The effect of gradually decline of salinity on haemolymph parameters of adult shrimp *Litopenaeus vannamei* (Boone, 1931)

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Abstract

The present study was conducted to evaluate the effects of salinity levels (40, 35, 30, 25, 20, 15, 10 and 5‰) on haemolymph parameters and survival of adult shrimp *Litopenaeus vannamei*. Shrimps were distributed in fiberglass tanks containing water with 40‰ salinity then salinity was gradually declined during 7 days at rate of 5‰ a day by adding clean fresh water. Haemolymph of three shrimps was daily sampled from each treatment in triplicates. The haemolymph parameters including total haemocyte count (THC), total plasma protein (TPP), differential haemocyte count (DHC) were measured. Significant differences were found between treatments in terms of THC values ($p<0.05$). The highest THC were observed in shrimps exposed to salinities of 25 and 5‰ ($p>0.05$) versus the lowest THC was measured in 35‰ one. No significant difference was found in TPP levels between examined salinities ($p>0.05$). Hyaline cell composed 67.8 to 68% of THC ($31.4 \times 10^6 \pm 0.79 \times 10^6$ cell ml^{-1}), but those of semi-granular and granular cells were 1.8 to 2% and 29.4 to 30%, respectively. Both semi-granular and granular cell counts were significantly lower in group 10‰ throughout the experiment period. However, hyaline cells count was found to be lower in shrimps exposed to 30‰, 25‰ and 20‰ salinities ($p<0.05$). The semi-granular cells showed higher values in shrimps exposed to salinities of 30‰ compared to other treatments ($p<0.05$). In salinities of 5‰ and 10‰, the values of granular cells were the lowest. It is suggested that a declining rate of 5‰ in salinity of water per day conducting for adaptation of *L. vannamei* could be appropriate to the shrimp, since THC and TPP levels can be recovered every 7 days through the salinity change.

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Introduction

Environmental factors including temperature, dissolved oxygen, salinity and pH have been recognized affecting the immune system in crustaceans (Le Moullac and Haffner, 2000). The Pacific white shrimp, *L. vannamei* is widely distributed in the eastern Pacific from Sonora (Mexico) to Tumbes (northern Peru), where water temperature and salinity varies in ranges between 15-33ºC and 5-45‰ respectively. So, this species is hyper-osmotic regulator at low salinity levels, hypo-osmotic regulator at high salinity limit with an iso-osmotic point about 715 mOsm kg^{-1} equivalent to 25‰ (Sowers *et al.,* 2006). Accordingly, the suitable salinity and temperature levels for growth and survival of *L. vannamei* ranging 10 to 35‰ and 20 to 30 ºC, respectively (Ponce-Palafox *et al.,* 1997). Another research Sowers et al. (2006) showed that salinity level in range of 15 to 20‰ improves the growth of *L. vannamei*. Although the good tolerance of the shrimp to environmental changes have been evidenced, the sudden changes in water parameters such as temperature, salinity and pH have been expressed reducing the immune level and increase the susceptibility to some bacterial and viral diseases (Pazir *et al.,* 2011; Wang and Chen, 2006). There are many studies regarding the effects of temperature, salinity and pH on the immune system and growth of *L. vannamei* (Jiang *et al.,* 2005; Kakoolaki *et al.,* 2011; Pan *et al.,* 2007; Perazzolo *et al.,* 2002); however, little works have investigated the impacts of sudden changes of these parameters during handling.

Due to annually incidences of white spot disease (WSD) and losses of shrimp farmers this species was primarily introduced to Iran since 2004 by Iranian Fisheries Sciences Research Institute (IFSRI) as an alternative species (Afsharnasab *et al.,* 2014). It has been introduced to Iran shrimp farming industry since 2006, when commercial shrimp farming mainly was based on *Fenneropenaeus indicus* for about 10 years with (Pazir *et al.,* 2011).

The area of this survey was located along the northern coasts of Persian Gulf where salinities of the shrimp farms were 20-57‰ (Kakoolaki *et al.,* 2015). In this area, *L. vannamei* juveniles and adults, usually are transferred from one region to another, with different water salinities, and sometimes due to insufficient adaptation, which resulted in sever mortalities. In these cases, it is supposed that drastic salinity changes may affect the immune capability of shrimps. Wang and Chen (2005), claimed that the shrimp relocated from 25‰ environment to lower salinity limits (5-15‰ salinity) decreased immune responses and resistance against *Vibrio alginolyticus* infection, which significantly resulted in lower growth and survival rate in *L. vannamei* (Gao *et al.,* 2016).

