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Transient enhancement of immune resistance functions in *Litopenaeus vannamei* through a low-dose octopamine injection

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ABSTRACT

Octopamine (OA) is known to play an important role in regulating invertebrate immune responses. In this study, we determined the effects of OA on immunity and physiological regulation in the white shrimp *Litopenaeus vannamei*. The total haemocyte count (THC), differential haemocyte count (DHC), phenoloxidase (PO) activity, respiratory bursts (RBs), superoxide dismutase (SOD) activity, and lysozyme, glucose, and lactate levels in plasma, and phagocytic activity and clearance efficiency in response to the pathogen, *Vibrio alginolyticus,* were measured when shrimp (11.1–13.0 g) were individually injected with saline or OA at 100 and 1000 pmol shrimp^{-1}. Results showed significant increases in THC, semigranular cells (SGCs), and PO activity per 50 μL of haemolymph at 0.5–4 h; granular cells at 0.5–2 h; respiratory bursts (RBs) at 0.5–1 h; phagocytic activity at 2–4 h; and clearance efficiency at 2–8 h, but PO activity per granulocyte at 0.5–2 h significantly decreased after the OA injection. All of the immune parameters had returned to control values by 8 h after receiving OA except granular cells at 4 h, RBs at 2 h, clearance efficiency at 16 h, and PO activity per granulocyte at 4 h. However, no significant differences were observed in hyaline cells, RBs per haemocyte, lysozyme and SOD activities, glucose, or lactate during the experimental period. An injection of OA also significantly decreased the mortality of shrimp challenged with *V. alginolyticus*. In another experiment, the immune-related genes of transglutaminase-I, lipopolysaccharide- and β-1,3-glucan-binding protein, prophenoloxidase-II, and peroxidase of shrimp that received 1000 pmol OA shrimp^{-1} for 1 h were significantly higher than those of shrimp that received the saline control. These results suggest that OA administration at ≤1000 pmol shrimp^{-1} mediates transient upregulation of immunity, which in turn promotes the resistance of *L. vannamei* to *V. alginolyticus*.

1. Introduction

The white shrimp, *Litopenaeus vannamei*, has become the primary species currently being cultured in Southeast Asian countries. It is known that the rapid degradation of environments in intensive culture ponds may result in increased incidences of diseases. Cultured shrimp consistently encounter various environmental stressors in intensive culture ponds. Commercial penaeid shrimp farming has been severely hit by epidemics associated with viruses and *Vibrio* infections, which have caused serious economic losses worldwide. Therefore, relationships between stress-induced neuroendocrine matter and immunity of shrimp are of primary concern.

Neuroendocrine hormones play critical roles in regulating homeostasis under stressful environments. Catecholamines (CAs), a class of biogenic amines, derived from tyrosine are required for many physiological and immunological functions, and behaviors in crustaceans [1–3]; they act as neurotransmitters, neuromodulators, and neurohormones and have been identified and quantitatively measured in nervous systems and the haemolymph of crayfish, *Pacifastacus leniusculus*, and other decapod crustaceans [4–6]. The release of CAs is the primary response to physiological stress in crustaceans, while subsequent induction of hyperglycemia and suppression of immunity are secondary responses [1,3].

Tyrosine is a precursor for the production of CAs, which can be metabolized to norepinephrine (NE) or octopamine (OA) via different pathways. OA biosynthesis requires tyrosine decarboxylase activity to convert tyrosine to tyramine and tyramine β-hydroxylase (TBH) activity to convert tyramine to OA [7]. Studies on invertebrates indicated that OA is involved in immunomodulation, and such studies were reviewed by Adamo [8]. OA was demonstrated to accelerate clearing of circulating bacteria from haemolymph, enhance phagocytosis, modulate activities of haemocytes, and increase resistance against pathogen infection in insects [9,10]. In our previous study, we documented that OA administration at ≤ 250.0 pmol prawn^{-1} causes the mediate a

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transient up-regulation in immunologic and physiologic responses to promote the resistance of *M. rosenbergii* to *L. garvieae*, which are thought to be mediated by α- and β-adrenergic-like octopamine receptors [11].

The defense mechanisms of crustaceans are mainly dependent on the innate immunity system that is activated when pathogen-related molecular patterns are recognised by soluble or by cell-surface pattern recognition proteins. Three types of circulating haemocytes are generally recognised as hyaline cells (HCs), semigranular cells (SGCs), and large granular cells (GCs) in crustaceans [12], which play critical roles in immunological defense functions [13,14]. The prophenoloxidase (proPO) system, which is contained in granulocytes, can be activated by several microbial polysaccharides through the non-self recognition system [15], leading to haemocyte degranulation [16], and it is involved in encapsulation, melanization, and recognition [17]. Phenoloxidase (PO) is the terminal enzyme in the proPO system and is considered a major immune indicator in crustaceans. Several reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen, and the hydroxyl radical (OH^-) are produced during phagocytosis as important components of defense responses of crustaceans to invading microorganisms, a process known as respiratory bursts (RBs) [18]. Superoxide anions are the first product released from RBs and are scavenged by superoxide dismutases (SODs) [19].

The aims of this present study were to examine (1) the effect of OA on the susceptibility of *L. vannamei* to *V. alginolyticus*, and (2) the immune responses and immune-associated gene transcription of *L. vannamei* injected with OA. Immune parameters of the total haemocyte count (THC), differential haemocyte count (DHC), PO activity, RBs, SOD activity, phagocytic activity, and the clearance efficiency of shrimp to *V. alginolyticus*, and immune-associated genes of lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP), serine protease (SP), proPO-I, proPO-II, peroxinectin (PE), α2-macroglobulin (α2-M), transglutaminase (TG)-I, TG-II, clottable protein (CP), crustin, lysozyme, and SOD messenger (m)RNA expressions were used as indicators. Furthermore, glucose and lactate levels of plasma were used to evaluate the effects of OA on physiologic responses.

2. Materials and methods

2.1. Litopenaeus vannamei

Shrimp, *L. vannamei*, obtained from an aquafarm of National Pingtung University of Science and Technology in Pingtung, Taiwan, were acclimated in an indoor concrete pond (5 × 5 × 1 m) with 12 tons of aerated seawater at 28 ± 1 °C and a salinity of 20‰ for 2 weeks before experimentation. Only healthy shrimp in the intermolt stage (stage C) were selected for this study. The molt stage was determined by examining the uropoda, in which partial retraction of the epidermis could be distinguished [20].

