nga, Lürling, Peeters, Roijackers, Scheffer and Nghia (2005) reported that stocking density significantly affected *P. monodon* survival, body-size and dry-weights over a 4-week experimental period during stage 1, but the negative effects in stage 2 was caused mainly by water quality deterioration but not by crowding stress. Stocking density affected the growth performance of Chinese shrimp mainly by influencing ingestion or activities of enzymes (PO, POD and hemolysin), DO concentration and stocking density played a crucial role in the production of Chinese shrimp farming (Li, Li, & Wang, 2006). In view of the importance of stocking density in shrimp culture，it is crucial to investigate the influence of stocking density on the growth and welfare of white shrimp. In this experiment, we set three stocking densities according to the actual shrimp culture practice, growth performance, feed utilization, antioxidant parameters and HSP70 levels were investigated. All treatment cages were submerged in the same pond to warrant the same water quality condition. Water quality in the pond was checked every day and adjusted to ensure the normal growth requirement of shrimp. Thereby preliminarily find out the effects

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of stocking density on shrimp growth, antioxidant ability and HSP70 level, provide instruction for the practice to ensure the optimal welfare and production of shrimp.

Materials and Methods

Experiment Setting

In this experiment, we set three stocking densities according to the shrimp culture practice under field conditions. Generally, the common tank size is about 670 m^2 in shrimp farm, stocking amount ranges 300000, 400000 and 500000 shrimps/tank, stands for 500, 667 and 833 shrimps/m^3, apply to our experiment cage is about 30, 40 and 50 shrimps/cage (64 L). Shrimp were obtained from Guangfeng Aquaculture Co. Ltd., Changbai island, Zhoushan city, Zhejiang province, China. 600 shrimps were selected and distributed randomly into 9 cages located in one shrimp ponds (667 m^2). The cage (40 cm × 40 cm × 40 cm) was made of polythene net, mesh size of the side net was 0.5 mm, while bottom mesh size was 0.25 mm to avoid leaking out of feed. The cages were submerged completely into the water with covers. Sand filtered seawater was used and disinfected with chlorinated lime at least 3 days before use.

Water temperature, dissolved oxygen, and pH in the net cages were recorded daily using water-quality checker HQ40d (HACH corporation, Love land, USA). Ammonia and nitrite was monitored throughout the feeding trial using spectrophotometric method. During the whole experiment, water temperature was maintained at 25 – 27 °C, dissolved oxygen 6.1 – 7.4 mg/L, pH 8.1 ± 0.2, salinity was 18‰ and total ammonia–nitrogen was 0.04 mg/L, nitrite was below 0.02 mg/L.

Feeding

Waterwheel was stopped for one hour during the feeding time. Remaining feed was siphoned after one hour, based on the amount of uneaten feed in the cage, the feeding ration was adjusted to exceed satiation level slightly. Uneaten feed was collected every time after feeding, dried and weighed for calculation of feed conversion ratio.

Survival rate, specific growth rate, feed conversion ratio and weight gain were calculated using the following equations:

Survival rate (%) = 100 × (Final number of shrimp／Initial number of shrimp),

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Specific growth rate (%/day) = 100 × [Ln (final mean body weight) – Ln (initial mean body weight)]／Time (days),

Feed conversion ratio = Total dry feed consumption／Net weight gain,

Weight gain= (Final whole weight-Initial whole weight)/Initial whole weight,

Sample and Analysis

At the end of 60 days feeding trial, the shrimps in each cage were weighed totally after starving for 24 h. Ten shrimps from each cage were randomly sampled for the assay of antioxidant parameters and HSP70 level. Shrimps during molting period were not sampled for biochemical analyses to minimize internal variations (Bonilla-Gómez, Chiappa-Carrara, Galindo, Jeronimo, Cuzon, & Gaxiola, 2012). Hemolymph was withdrawn from the pericardial cavity using a 1 mL syringe, then gathered into 1.5 mL Eppendorf tubes and centrifuged at 4 °C, 3000 r/min for 10 min. The supernatant was collected and stored at −80 °C until analysis of antioxidant parameters and HSP70. Then the muscle and hepatopancreas of the shrimps were excised, washed and pooled into 2 mL Eppendorf tubes and stored at −80 °C for analysis of antioxidant parameters and HSP70.

Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), malondialdehvde (MDA) and HSP70 protein level was analyzed using elisa kits（48T, provided by Shanghai Changjin Biotechnology Co., Ltd., China）according to the manufacturer's instruction. All samples were analyzed individually. All assays were run in triplicate.

Statistical Analysis

Data were presented as the mean ± standard deviation (SD) of triplicates. Statistical analysis was performed using SPSS statistical software (Version 19.0, SPSS Inc., USA). All data were subjected to One-Way Analyses of Variance (ANOVA). Duncan's multiple range test was used for determining the statistical significance among groups. Mean values were considered significantly different if the *P* value was less than 0.05.

Results

Effects of Stocking Density on Shrimp Growth, Feed utilization and Survival Rate

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Specific growth rate and weight gain of shrimps in S30 group was significantly higher than that of S40 and S50 group ($P < 0.05$, Table 1). Feed conversion ratio of shrimps in group S30 and S40 was better compared to that of the S50 group ($P < 0.05$), and there was no difference between group S30 and S40 ($P > 0.05$). The final average size decreased with increasing stocking density, and the final weight of shrimp in S50 group was significantly lower than that of the S30 group ($P < 0.05$). Survival rate decreased with increasing density, but there was no statistical difference among the treatments ($P > 0.05$).

Effects of Stocking Density on HSP70 in Muscle, Hemolymph and Hepatopancreas of *L. vannamei*

HSP70 increased with increasing stocking density in hepatopancreas and the difference was significant among three groups ($P < 0.05$, Fig. 1). In muscle, HSP70 of group S40 was higher than that of the group S30 ($P < 0.05$). However, there was no difference in HSP70 level in hemolymph ($P > 0.05$).

Effects of Stocking Density on Antioxidant Ability of *L. vannamei*

SOD activity of hemolymph in group S30 was higher than that of the other two groups ($P < 0.05$, Fig. 2), and there was no difference between group S40 and S50. SOD activities decreased with increasing density in hepatopancreas ($P < 0.05$). There was no difference among three groups in muscle ($P > 0.05$).

There was no difference in CAT activities among three groups in muscle and hepatopancreas ($P > 0.05$, Fig. 3). CAT activity of S30 group was higher than that of S40 and S50 in hemolymph ($P < 0.05$) while there was no difference between S40 and S50 ($P > 0.05$).

GSH contents decreased in muscle, hemolymph and hepatopancreas with increasing stocking density (Fig. 4). Significant differences were obtained between all groups in hemolymph ($P < 0.05$). GSH content in muscle in group S30 and S40 was higher than that of group S50 ($P < 0.05$), and there was no difference between group S30 and S40. GSH content in hepatopancreas in group S30 was higher than that of group S40 and S50 ($P < 0.05$).

MDA content tended to increase in muscle, hemolymph and hepatopancreas with increasing

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stocking density (Fig. 5). Significant differences were obtained between all groups in hemolymph ($P < 0.05$). MDA content of S50 group was higher than that of S30 and S40 in muscle, while MDA contents in S40 and S50 were higher than that of S30 in hepatopancreas ($P < 0.05$).