In the present study, the effect of salinity fluctuations was investigated on the immune parameters of *L. vannamei* to determine the safe range of salinity changes for handling and adaptation of the shrimps during transportation between regions with different salinity levels.

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Materials and methods

Animals and experimental conditions

This study was carried out in Iran shrimp Research Institute located in Boushehr province, Iran, during March to April 2018. Sub-adults of *L. vannamei* (mean total weight: 32.87 ± 0.36 g; mean total length: 14.69 ± 0.05 cm) were obtained from private shrimp farms. All the shrimps were in the inter-molt stage and the molt stage was identified by examination of uropod in which partial withdrawal of epidermis could be revealed (Robertson *et al.,* 1987). Fiber glass cylindrical tanks (4000 l) were used to stock shrimps at density of 35 pcs/m^2 in triplicates. Shrimps were fed with formulated pellet (Havoorash Company, Boushehr, Iran; moisture content: 5-10%; crude protein: 40-45%; crude lipid: 7-11%; crude fiber: 1.5-2%; ash content: 8-13%).

Environmental parameters

During the experiment, water temperature, dissolved oxygen and pH were daily measured by HACH (HQ40d) Multi-Parameter. The physicochemical values were obtained as 27 ± 1 ºC, 5.02 ± 0.32 mg mL^{-1} and 7.8-8.2 respectively. Water salinity level was maintained at 40‰ (salinity is normally using in the area) at the beginning of the study, and gradually declined by adding fresh water at a rate of 5% a day to obtain 35, 30, 25, 20, 15, 10 and 5‰ water salinity during 7-days experiment period by adding freshwater at 8:00 o'clock. The salinity was measured using a Refract meter (ATAGO S/Mill, Japan). To assay immune parameters, sampling was carried out with 150 shrimps for each treatment in triplicates.

Sampling and immune parameters assay

Haemolymph were sampled daily and randomly obtained from the shrimp 4 h after water exchanges. The haemolymph samples (100 µl) were taken from ventral sinus situated at the first abdominal segment and put into a 1 ml sterile syringe (25 gauge) prefilled with 0.6 ml of ice-cold shrimp anticoagulant buffer (30 mMtri-sodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.115 M Glucose, pH 7.55, osmolality 780 mOsm kg^{-1}) mixed with 0.4 ml haemolymph and placed into a 1.5 ml eppendorf tube, and then kept at 4 °C (Vargas-Albores *et al.,* 1993).

Total haemocyte count assays

The haemolymph samples were separated into two parts and incubated at 4 ºC. A drop of anticoagulant – haemolymph combination was positioned on a haemocytometer to count haemocyte count (THC) under a light microscope at 40X (Cetti, triton II, England). Cells were counted on both sides of the grids, and then THCs were measured using the following equation:

$$THC=\frac{\text{number of cells counted}}{\text{proportion of chamber counted}}\times\frac{\text{volume of sample dilution}}{\text{volume of original mixture in sample}}\times 10^6 \text{ (cells ml}^{-1}\text{)}$$

Total plasma protein measurement

To assay the total plasma protein (TPP), the haemolymph was centrifuged (2500 rpm for 15 min at 4 ºC) and supernatant was subjected to assay TPP according to a modified Biuret method (Ghaednia *et al.,* 2012), using bovine serum albumin (BSA) as standard.

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Differential haemocyte count assay

To determine the differential haemocyte counting (DHC), a 0.2 ml samples of haemolymph were taken from shrimps (individually) into a sterile syringe (1 ml) containing 0.2 ml fixative (10% formalin in 0.45 M NaCl) based on the aforesaid method. After 10 min, 20 µl of the fixed haemolymph suspension was spread on a microscope glass slide. For counting of haemocytes (H), the smear was completely dried at room temperature for 20 min and then was submerged in May Grünwald stain. After 5 min, Phosphate buffer (1.15 M, pH 6.6) was added for 5 min with slow blowing. The mixture was then removed and the slide deepened in Giemsa stain for 15 min. After that, the slide was rinsed with distilled water for 10 min, and covered by a covering glass (Kakoolaki *et al.,* 2010). Three main different types of haemocytes were determined following the method of Johansson et al. (2000); Kondo (2003) as: hyaline cells (HCs), semi-granular cells (SGCs) and granular cells (GCs) (Fig. 1). About 200 cells were counted on each slide, totally. The DHC was measured using the following equation:

$$DHC\% = \frac{\text{number of different haemocyte type}}{\text{total haemocyte cell count}} \times 100$$

The proportion of GH in total haemocyteswas recorded and used to calculate the total number of haemocytes (i.e. count/200 × THC).