Five studies were conducted. For the susceptibility experiment, test and control groups were comprised of 10 shrimp each in triplicate. To determine immunological parameters, experiments were carried out in six replicate test groups consisting of one shrimp each in 20-L PVC tanks containing 10 L of aerated seawater (20‰). The same experimental treatment was carried out to determine immune-related gene expressions and physiological parameters. For studies of phagocytic activity and clearance efficiency, another six prawns were used in each of the test and control groups. No significant difference in weight was observed among the treatments. Shrimp were fed twice daily with a formulated shrimp diet (Grobest Feeds Corp., Pingtung, Taiwan) during acclimation and the experiments, and the water temperature was maintained at 28 ± 1 °C and pH at 7.6–8.4.

2.2. Vibrio alginolyticus

Vibrio alginolyticus (CH003), a known pathogenic strain, which was isolated from diseased shrimp, *L. vannamei*, that displayed symptoms of anorexia, inactivity, poor growth, and necrotic musculature, was used for the study [21]. Stocks were plated on tryptic soy agar (TSA, Difco) supplemented with 2% NaCl for 24 h at 28 °C, and then transferred to 10 mL tryptic soy broth (TSB, Difco) supplemented with 2% NaCl, where they remained for 24 h at 28 °C, after which they were centrifuged at 7155 × *g* for 15 min at 4 °C. The supernatant was removed, and the bacterial pellet was suspended in a saline solution (0.85% NaCl) at concentrations of 10^7 and 10^9 colony-forming units (cfu) mL^{-1} as respective stock bacterial suspensions for the susceptibility study and for the phagocytic activity and clearance efficiency studies.

2.3. Effect of octopamine on the susceptibility of shrimp to V. alginolyticus

OA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile saline (0.85% NaCl) to concentrations of 5×10^{-5} and 5×10^{-6} mol L^{-1} before injection. Twenty microliters of an OA solution was injected into the ventral sinus of the cephalothorax of individual *L. vannamei* (11.2 ± 0.6 g) to reach respective doses of 100 and 1000 pmol $shrimp^{-1}$ in the initial stage. At 1 h after the injection, a challenge test was conducted by injecting 20 μL of a bacterial suspension (10^7 cfu mL^{-1}) resulting in 2×10^5 cfu $shrimp^{-1}$ into the ventral sinus of the cephalothorax. Shrimp that received 20 μL of sterile saline and then *V. alginolyticus* at 2×10^5 cfu $shrimp^{-1}$ served as the saline-challenged controls. Shrimp that received OA at 1000 pmol $shrimp^{-1}$ and then were injected with 20 μL of sterile saline served as the unchallenged control (Table 1). Test and control prawn (10 shrimp $aquarium^{-1}$) were kept in 60-L glass aquaria containing 40 L of seawater at 28 °C and a salinity of 20‰. Therefore, there were four treatments and each treatment was conducted with 30 shrimp. The experiment lasted 168 h.

2.4. Effect of octopamine on immune parameters of L. vannamei

Litopenaeus vannamei (12.5 ± 0.8 g) was individually injected with 5×10^{-5} and 5×10^{-6} mol L^{-1} of an OA solution into the ventral sinus of the cephalothorax to reach respective doses of 100 and 1000 pmol $shrimp^{-1}$. Shrimp that received 20 μL of saline served as the control. There were three treatments (saline (0), 100, and 1000 pmol

Table 1
Effect of octopamine on the susceptibility of white shrimp, *Litopenaeus vananmei*, challenged with *Vibrio alginolyticus*.

Octopamine (pmol $shrimp^{-1}$)	Bacterial dose (cfu $shrimp^{-1}$)	No. of shrimp	Survival rate (%), time after challenge (h)					
			6	24	48	72	120	168
1000	saline	30	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
saline	2×10^5	30	83.3 ± 5.8^a	70.0 ± 0^b	60 ± 0^b	56.7 ± 5.8^b	56.7 ± 5.8^b	56.7 ± 5.8^b
1000	2×10^5	30	10 ± 0^a	83.3 ± 5.8^a	80 ± 0^a	80 ± 0^a	80 ± 0^a	80 ± 0^a
100	2×10^5	30	10 ± 0^a	80.0 ± 0^a	76.7 ± 5.8^a	76.7 ± 5.8^a	76.7 ± 5.8^a	76.7 ± 5.8^a

Data in the challenge groups in the same column with different superscripts are significantly different. ($p < 0.05$) among treatments.
Values are mean ± S.E. (n = 30 shrimp in each case).

shrimp^{-1}) with six sampling times (0.5, 1, 2, 4, 8, and 16 h) for immune parameter determination. Six shrimp from each treatment and time were used for the studies. In addition, another six prawn with no treatment were used as the initial group.

At the beginning (0 h) and at 0.5, 1, 2, 4, 8 and 16 h after the injection, haemolymph was withdrawn from the ventral sinus of each prawn into a 1-mL sterile syringe (25 gauge), and was diluted 10-fold in anticoagulant solution (trisodium citrate 30 mM, sodium chloride 0.34 M, and EDTA 10 mM, pH 7.55, with the osmolality adjusted to 780 mOsm kg^{-1} with 0.115 M glucose). A drop of the anticoagulant-haemolymph mixture was loaded in a haemocytometer to enumerate the THC and DHC (Leica DMIL, Leica Microsystems, Wetzlar, Germany). One milliliter of the haemolymph mixture was centrifuged at 400 × *g* and 4 °C for 20 min, and the supernatant as plasma was used for the lysozyme activity assay. The haemocyte pellet was washed twice with cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, and 0.26 M magnesium chloride; pH 7.0). The resultant haemocyte pellet was then used for the PO activity assay. Another 100 μL of the haemolymph mixture was used for the RB analysis. The remainder of the diluted haemolymph mixture was used for SOD activity assay.

PO activity in haemocytes was spectrophotometrically measured at 490 nm by recording the formation of dopachrome produced from L-3,4-dihydroxyphenylalanine (L-DOPA, D-9628, Sigma, St. Louis, MO, USA) according to the method of Mason [22] and Hernández-López et al. [23]. L-DOPA and trypsin respectively served as the substrate and an elicitor. The procedure was formerly described in detail [24]. The optical density (OD; 490 nm) was expressed as dopachrome formation in haemocytes per 50 μL of haemolymph or per 10^7 granulocytes (the sum of GCs and SGCs).