Discussion

Shrimps were usually stocked at high densities under industrial production condition. In our experiment, three most used stocking densities in practice were set as research objects. Results showed stocking density had great relation with the growth of shrimp. Specific growth rate and weight gain in S30 group was significantly higher than that of S40 and S50 group. And average final size of shrimp decreased with increasing stocking density. Similar results were observed in previous researches. Cao, Wang, Yan and Ma (2006) found there was a decreasing trend in feed intake, feed efficiency, growth and survival rate with increasing stocking density. Zhang (2008) found the growth of shrimp was significantly affected by stocking density. Reduced growth and survival of shrimp cultured at high densities was thought to result from an increase in competition for the same space and natural food sources and higher events of cannibalism (Arnold, Sellars, Crocos, & Coman, 2005). There was also other research thought stocking density affected the growth performance of shrimp mainly by influencing ingestion or activities of enzymes (Li et al., 2006). However, Arnold, Coman, Jackson and Groves (2009) found that growth and survival was not affected by stocking density, greater production outputs were achieved at the higher density. Higher stocking density produced slightly higher yields while larger shrimp were associated with lower stocking density (Mena-Herrera et al., 2006). This difference may be caused by different breeding conditions. Increasing stocking density may increase production through better management of water quality and feeding protocol.

HSP70 could response to various environment stress. The HSP70 level of *Litopenaeus vannamei* increased under rapid temperature changes (Guo, Wang, Dong, & Huang, 2010). Ding, Wang, Sun, Guo and Dong (2009) found salinity fluctuation frequency affected HSP70 expression in juvenile Chinese shrimp. Virus infection promoted changes in the expression of Hsp70 in *Litopenaeus vannamei* (Valentim-Neto, Moser, Fraga, & Marques, 2014). Transcription of LvHSP70 was induced in haemocytes and hepatopancreas of *Litopenaeus vannamei* after different bacteria injection (Zhou, Wang, He, Zheng, Wang, Xin, Liu, & Wang, 2009). Previous study indicated HSP70 could work as

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stress biomarker for crowding too. Li et al. (2006) found that increasing stocking density induced the expression of stress related protein HSP70 in rainbow trout. Aksakal, Ekinci, Erdogan, Beydemir, Alm, & Ceyhun (2011) found increasing stocking density elevated mRNA levels of HSP70 in rainbow trout. HSP70 mRNA level in liver of tilapia increased obviously after 48h acute crowding stress (Qiang, Yang, He, Wang, Xu, & Zhu, 2014). But we lack of relevant data of the HSP70 response for white shrimp under crowding stress. The HSP70 level in three tissues investigated in our experiment increased with increasing stocking density, which indicated it was stressful to shrimp under high stocking density. In the former study on black tiger shrimp by Nga et al. (2005), the negative effects in stage 2 were caused mainly by water quality deterioration. In view of this, we excluded the effects of water quality by submerging all the cages in the same pond, and water quality was maintained at a level that could ensure the normal requirement of shrimp, so the results observed in our experiment necessarily meant crowding stress affected the HSP70 level of shrimp.

Saroglia and Liu (2012) found increasing stocking density could cause undesirable influences for aquatic animal by disrupting the physiological balance. Crowding could result in oxidative stress in aquatic animals. Aksu, Kutluyer, Can, Erişir and Benzer (2016) found oxidative stress biomarkers were negatively influenced by increased quantitative changes in stocking density. Tu, Silvestre, Bernard, Douny, Phuong, Tao, Maghuin-Rogister and Kestemont (2008) suggested that hepatopancreas lipid peroxidation and CAT activity could be proposed as biomarkers to point out the general stress status of the black tiger shrimp cultivated in intensive and extensive systems. SOD activity of GIFT strain tilapia was significantly affected by stocking density (Kpundeh, He, Qiang, Hong, & Xu, 2014). Aksakal et al. (2011) found increasing stocking density caused inhibition of antioxidant enzymes. Liu (2006) found crowding stress increased the free radical level and inhibited antioxidant enzymes activity in Chinese sturgeon. Many other factors could also cause oxidative stress to shrimp, such as chemical compounds, pathogens, water quality variation, and so on. Pacific white shrimps exposed to bacterial pathogens exhibited higher antioxidant enzyme activities and higher oxidative stress level in the digestive gland (Castex, Lemaire, Wabete & Chim, 2009). Environmental hypoxia and reoxygenation increased reactive oxygen species (ROS) production in Pacific white shrimp (*Litopenaeus vannamei*), activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in extracts of muscle and hepatopancreas from white shrimp increased (Parrilla-Taylor &

