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Effect of salinity changes on haemocyte of pearl oyster *Pinctada martensii*Ming-Qiang Chen^{1,2}, Gang Yu^{1,2}, Zhen-Hua Ma^{1,2,3*}, You-Ning Li^{1*}, Kong-Wu Xing¹, Li Wang³¹Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya 572018, Hainan, China²Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, 231 Xingang Road West, Haizhu District, Guangzhou 510300, Guangdong, China³Sansha Meiji Fisheries Development Co., Ltd., Haikou 570311, Hainan, China

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ABSTRACT

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Pearl oyster *Pinctada martensii***Objective:** To evaluate the impact of salinity changes in the rearing environment on the haemocyte activities of pearl oyster *Pinctada martensii* (*P. martensii*).**Methods:** Three salinity treatments including 12‰, 22‰, and 32‰ were tested. Haemocyte mortality rate and neutral red retention (NRR) assay were used to investigate the impact of rearing salinity on the haemocyte activities of pearl oyster.**Results:** Results from the present study indicated that salinity significantly affected the haemocyte mortality rate of *P. martensii*. The highest haemocytes mortality rate was observed in the 12‰ treatment, and the lowest mortality rate was observed in the 32‰ treatment. NRR time was also significantly affected by the salinity. The highest NRR time was observed in the 32‰ treatment, and the lowest NRR time was observed in the 12‰ treatment.**Conclusions:** Results from the present study indicated that salinity < 22‰ had significant impact on the haemocyte mortality rate and NRR time. Reducing the environmental salinity will pose an additional stress and may also reduce the defense capacities of *P. martensii* and make them more susceptible to parasites and bacteria.

1. Introduction

Haemocytes in mollusks can produce mediators for stress and main immune responses[1-3]. Previous studies have confirmed that haemocyte densities are sensitive to stresses, and regulated by infection, temperature and salinity fluctuations, and wounding[4-6]. Increases of haemocyte densities in mollusks are commonly considered as a consequence of either proliferation or movement of cells from tissues into the haemolymph, while decreases in haemocyte densities are thought to be a result of cell lysis or

reduced cell movement from tissues to haemolymph[7].

Lysosomes are polymorphic, hydrolytic enzyme-containing organelles[8] in cells. Neutral red is a cytotoxic weak base agent, and has been used to assess the lysosomal membrane integrities. Evidences indicate that the lysosomes in unstressed cells will retain neutral red dye for a long period while those in stressed cells lose neutral red dye at a quicker rate[9,10]. Therefore, correlation between the neutral red retention (NRR) time and stress status on animals can consequently be predicted[11]. After decade's optimization, the NRR assay has been applied to measure the impact of environmental parameters on lysosomal membrane stability in several molluscan species[12-15].

Pearl oyster *Pinctada martensii* (*P. martensii*) is a commercially important species widely cultured in the coastal area of South China Sea. Temperature and salinity have been considered as the major environmental factors affecting the immunity capacity of shellfish[16]. Previous study has demonstrated that environmental factors such as temperature and salinity can interrupt the haemocyte activities in shellfish[6]. Such interruption can directly affect the primary immune system in shellfish. In China, *P. martensii* is

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mainly cultured in the coastal zone where dramatic salinity changes often occurred within 12–24 h after heavy rain. The present study was designed to evaluate the impact of salinity changes in the rearing environment on the haemocyte activities of pearl oyster. Results from the present study will provide some biological insights into the immune response of *P. martensii* to environmental changes.

2. Materials and methods

The pearl oysters used in this study were the 4th generation of artificial bred pearl oyster in Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute. The shell length, width and height were (53.1 ± 1.5) mm, (19.7 ± 1.4) mm and (52.7 ± 1.6) mm (mean \pm SD, $n = 60$), respectively. The pearl oysters were transferred from pearl oysters farm, and acclimated in 30 °C water for 2 days in the wet lab of Tropical Aquaculture Research and Development Center before being used in the experiment. During the acclimation and experiment period, the pearl oysters were held in 500 L concrete tanks and fed with mixed microalgae (*Chaetoceros* and *Platymonas*, 1:1 by density).

In this study, two salinity treatments (12‰, 22‰) were tested, and the salinity of 32‰ was used as the control. After 2-day acclimation, salinity was gradually reduced by adding fresh water to the rearing tanks to the target salinity in 12 h. Upon reaching the target salinity and after acclimation at 12‰, 22‰ or 32‰ for 12 h, a 0.2 mL haemolymph per individual was drawn from the heart using a 29G \times 1 mL syringes, and then placed into a 2 mL siliconized Eppendorf tube containing 0.2 mL of 0.1% trypan blue (Sigma) solution and gently mixed. After 5 min, 0.1 mL hemolymph was added onto a slide and observed under a compound microscope (600 \times magnifications). Two hundred cells per slide were examined and haemocytes stained blue were recorded as dead. The percentage of mortality was then calculated individually.