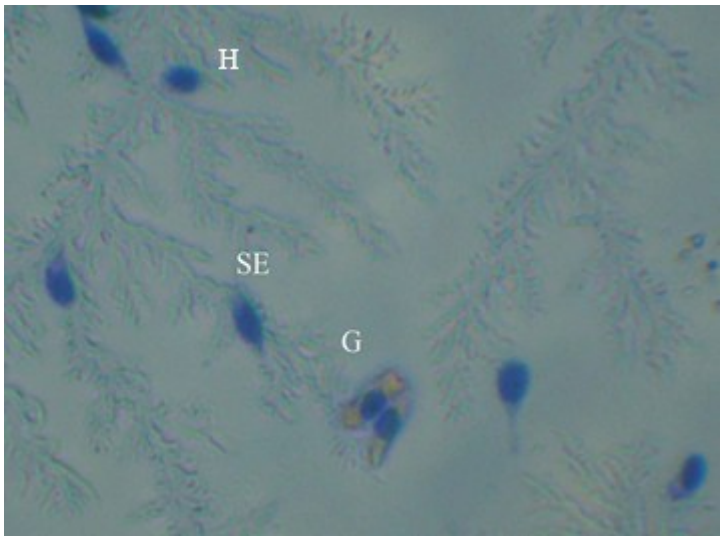

Figure 1. Hemocyte cell differentiation of shrimp treatments (magnification: 400X), Granular Cell (G), Semigranular cell (SE), Hyaline cell (H).

Survival rate

The survival rate of shrimp was measured by through following equation:

$$\text{Survival (\%)} = (N_t / N_0) \times 100$$

Where N_t and N_0 are the numbers of live shrimps at the end and beginning (*0*) of the experiment (*t*) respectively.

Data Analysis

Data were analyzed using SPSS 18.0 software and presented as means ± standard deviation. The significant differences between groups were calculated using one-way analysis of variance (ANOVA), followed by Tukey test to compare the means of groups, two by two.

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Results

Water quality

The water quality parameters including temperature (23-25 °C), dissolved oxygen (4.5-5.2 mg mL^{-1}) and pH (7.8-8.1) were kept, and at normal ranges during the experiment period. The water-quality parameters such as NO_2-N, NH_3-N and PO_4-P were not significantly different among the treatments ($p>0.05$).

Immune parameters

There were significant differences in THC values between salinity treatments (Fig 2, $p<0.05$). The THC levels decreased by a decline in salinity from 40‰ to 35‰ and 30‰. After an increase in 25‰ salinity, the levels of THC returned to initial levels in salinities of 20‰, 15‰ and 10‰. In salinity of 5‰, THC levels increased again reached THC levels in 25‰ salinity treatment.

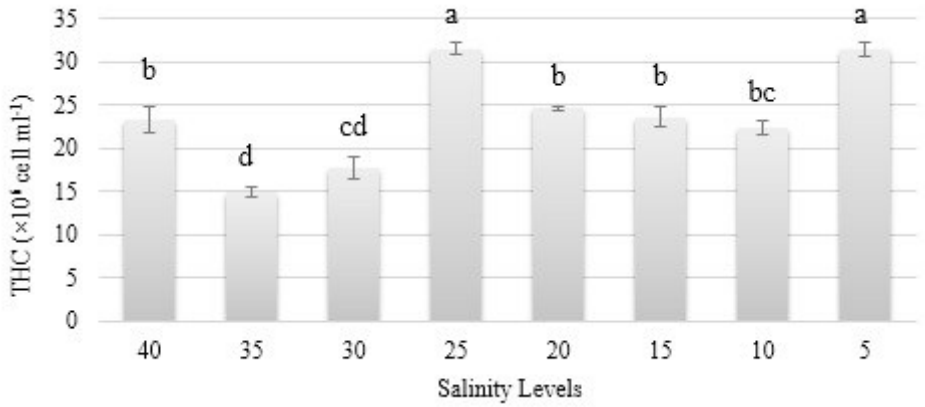

Figure 2. Mean (± S.E.) total haemocyte count of *L. vannamei* at different salinity levels. Bars with different letters are significantly different ($p<0.05$) among treatment.