Zenteno-Savín, 2011). Wang, Zhou, Wang, Tian, Zheng, Liu, Mai, Wang (2009) found acute pH increase and decrease could cause oxidative stress and cooperatively activate expression of CAT and GPx mRNA of Pacific white shrimp. Stress induced by acute temperature decrease from 23 to 12 also produced oxidative stress and lipid peroxidation in *L. vannamei* (Qiu, Wang, Wang, Liu, & Wang, 2011). In our experiment, MDA level increased, GSH contents decreased obviously in hemolymph, as well as SOD activity, which indicated obvious oxidative stress to shrimp. Oxidative stress and tissue damage via inactivation of antioxidant enzymes in infected shrimps could result in system failure and sudden death (Parrilla-Taylor, Zenteno-Savín, & Magallón-Barajas, 2013). So this could result in slow growth and low survival rate of shrimp under high stocking density.

In conclusion, our results showed that increasing stocking density led to low survival rate and growth rate. High density also compromised shrimp welfare, therefore increased the risk of disease outbreak and difficulty of management. So we should consider the actual conditions of aquafarms and decide specific stocking density in practice. There is still great potential to increase shrimp production when the shrimps are cultured under a suitable density.

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Table 1. Effects of stocking density on growth and feed utilization of white shrimp.

Parameters	Stocking density		
	30	40	50
Initial weight(g)	1.9±0.2	2.0±0.3	1.9±0.2
Final weight(g)	6.7±0.6[a]	6.2±0.8[ab]	5.8±0.5[b]
[1]SGR (% /day)	2.05±0.11[a]	1.89±0.06[b]	1.86±0.09[b]
[2]WG (%)	185.0±9.7[a]	146.4±8.4[b]	140.2±8.8[b]
[3]FCR	1.59±0.13[a]	1.65±0.21[a]	1.77±0.14[b]
[4]Survival rate (%)	83.3±3.3%	79.2±3.8%	78.7±4.2%

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Each value represents mean ± S.D. (n=3).Values in the same row with different superscripts are significantly different (P<0.05).

[1]SGR: specific growth rate= [ln (final mean weight) – ln (initial mean weight)] ÷ Time interval (days) ×100

[2]Weight gain (%) = 100 × (final body weight − initial body weight)／initial body weight

[3]FCR: feed conversion rate=Dry feed consumption/Net weight gain

[4]Survival rate (%) = final number of shrimp/initial number of shrimp× 100.

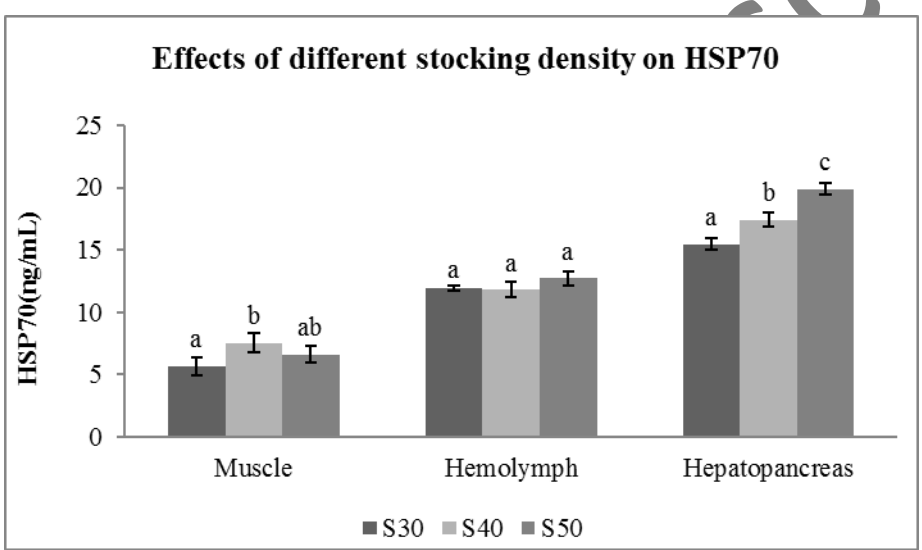

Figure 1. Effects of stocking density on HSP70 level in muscle, hemolymph and hepatopancreas of *L. vannamei*. Each bar represents mean ± S.D. Bars with different letters are significantly different (P < 0.05).