The NRR assay used in this study was based on the methods developed by Lowe *et al.*[17] and Zhang *et al.*[15]. The neutral red stock solution consisted of 2.28 mg of neutral red powder (Sigma) dissolved in 10 mL of dimethylsulphoxide. The working solution (0.002 mg/mL) was prepared by diluting 17 μ L of the stock solution with 2 mL of sterilized seawater. The hemolymph was collected individually using the same method as described in the previous section, which was completed within 20 s to minimize the potential sampling effects. The hemolymph was then placed into a siliconized Eppendorf tube containing 0.2 mL of sterilized seawater and gently mixed. A 40 μ L mixture of the hemolymph and sterilized seawater was then placed onto a microscope slide. The slide was then placed into a 10 °C light-proof humidity chamber to allow the cells to adhere to the slide. After 15 min, the slide was removed from the chamber and the excess hemolymph was removed. A volume of 20 μ L of neutral red working solution (10 °C) was then added to the cell layer and incubated in the chamber for another 15 min. A cover slide was then placed onto the slide and the haemocytes were examined using a compound microscope at a 600 magnification. The slide was examined at a 15 min interval for the first 60 min and then every 20 min. A total of 30 granulocytes were examined at each time interval. Once over 50% of the haemocytes lost neutral

red dye from their lysosomes, the experiment was stopped. The previous examination time was recorded as the NRR time.

The data in this paper were expressed as mean \pm SD, and tested by One-way ANOVA (PASW Statistics 18.0, Chicago, SPSS Inc.). All the data were tested on their normality and showed normal distribution. ANOVA was then applied. A probability level of $P < 0.05$ was considered to be statistically significant.

3. Results

In the present study, salinity significantly affected the haemocyte mortality rate of *P. martensii* ($P < 0.05$). Upon reaching the target salinity (0 h, Table 1), the highest haemocyte mortality rate was observed in *P. martensii* held in the salinity of 12‰. The haemocyte mortality rate in *P. martensii* was not significantly different when held at 22‰ and 32‰ ($P > 0.05$). After 12 h further acclimation, the highest haemocyte mortality rate was observed in *P. martensii* held in the salinity of 12‰, and the lowest haemocyte mortality rate was observed in 32‰ salinity treatment.

Table 1

Haemocyte mortality rate of *P. martensii* held at 12‰, 22‰ and 32‰.

Acclimation time	Haemocyte mortality rate (%)		
	12‰	22‰	32‰
0 h	11.85 \pm 1.21 ^a	8.42 \pm 2.33 ^b	6.89 \pm 1.98 ^b
12 h	28.12 \pm 4.57 ^a	14.39 \pm 5.28 ^b	7.43 \pm 1.68 ^c

Different letters in the same row represent significant difference ($P < 0.05$).

In this study, NRR time was significantly affected by the environmental salinity ($P < 0.05$, Table 2). When reaching the target salinity, the lowest NRR time was observed in 12‰ salinity treatment, while the NRR time was not significantly different between 22‰ and 32‰ salinity treatments ($P > 0.05$). After 12 h further acclimation, the lowest NRR time was observed in 12‰ salinity treatment, and the highest NRR time was recorded in 32‰ salinity treatment ($P < 0.05$).

Table 2

NRR time of *P. martensii* held at 12‰, 22‰ and 32‰.

Acclimation time	NRR time (min)		
	12‰	22‰	32‰
0 h	66.67 \pm 11.55 ^a	93.33 \pm 11.55 ^b	113.33 \pm 11.55 ^b
12 h	50.00 \pm 8.66 ^a	86.67 \pm 11.55 ^b	106.67 \pm 11.55 ^c

Different letters in the same row represent significant difference ($P < 0.05$).

4. Discussion

Salinity variation has been found inducing the haemocyte mortality in shellfish species such as Pacific oyster *Crassostrea gigas* and American oyster *Crassostrea virginica*[6,18]. In the present study, haemocyte mortality rate increased with the decreasing of environmental salinity. Haemocytes in bivalve are the main line of defense against foreign materials[19]. Increase of haemocyte mortality in *P. martensii* at low salinity will reduce its immune capacity and affect the healthy condition of *P. martensii*.

Neutral red is a lipophilic compound which can freely permeate the membrane of cell[10]. Generally, lysosomes in unstressed cells are impermeable to various substrates. When cells are under stress,

the lysosomal membrane will be destabilized leading to increase of permeability to substrates[20]. Neutral red is a weak cytotoxic compound that is considered as an additional stressor to the cells[17]. Lysosomes in unstressed cells can accumulate and retain the neutral red dye for a long period of time. While destabilizing the neutral red dye will leak into the cytosol of the cell through the damaged membranes[17,20,21].

Evidence has indicated that hyposalinity can induce destabilization of lysosomal membrane[22]. In the present study, NRR time of *P. martensii* was significantly affected by the environmental salinity. The NRR time of *P. martensii* in 12‰ was nearly twice lower than that in 32‰. This may suggest that under low salinity rearing environment, the membranes of lysosome in the haemocytes of *P. martensii* were suffering hyposalinity stress. Our present results are consistent with the finding reported by Nicholson[22], suggesting that reducing salinity has significant impact on the haemocyte of *P. martensii*.

In summary, the present study investigated the impact of environmental salinity on the haemocyte of *P. martensii*. Results from the present study indicate that salinity < 22‰ has significant impact on the haemocyte mortality rate and NRR time. Reducing the environmental salinity will pose an additional stress and may also reduce defense capacities of *P. martensii* and make them more susceptible to parasites and bacteria.

Conflict of interest statement

We declare that we have no conflict of interest.

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