RBs of haemocytes were quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) formation as described previously [19,25]. The OD at 630 nm was measured for triplicate reactions using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). RBs are expressed as NBT reduction in haemocytes per 10 μL of haemolymph or per 10^7 haemocytes.

For SOD activity examination, the haemocyte lysate supernatant (HLS) was prepared as described previously [26]. SOD activity was measured by its ability to inhibit superoxide radical-dependent reactions using a Ransod Kit (Randox, Crumlin, UK) based on previously described methods [27]. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units (mg protein)$^{-1}$. Protein was quantified by a method described by Bradford [28] using a Bio-Rad protein assay kit (no. 500-0006, Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as the standard.

Plasma lysozyme activity was modified as described by Ellis [29] and Obach et al. [30]. Briefly, 10 μL of individual plasma was mixed with 200 μL of a *Micrococcus luteus* (Sigma) suspension at 0.2 mg mL^{-1} in 0.05 M sodium phosphate buffer (pH 6.2). The mixture was incubated at 27 °C, and its OD was detected after 1 and 6 min at 530 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} mL^{-1} serum. Lysozyme concentrations were calculated from a standard curve of known lysozymes from chicken egg white (L4631-1VL, Sigma) concentrations.

2.5. Phagocytic activity and clearance efficiency of L. vannamei to V. alginolyticus

Litopenaeus vannamei received saline or OA as described above except sampling times were at 2, 4, 8, and 16 h. Tests were carried out on six shrimp (replicates) at each treatment and sampling time. For phagocytic activity and the clearance efficiency assay, 20 μL of a bacterial suspension (10^9 cfu mL^{-1}) resulting in 2×10^7 cfu shrimp^{-1} was injected into the ventral sinus of each shrimp 2 h after it had received saline or OA. After injection of the bacterial suspension, the shrimp were kept for 1 h in a separate tank containing 40 L of water at 28.0 ± 1.0 °C. Then, 200 μL of haemolymph was collected from the ventral sinus and mixed with 200 μL of a sterile anticoagulant solution. This mixture was divided into two equal subsamples: one to measure phagocytic activity and the other to measure the clearance efficiency. Phagocytic activity was evaluated as previously described by Weeks-Perkins et al. [31]. Briefly, 200 μL of the diluted haemolymph sample was mixed with 200 μL of 0.1% paraformaldehyde for 30 min at 4 °C to fix the haemocytes. They were then centrifuged at 400 × *g* and 4 °C, washed, and resuspended in 0.4 mL of a saline solution. A 50-μL sample of the suspension was spread on a glass slide. The slide was placed in a cytospin centrifuge (Model Cytospin 3, Shandon, UK) and centrifuged at 113 × *g* for 3 min. The slide was then air-dried, stained with Liu's staining method, and observed under a light microscope. In total, 200 haemocytes were counted, and haemocytes engulfing stained bacteria were phagocytic haemocytes. Phagocytic activity, defined as the percentage phagocytosis (PR), was expressed as:

$$PR = [(\text{phagocytic haemocytes}) (\text{total haemocytes})^{-1}] \times 100.$$

The clearance efficiency was measured following the method of Adams [32]. Diluted haemolymph was further diluted with a sterile saline solution. Three 50-μL portions of each diluted haemolymph sample were spread on separate TSA plates and incubated at 28 °C for 20 h before the colonies were counted using a colony counter. The number of colonies of shrimp receiving saline was expressed as the control group, and the numbers of colonies of shrimp receiving OA after 2, 4, 8, and 16 h were expressed as the test groups. The clearance efficiency toward *V. alginolyticus*, defined as the percentage inhibition (PI), was calculated as:

$$PI = 100 - [(\text{cfu in test group}) (\text{cfu in control group})^{-1}] \times 100.$$

2.6. Estimation of glucose and lactate of L. vannamei

Shrimp (11.8 ± 0.7 g) that received saline or OA and then haemolymph were collected and treated the same as those described above at sampling times of 0.5, 1, 2, 4, 8, and 16 h, except the osmolality was adjusted with NaCl instead of glucose. The plasma glucose concentrations were measured using a coupled glucose oxidase and peroxidase reaction with a Glucose Kit (Randox, GL2623). The OD at 505 nm was measured by an ELISA plate reader, and the glucose concentration was calculated from a known glucose concentration standard curve.

Plasma lactate concentrations were measured using the colorimetric lactate oxidase and peroxidase method with a Lactate Kit (Fortress Diagnostic, Crumlin, UK, BXC0621A). The OD at 550 nm was measured with an ELISA plate reader, and the lactate concentration was calculated from a known lactate concentration standard curve.

2.7. Analysis of immune-related gene expressions

Litopenaeus vannamei (12.3 ± 0.5 g) injected with saline or OA at 1000 pmol shrimp^{-1} for 1 h was used in these tests. Each haemolymph sample was withdrawn and diluted as stated above. The diluted haemolymph was centrifuged at 400 × *g* and 4 °C for 20 min. The resulting haemocyte pellet was used for total RNA isolation to determine transcript levels of immune-related genes of the LGBP, PE, TG-I, TG-II, α2-M, clottable protein, crustin, lysozyme, SP, as well as the SOD, POI, and POII genes.

mRNA transcripts were measured using a SYBR green I real-time reverse-transcription polymerase chain reaction (RT-PCR) assay in an ABI PRISM 7900 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA), and details of the measurement procedures were previously described [33], and checked following

Table 2
Specific primers used in the experiment.