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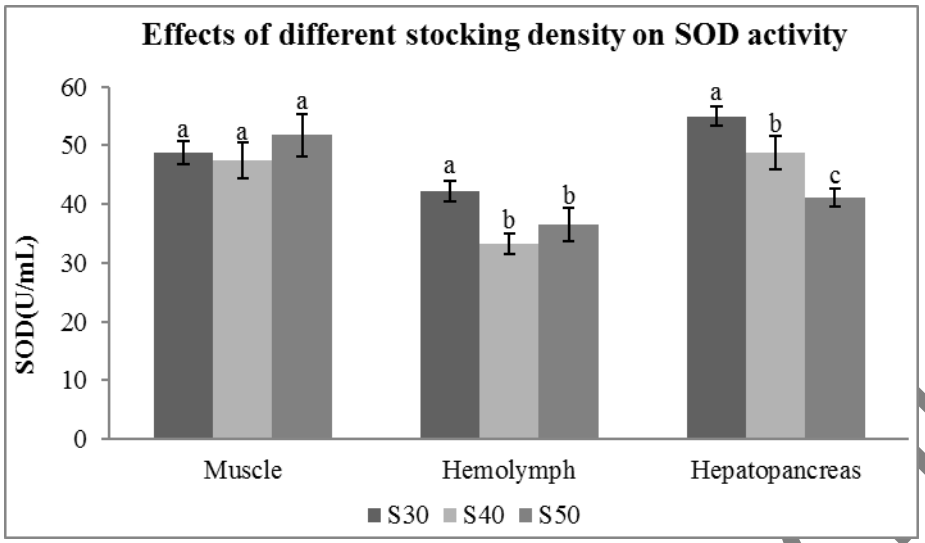

Figure 2. Effects of stocking density on SOD activities in muscle, hemolymph and hepatopancreas of *L. vannamei*. Each bar represents mean ± S.D. Bars with different letters are significantly different ($P < 0.05$).

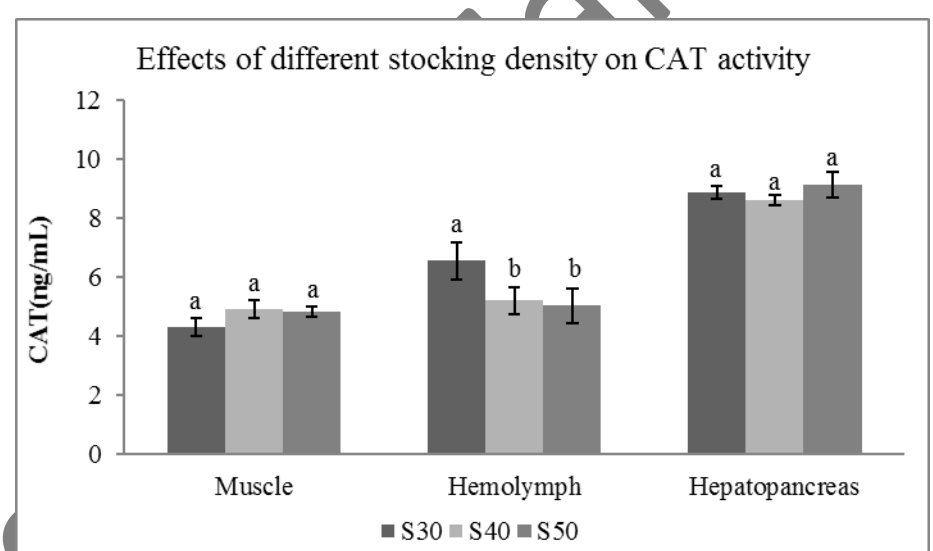

Figure 3. Effects of stocking density on CAT activities in muscle, hemolymph and hepatopancreas of *L. vannamei*. Each bar represents mean ± S.D. Bars with different letters are significantly different ($P < 0.05$).