Also, no significant difference ($p>0.05$) was observed in THC levels between the shrimps exposed to 40‰, 20‰, 15‰ and 10‰. According to differential haemocyte counting data, there were significant differences ($p<0.05$) in hyaline, semi-granular and granular cells between salinity treatments. Hyaline cell composed 67.8 to 68% of THC (Fig 3), but semi-granular and granular cells composed 1.8 to 2% and 29.4 to 30% of THC, respectively. Both semi-granular and granular cell counts were significantly ($p<0.05$) lower than other treatments throughout the experiment period. However, hyaline cells count were found to be lower in shrimps exposed to 30‰, 25‰ and 20‰ salinities (Fig 3, $p<0.05$). The lowest percent of hyaline cells were observed in shrimps exposed to salinities 25‰ and 30‰ (Fig 3, $p<0.05$). The semi-granular cells showed higher values in shrimps exposed to salinities of 30‰ and compared to other treatments (Fig 3, $p<0.05$). In salinities of 5‰ and 10‰, the values of granular cells were lower compared to other treatments. Furthermore, the highest values of granular cells were observed in shrimps exposed to 25‰ salinity (Fig 3, $p<0.05$).

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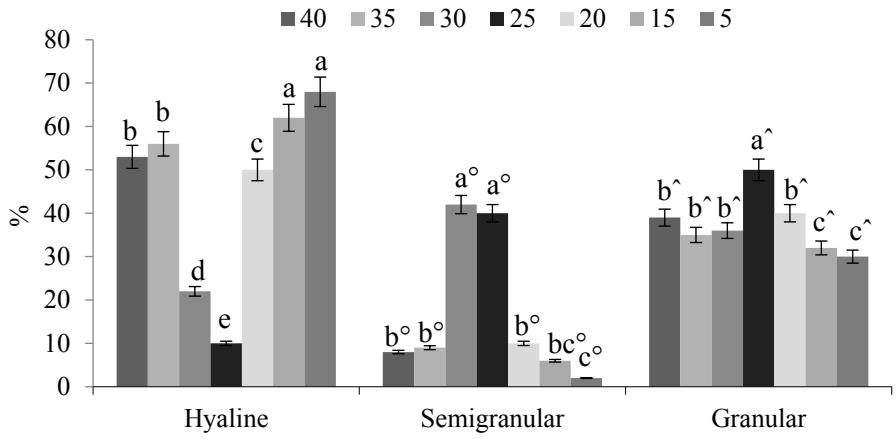

Figure 3. Percent of different haemocyte cells of *L. vannamei* exposed to different salinity levels.

TPP levels in shrimps exposed to 35‰ was significantly lower compare to those exposed to 40‰ (Fig 4, $p<0.05$). Afterwards, TPP levels elevated gradually and reached 115 mg mL^{-1} at 5‰ salinity. No significant differences were observed in TPP levels between the shrimps exposed to <35‰ salinity (Fig 4, $p>0.05$).

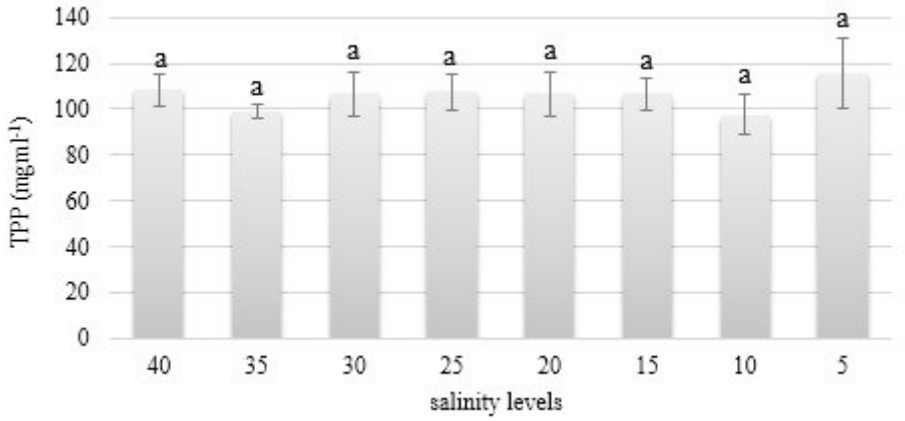

Figure 4. Mean (± S.E.) total protein plasma of *L. vannamei* at different salinity levels. Bars with different letters are significantly different ($p<0.05$) among treatment.

Survival rate

A mean survival rate of 88% was recorded for all salinity treatments during experiment period with no significant differences by salinity graduation ($p>0.05$).