Primer		Sequence (5′→3′)	Reference/GeneBank
α2-M	F	GCACGTAATCAAGATCCG	DQ988330
	R	CCCATCTCATTAGCACAAAC	
Q-CP	F	CGCATCCAGACGGAGTTCTT	DQ984182
	R	GCTGTTGTGCATCGAACTTTG	
Q-Crustin	F	CTGGCCTCGATAAGTGTTGCT	AY486426
	R	GGAGGCTTGCACACGTGTT	
Q-Lysozyme	F	CTGGTGCGGAAGCGACTAC	[52]
	R	CGGACATCAGATCGGAACATG	
Q-LGBP	F	CATGTCCAACTTCGCTTTCAGA	[53]
	R	ATCACCGCGTGGCATCTT	
Q-PE	F	TGGACCTCGCGGGAGAT	[54]
	R	GACCGATAGCCACCATGCTT	
Q-SOD	F	TCATGCTTTGCCACCTCTC	KC787355
	R	CCGCTTCAACCAACTTCTTC	
Q-PO-I	F	GCCTTGGCAACGCTTTCA	[33]
	R	CGCGCATCAGTTCAGTTTGT	
Q-PO-II	F	GAGAGGCTGAACCGAGACTGA	[55]
	R	AAGAAAACGGCCCCCAATT	
Q-SP	F	CGTCGTTAGGTTAAGTGCGTTCT	AY368151
	R	TTTCAGCGCATTAAGACGTGTT	
Q-TG-I	F	GAGCTTCAAGATCGAGGATCGA	[56]
	R	GCTGGTGTTCGTAGCGGTTATC	
Q-TG-II	F	GCCCGAGGACAGGGAAA	[56]
	R	GCGGTGGTCAGTGAATCCA	
Q-β-actin	F	CATCACCAACTGGGACGACATGGA	[57]
	R	GAGCAACACGGAGTTCGTTGT	

directions of Bustin et al. [34]. Specific primer pairs used for the quantitative RT-PCR are outlined in Table 2. After amplification, data acquisition and analysis were performed using Sequence Detection Software (SDS vers. 2.1, Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was chosen as the calculation method [35]. The difference in the cycle threshold (C_T) value of the tested gene and its housekeeping gene (β-actin), called ΔC_T, was calculated. $\Delta\Delta CT$ = (ΔC_T of OA-injected shrimp for the tested gene) - (ΔC_T of the saline control).

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze the data. When the ANOVA identified differences among groups, a multiple-comparisons (Duncan) test was conducted to compare significant differences among treatments using the SAS computer software (SAS Institute, Cary, NC, USA). Percent data were normalized using an arc-sine-transformation before analysis. A statistically significant difference required that $p < 0.05$.

3. Results

3.1. Effects of OA on the susceptibility of L. vannamei to V. alginolyticus

All of the unchallenged control shrimp that received OA at 1000 pmol shrimp^{-1} and were then injected with saline survived. Among the test groups, survival rates of shrimp that received OA at 100 and 1000 pmol shrimp^{-1} were significantly higher than those of saline-challenged control shrimp at 24–168 h. After 168 h of challenge, survival rate of shrimp received OA at 100 and 1000 pmol shrimp^{-1} significantly increased by 23.4% and 20.3%, respectively, compared to that of the saline-challenged control. However, no significant difference in survival rates was observed between shrimp that received OA at 100 and 1000 pmol shrimp^{-1} (Table 1).

3.2. Effect of OA on the immunological and physiological parameters of L. vannamei

For shrimp that received OA at 100 and 1000 pmol shrimp^{-1}, THC, SGCs, and GCs were significantly higher at 0.5–4, 0.5–4, and 0.5–2 h, respectively, than those of shrimp that received the saline control, and THCs were significantly higher by 47.7% and 43.7% at 0.5 h, 50.1% and 44.6% at 1 h, 23.1% and 19.3% at 2 h, and 14.6% and 30.0% at 4 h, respectively; SGCs were significantly higher by 116.0% and 115.6% at 0.5 h, 109.7% and 78.1% at 1 h, 73.9% and 61.4% at 2 h, and 33.3% and 32.4% at 4 h; and GCs were significantly higher by 83.3% and 85.5% at 0.5 h, 89.8% and 96.7% at 1 h, and 70.9% and 64.3% at 2 h, respectively, compared to shrimp that received the saline control. At 8 h

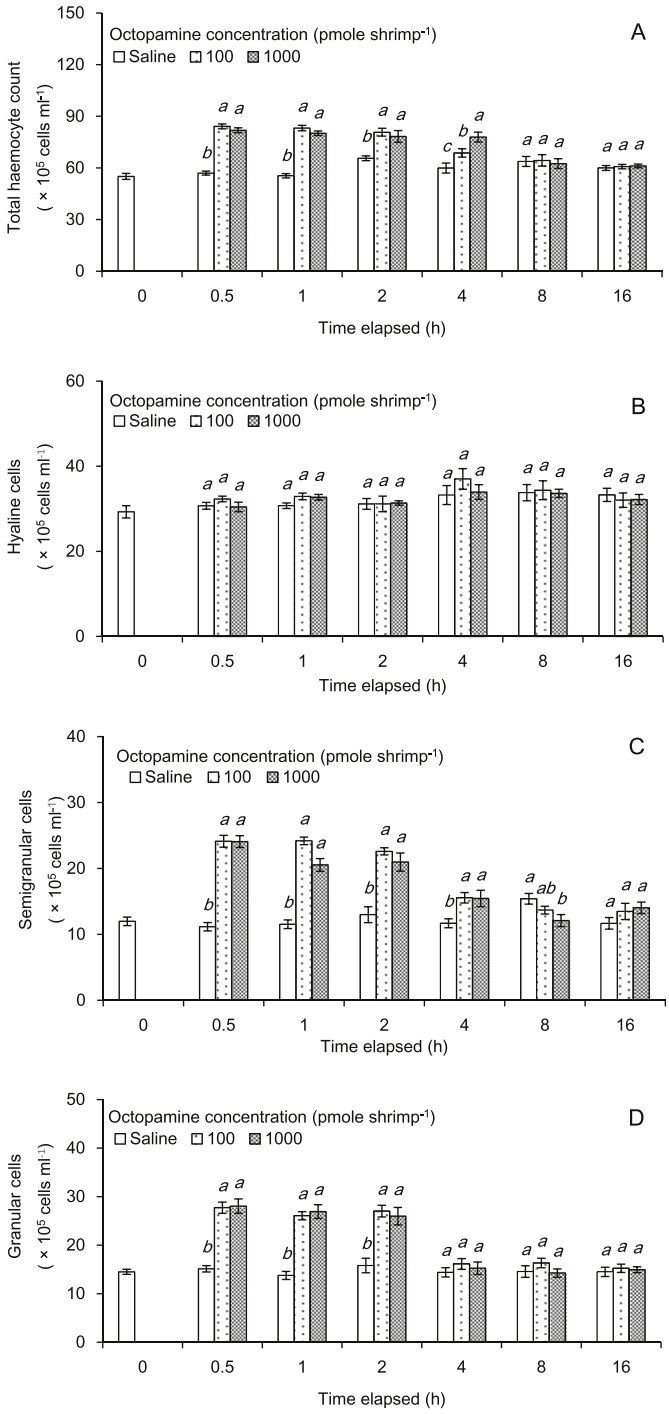

Fig. 1. Total haemocyte count (A), hyaline cells (B), semigranular cells (C) and granular cells (D) of *Litopenaeus vannamei* received saline or octopamine at 100 or 1000 *p*mol shrimp^{-1}. Each bar represents the mean value from 6 samples with the standard error. Bars with different letters significantly differ ($p < 0.05$) among treatments at the same sampling time.