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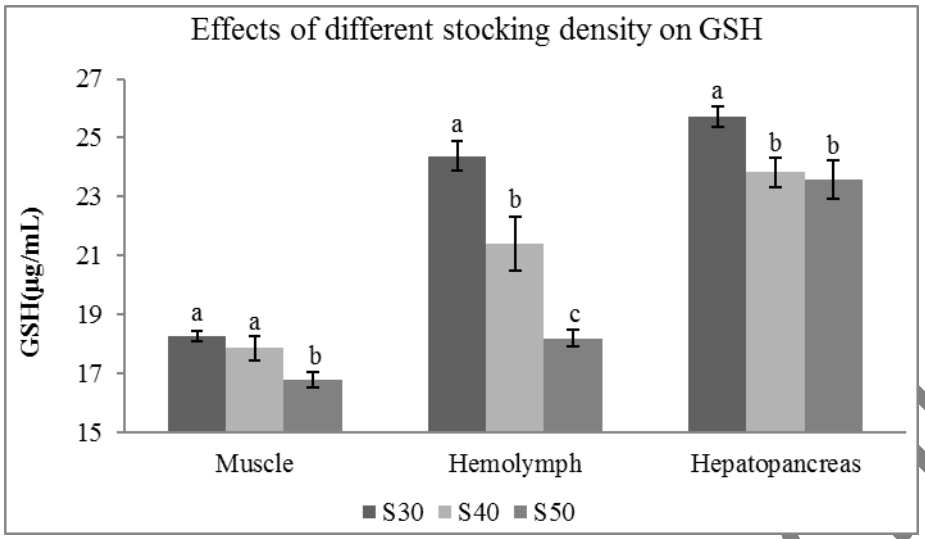

Figure 4. Effects of stocking density on GSH contents in muscle, hemolymph and hepatopancreas of *L. vannamei*. Each bar represents mean ± S.D. Bars with different letters are significantly different ($P < 0.05$).

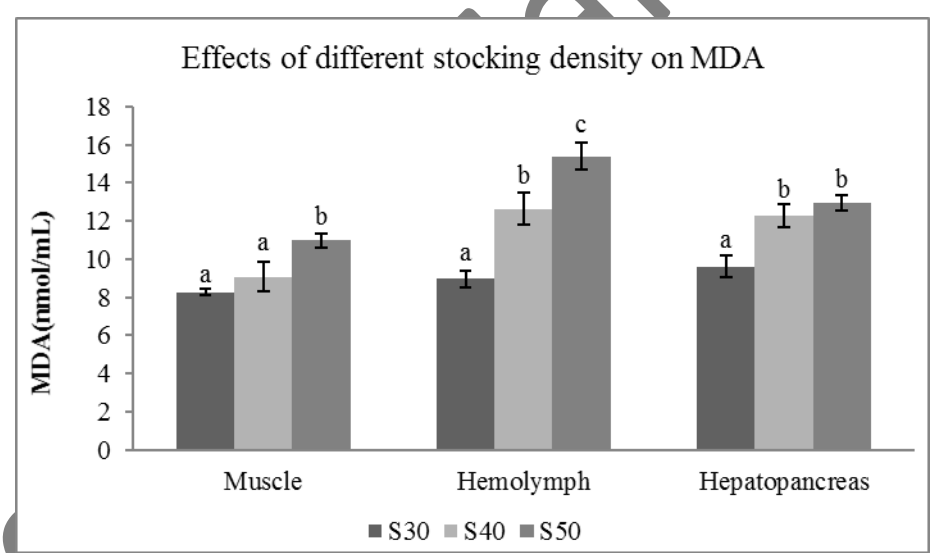

Figure 5. Effects of stocking density on MDA contents in muscle, hemolymph and hepatopancreas of *L. vannamei*. Each bar represents mean ± S.D. Bars with different letters are significantly different ($P < 0.05$).