Discussion

In decapods, the number of haematocytes can be altered by environmental parameters including temperature, salinity, dissolved oxygen, pH and ammonia (Verghese *et al.,* 2007). It is known that fluctuations in normal environmental factors affect growth and survival of shrimps (Verghese *et al.,* 2007). Salinity is major important environmental factor affecting the survival, growth, physiology and immunology of shrimps (Lu-Qing *et al.,* 2005). In the present study, the THC levels decreased when the salinity decreased from 40‰ to 30‰, increased in 25‰ salinity and reached initial levels in salinities of 20‰, 15‰ and 10‰. In addition, after a decline in

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TPP levels in shrimps exposed to 35‰, TPP elevated gradually and reached 115 mg mL^{-1} at 5‰ salinity. In coincidence with results of this study, Lu-Qing et al. (2005) observed decline in haemocyte count in the shrimp *Marsupenaeus janponicus* when transferred from 30 ‰ salinity to 5‰ during 6 days, however immune parameters including phenol oxidase activity and antibacterial activity increased during this period. By the 6^{th} day of exposure, all parameters returned to their initial levels in control group. Wang and Chen (2006) reported that *L. vannamei* was more susceptible to *Vibrio alginolyticus* when they were transferred from 25‰ salinity to 5‰ during 24h, while susceptibility of tiger shrimp *Penaeus monodon* to increase with decline in water salinity. Also, the susceptibility of *L. vannamei* to *V. alginolyticus* increased in shrimps exposed to water with low salinities (Liu *et al.,* 2004). Similar results also observed when *P. monodon* exposed to low salinities and then challenged with *P. damselae* sub sp. In this regard, significant reductions were found in immune parameters including THC, HC, phenoloxidase activity, SOD activity, phagocytosis activity and clearance efficiency (Wang and Chen, 2006). A short-term (10h) decline in salinity from 30 to 15‰, reduced the bactericidal and antibacterial activity of *F. chinensis* and *L. vannamei* (Pan and Jiang, 2001) in *F. paulensis*, although THC levels decreased with declining in salinity from 34 to 22%, the THC levels recovered after 7 days (Perazzolo *et al.,* 2002). Esparza-Leal et al. (2019) exhibited that the total haemocyte count (THC) of shrimp declined in a time-dependent manner at all salinity levels, indicating a reduction in resistance of shrimp against infections, since a low value of THC shows an alarm of the immune system.

All above results clearly show that shrimps are capable to copy with sudden changes of water salinity, however, the recovery duration may be different depending of species. In addition, salinity stress may reduce the immunity system in shrimps, which this may be related to the decreased levels of THC during stress (Jia *et al.,* 2014).

The decreased levels of THC also reported in relation to life cycle of shrimps and other environmental stressors such as temperature (Cheng *et al.,* 2005). These reduced THC was in coincidence with apparent decreases in immune system, indicating the effects of life cycle and temperature stress on immune system of shrimps (Lin *et al.,* 2012).

In the present study, the THC levels decreased when salinity decreased from 40‰ to 35‰ and 30 ‰. However, a significant increase in THC levels at 25‰ salinity was observed, which may be due to the haemocyte proliferation rate, or the migration of THC from other tissues to the circulatory fluids or osmosis equilibrium between haemolymph and the environment (Vargas-Albores *et al.,* 1998).

In this study, little changes were observed in TPP levels of *L. vannamei* during salinity challenge. Nevertheless, a significant decline was found after reducing salinity from 40‰ to 35‰. Afterwards, TPP levels elevated gradually and reached 115 mg mL^{-1} at 5‰

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salinity, indicating the gradual adaptation of the shrimps to salinity changes with time. In *P. californiensis*, although salinity had no effect on TPP levels, the total haemocytic pro-PO increased as salinity was elevated (Vargas-Albores *et al.,* 1998). Another study, exhibited that a rapid reduction in salinity level remarkably affected ATPase, SOD, ACP, and AKP activities. Osmotic, metabolism, and immune-corresponded enzyme attributes of *L. vannamei* are sensitive parameters to respond to sudden decrease of salinity (Shen *et al.,* 2020). According to the results of this study, decreasing the salinity for sub-adult of *L. vannamei* should be gradually and not change more than 5‰ per day. When the water salinity reduces, shrimps will act osmosis adaptation, by haemocyte cell proliferation and migrating them from other tissues to the circulatory fluid to improve the health status.

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Conflicts of interest

None of the authors has any conflicts of interest to declare.

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