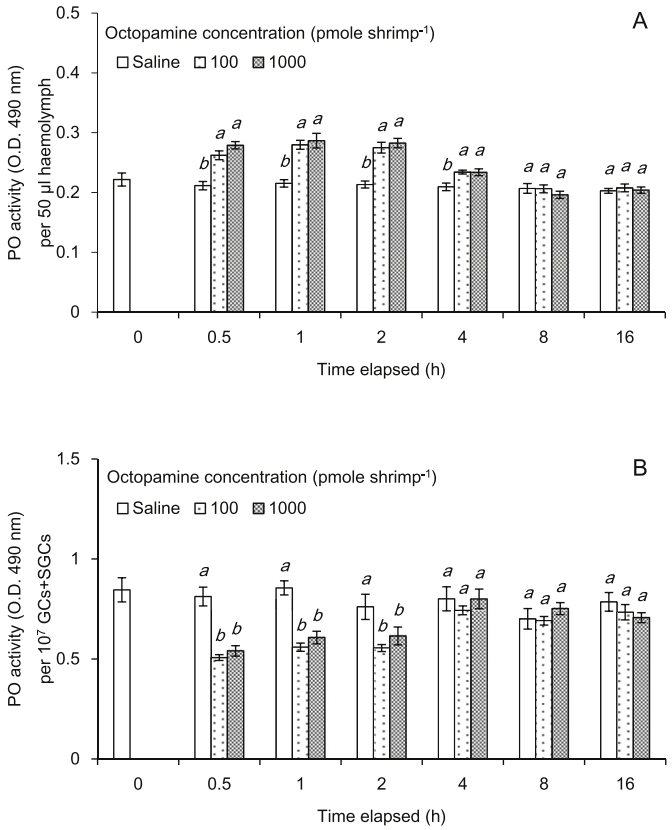

Fig. 2. Phenoloxidase (PO) activity in haemocyte of per 50 µL haemolymph (A) and in per 10^7 granulocytes (granular cells (GCs) + semigranular cells (SGCs)) (B) of *Litopenaeus vannamei* received saline or octopamine at 100 or 1000 *p*mol shrimp^{-1}. Statistical descriptions are the same as those in Fig. 1.

after the injection, SGCs of shrimp that received OA at 1000 pmol shrimp^{-1} were significantly lower than those of shrimp that had received the saline control. However, no significant differences were observed in HCs among the three treatments at 0.5–16 h, in THCs at 8–16 h, in GCs at 4–16 h, or in SGs at 16 h (Fig. 1AD).

PO activities in haemocytes per 50 µL of haemolymph of shrimp that received OA at 100 and 1000 pmol shrimp^{-1} were significantly higher than those of shrimp that received the saline control at 0.5–4 h, and had significantly increased by 24.0% and 31.9% at 0.5 h, 30.0% and 33.1% at 1 h, 28.9% and 32.5% at 2 h, and 11.8% and 11.6% at 4 h, respectively, compared to those of saline-injected shrimp. On the contrary, PO activities per granulocyte (SGC + GC) of shrimp that received 100 and 1000 pmol shrimp^{-1} were significantly lower than those of shrimp that received the saline control at 0.5–2 h, and had significantly decreased by 37.6% and 33.5% at 0.5 h, 34.7% and 29.1% at 1 h, and 27.1% and 19.1% at 2 h, respectively, compared to shrimp that had received the saline control. However, no significant differences in PO activity per 50 µL of haemolymph and per granulocyte were observed among the three treatments at 8–16 h or at 4–16 h, respectively, and between the two OA-injected shrimp at 0.5–4 h or at 0.5–2 h, respectively (Fig. 2A and B).

RBs in haemocytes per 10 µL of haemolymph had significantly increased by 34.5% and 62.4% at 0.5 h in shrimp that received OA at 100 and 1000 pmol shrimp^{-1}, respectively, and by 51.7% at 1 h for shrimp that received OA at 1000 pmol shrimp^{-1}, compared to saline-injected shrimp. However, no significant differences in RBs per 10 µL of haemolymph were observed among the three treatments at 2–16 h or between the two OA-injected shrimp groups at 0.5–1 h (Fig. 3A). No significant differences in RBs per haemocyte, or SOD activities of shrimp were observed among the three treatments at 0.5–16 h (Fig. 3B and C).

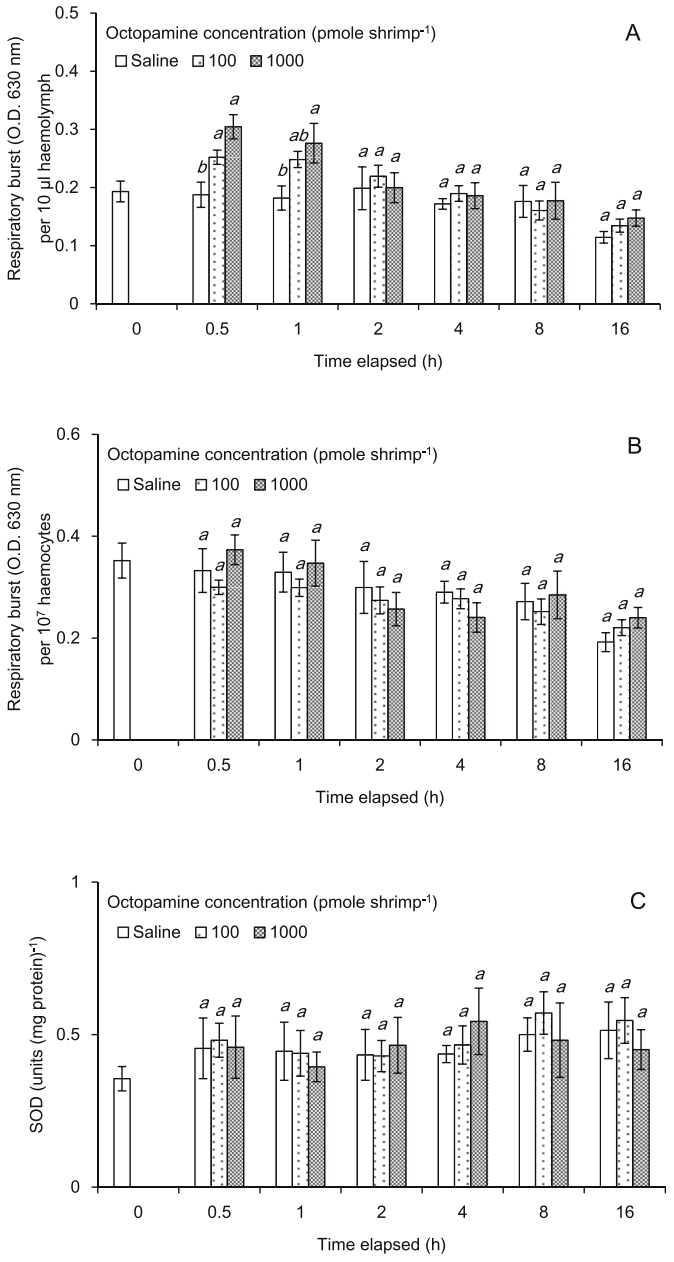

Fig. 3. Respiratory burst in haemocyte of per 10 µL haemolymph (A) and in per 10^7 haemocytes (B), and superoxide dismutase (SOD) activity (C) of *Litopenaeus vannamei* received saline or octopamine at 100 or 1000 *p*mol shrimp^{-1}. Statistical descriptions are the same as those in Fig. 1.

Phagocytic activities and clearance efficiencies of shrimp that received OA at 100 and 1000 pmol shrimp^{-1} were significantly higher than those of shrimp that received the saline control at 2–4 and 2–8 h, respectively. For shrimp that received OA at 100 and 1000 pmol shrimp^{-1}, phagocytic activities had respectively increased by 40.2% and 43.4% at 2 h and by 27.1% and 34.0% at 4 h, and clearance efficiencies had increased by 70.9% and 80.9% at 2 h, by 58.3% and 68.8% at 4 h, and by 38.6% and 42.5% at 8 h, respectively, compared to those of shrimp that received the saline control. However, no significant differences in phagocytic activities or clearance efficiencies were observed among the three treatments at 8–16 and 16 h, or between the two OA-injected shrimp groups at 2–4 and 2–8 h, respectively (Fig. 4A and B). No significant differences in lysozyme activity (Fig. 4C), and glucose or lactate levels (Fig. 5A and B) in plasma were observed among the three treatments at 0.5–16 h.

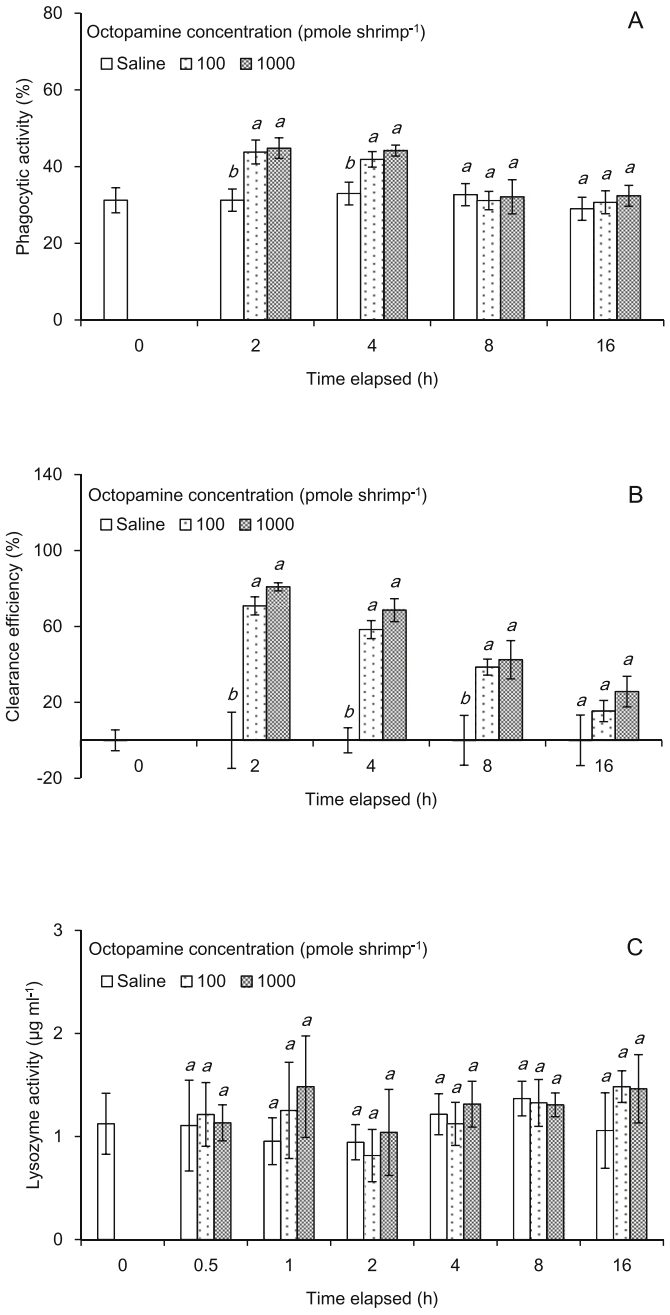

Fig. 4. Phagocytic activity (A), clearance efficiency (B) and lysozyme activity of *Litopenaeus vannamei* received saline or octopamine at 100 or 1000 *p*mol shrimp^{-1}. Statistical descriptions are the same as those in Fig. 1.

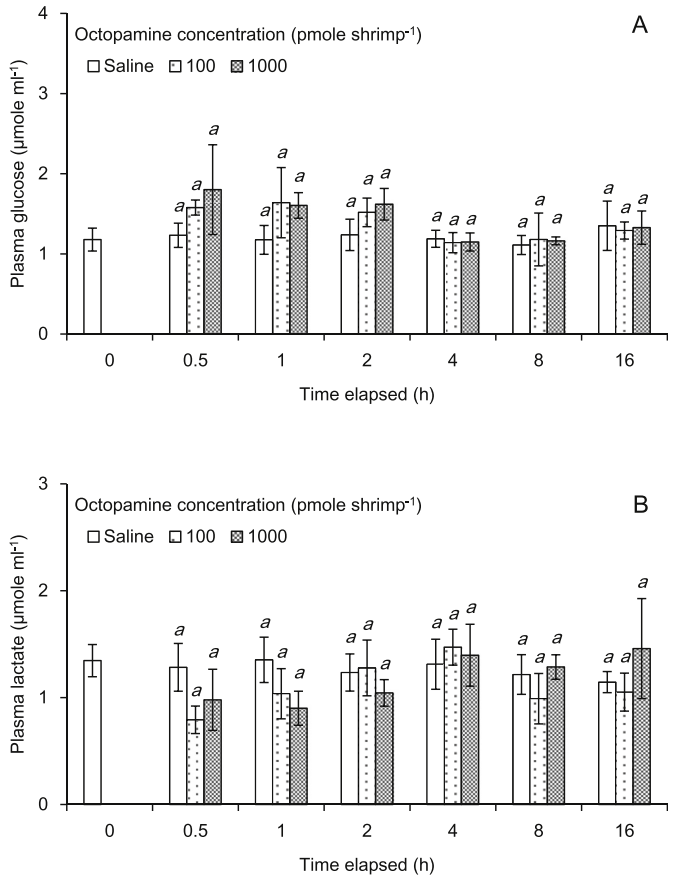

Fig. 5. Haemolymph glucose (A) and lactate (B) of *Litopenaeus vannamei* received saline or octopamine at 100 or 1000 *p*mol shrimp^{-1}. Statistical descriptions are the same as those in Fig. 1.

3.3. Effects of OA on immune-related gene expressions

After 1 h, LGBP, proPO-II, PE, and TG-I mRNA expressions in haemocytes of shrimp that received OA at 1000 pmol shrimp^{-1} were significantly higher than those of shrimp that received the saline control, and had increased by 304.3%, 391.8%, 191.0%, and 350.9%, respectively, compared to those of shrimp that received the saline control. However, no significant differences in SP, proPO-I, α2-M, TG-II, CP, crustin, lysozyme, or SOD transcripts were observed between shrimp that received saline and OA at 1000 pmol shrimp^{-1} (Fig. 6).

4. Discussion

The effects of stress-induced hormones/neurohormones on immunity are complex in invertebrates [8]. In insects, a transient increase in haemolymph OA of up to 10-fold over baseline levels was observed during acute stress [36,37]. Cockroaches, *Periplaneta americana*, exposed to a 50% lethal dose (LD_{50}) of *Staphylococcus aureus* in conjunction with either 0.1 mM OA or the OA agonist, clonidine, had higher survival rates compared to the saline-treated control [38]. OA in haemolymph was also significantly elevated by bacterial infection, and this elevation appeared to enhance immune function in the cricket, *Gryllus texensis* [39]. In our previous study, *M. rosenbergii* that had received OA at 25.0 or 250.0 pmol prawn^{-1} showed increased resistance to *L. garvieae* [11]. In the present study, we found that treatment with OA at 100 and 1000 pmol shrimp^{-1} significantly increased resistance to *V. alginolyticus*. This fact suggests that OA elevation in haemolymph by an injection may promote the resistance abilities of *M. rosenbergii* and *L. vannamei*.

In invertebrates, haemocytes play important roles in regulating physiological and immunological functions. OA is considered a bifunctional molecule which both acts as a modulator of haemocyte activity and mediates bacterial attachment to haemocytes [10]. Diehl-Jones et al. [40] indicated that OA stimulates the locomotory activity of plasmatocytes by altering the actin-cytoskeleton rearrangement. In *Spodoptera exigua*, upregulation of the circulating haemocyte population in response to bacterial challenge is mediated by OA and 5-hydroxytryptamine via Rac1 signals [41]. In our previous study, OA-injected *M. rosenbergii* exhibited significant increases in THC, HCs, SGCs, and GCs at 0.5, 1, and/or 2 h after the injection, compared to the saline-injected and initial control [11]. In *Penaeus monodon*, hyaline cells are young, immature haemocytes of both large- and small GCs that are produced in hematopoietic tissue (HPT) and can be released into the haemolymph, and large GCs mature and accumulate in connective

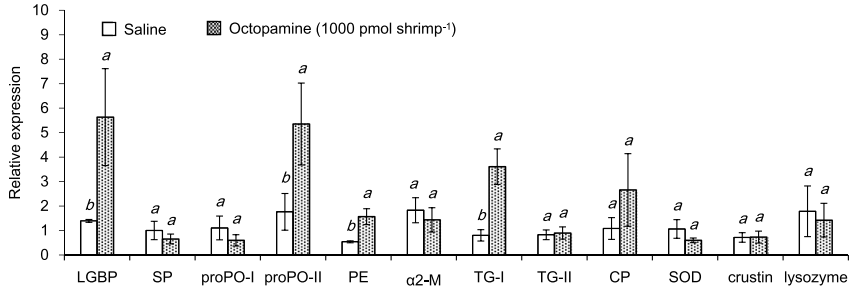

Fig. 6. Immune-related gene expressions of *Litopenaeus vannamei* received saline or octopamine at 1000 *p*mol shrimp^{-1}. Statistical descriptions are the same as those in Fig. 1.

tissues and are easily released into the haemolymph [42]. In the present study, *L. vannamei* that received OA at 100 and 1000 pmol shrimp^{-1} exhibited significant increases in the THC at 0.5–4 h, in SGCs at 0.5–4 h, and in GCs at 0.5–2 h, but decreases in the SGCs at 8 h after the injection compared to the saline-injected and initial control. These results suggest that OA enhanced mobilization of fixed haemocytes and regenerated haemocytes release into the circulatory system, which resulted in elevation of the THC and granulocytes in OA-injected shrimp, and the THC increase resulted from an increase in granulocytes (GCs and SGCs). However, the young haemocytes to fix in connective tissues for maturation resulted in circulating SGCs decrease at follow-up responses of the transient elevation.

Huang et al. [43] indicated that strong PmTG-I signals were detected in young haemocytes in HPT of *P. monodon*, which decreased when haemocytes matured and were released into the haemolymph. In *L. vannamei*, LvTG-I and LvTG-II are abundantly expressed in haemocytes, and LvTG-I expression in hyaline cells was higher than in GCs [44,45]. In addition, LvTG-II mRNA was highly expressed in haemocytes, but was relatively less expressed in other tissues. Those results suggest that shrimp TG-I expression in haemocytes possibly takes place in early developmental stages but is downregulated in later developmental stages (after maturing and being released into the haemolymph), and which is simultaneously substituted for STGII [44]. In the present study, the LvTG-I of haemocytes was significantly higher in *L. vannamei* that received OA at 1000 pmol shrimp^{-1} for 1 h than that of the saline control, but the level of LvTG-II remained the same. Elevation of LvTG-I implies that regenerated haemocytes were the majority of the increased circulating haemocytes through OA induction.

The proPO system that exists in granular haemocytes plays a major role in innate immunity in crustaceans; it is involved in non-self recognition, haemocyte communication, and the production of melanin, and can be regulated by biogenic monoamine [24]. In *Pacifastacus leniusculus*, proPO mRNA was not expressed in HPT, but haemocytes released into the circulation expressed proPO [46]. In *L. vannamei*, Yeh et al. [47] indicated that the function of proPO-II contributes earlier to the acute-phase defense mechanism, and proPO-I is the major type of proPO involved in follow-up defense in white shrimp challenged with *V. alginolyticus*. In the present study, when *L. vannamei* was injected with saline and OA, PO activity in haemocytes per 50 μL of haemolymph was significantly higher in shrimp that received OA after 0.5–4 h than those of shrimp which received the saline control. In the meantime, PO activity per granulocyte of shrimp that received OA was significantly lower than that of shrimp which received the saline control, and expression patterns of granulocytes at 0.5–2 h disagreed with the PO activity per granulocyte and agreed with the PO activity in haemocytes per 50 μL of haemolymph; the same responses were observed in *M. rosenbergii* injected with saline and OA at 25.0 or 250.0 pmol prawn^{-1} [11]. In addition, proPO system-related genes of LGBP, proPO-II, and PE in shrimp that received OA at 1000 pmol shrimp^{-1} after 1 h were significantly higher than those of shrimp that received the saline control, but genes of SP and proPO-I did not change. These results suggest that OA promoted the release of regenerated granulocytes which incompletely expressed proPO (proPO-II expression only), and mobilization of granulocytes in connected tissues also induced LGBP and PE expressions to enhance proPO system regulation, resulting in elevation of PO activity in haemocytes per unit of haemolymph, but the mean per granulocyte deceased to maintain homeostasis. In addition, elevated proPO-II is in agreement with the function of proPO-II which contributes earlier to the acute-phase defense mechanism [47].

The production of species of reactive oxygen intermediates (ROIs) during phagocytosis is a key cellular defense mechanism; these are needed to kill invading pathogens, and begin with NADPH oxidase in phagocytes. Superoxide anions are the first product released from RBs, and can be scavenged by SOD. Clearance from the circulation is induced by humoral factors such as agglutinins, lectins, cytotoxic factors [48], and antimicrobial factors [49]; this causes bacterial aggregation in the circulating haemolymph, and enhances the clearance rate [42]. In insects, OA is known to mediate haemocyte phagocytosis and nodule formation, which are able to increase the survival of cockroaches exposed to *S. aureus* [9,10]. In *M. rosenbergii*, injection of prawn with OA at 25.0 or 250.0 pmol prawn^{-1} notably promoted the clearance efficiency, phagocytosis, and upregulation of ROIs [11]. In the present study, shrimp that received OA at 100 or 1000 pmol shrimp^{-1} exhibited increased clearance efficiency at 2–8 h, phagocytic activity at 2–4 h, and O_2^- in haemocytes per unit of haemolymph at 0.5–1 h, which correlated with increased resistance of *L. vannamei* to *V. alginolyticus*. These facts suggest that OA promotes humoral and cellular immune responses, resulting in elevated resistance against pathogens in shrimp injected with OA at 100 or 1000 pmol shrimp^{-1}, and the elevation in O_2^- per unit of haemolymph resulted from an increase in the THC.

Haemolymph glucose levels are generally considered a good physiological indicator of stress conditions in animals. Glucose is oxidized by glycolysis, an energy-generating pathway that converts it to pyruvate. In the absence of oxygen, pyruvate is converted to lactate, and when oxygen is present, pyruvate is further converted into CO_2 and H_2O. Elevated blood glucose can result from reduced utilization of glucose or stimulation of gluconeogenesis and/or glycogenolysis. Kuo and Yang [3] indicated that *M. rosenbergii* administered OA at 2 nM prawn^{-1} (2000 pmol prawn^{-1}) by injection exhibited notably induced elevation of glucose in haemolymph. Lactate can be converted to pyruvate in the presence of oxygen, which can then be converted into glucose. In our previous study, *M. rosenbergii* injected with OA at 25.0 or 250.0 pmol prawn^{-1} had significantly higher glucose, but significantly lower lactate in plasma [11]. In the present study, although neither plasma glucose nor lactate significantly differed during the experimental period, glucose slightly increased at 0.5–2 h and lactate slightly decreased at 0.5–1 h, after shrimp were injected with OA at 100 or 1000 pmol shrimp^{-1}. These facts suggest that OA induced gluconeogenesis and/or glycogenolysis to provide a metabolic energy source under aerobic glycolysis for the energy demand following stress-induced CA release in prawn and shrimp.

The immunosuppressive or immunoenhancing effect of OA is

considered to be dose-dependent [8]. Injection of OA prior to a bacterial challenge resulted in increased mortality in the cricket, *Gryllus texensis* [50], which is contrary to experimental results of Baines et al. [9] in a cockroach. According to methods of Châtel [51], using an ELISA, OA levels in the haemolymph of *M. rosenbergii* and *L. vannamei* were 4030.6 ± 0.03 and 4880.0 ± 0.10 pmol ml^{-1}, respectively (unpublished data). Therefore, both *M. rosenbergii* and *L. vannamei* that received OA at a lower dose than the physiological baseline exhibited significantly enhanced immune responses and resistance ability. Further study is needed to verify biphasic effects of OA on immune function of shrimp.

In conclusion, the present study documented that *L. vannamei*, which had received OA at ≤ 1000 pmol shrimp^{-1} showed upregulation of humoral and cellular immune responses, and immune-related gene expressions of LGBP, proPO-II, PE, and TG-I and consequently exhibited increased resistance to *V. alginolyticus*.

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