Fish and Shellfish Immunology 92 (2019) 188–195

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

The intracellular signaling pathway of octopamine upregulating immune resistance functions in *Penaeus monodon*

Kuan-Fu Liu[a], Hsin-Wei Kuo[b], Chin-Chyuan Chang[b], Winton Cheng[b,*]

[a] Tungkang Biotechnology Research Center, Fisheries Research Institute, C.O.A, Pingtung, 92845, Taiwan, ROC
[b] Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung, 91201, Taiwan

ARTICLE INFO

Keywords:
Octopamine
Penaeus monodon
Immune resistance
Ca^{2+} and cAMP second messenger pathways

ABSTRACT

Octopamine (OA), a biogenic monoamine, is known to mediate several immune responses. This study analyzed the effects of OA on immunological regulation in the tiger shrimp *Penaeus monodon*. The immune parameters including total haemocyte count, differential haemocyte count, phenoloxidase activity, respiratory bursts, superoxide dismutase activity, and phagocytic activity and clearance efficiency in response to the pathogen, *Photobacterium damselae*, were determined when shrimp were individually injected with saline or OA at 100 or 1000 pmol shrimp^{-1}. In addition, the intracellular second messengers in haemocyte such as Ca^{2+} and adenosine 3′,5′-cyclic monophosphate (cAMP) were examined in shrimp receiving saline or OA at 1 or 10 nmol shrimp^{-1}. Results showed that all of the immune parameters significantly increased at 2–4 h in OA-injected shrimp except hyaline cells in 100 pmol shrimp^{-1}-injected shrimp at 4 h, but phenoloxidase activity per granulocyte significantly decreased at 2–4 h. However, these had returned to saline control levels after receiving OA for 8 h except differential haemocyte count and phenoloxidase activity per granulocyte for 16 h. An injection of OA also significantly increased the survival rate of shrimp challenged with *Pho. damselae*. Shrimp receiving OA at 1 and 10 nmol shrimp^{-1} significantly increased the intracellular Ca^{2+} concentration ([Ca^{2+}]i) at 30–60 min and 30 min, and cAMP concentration [cAMP]i) at 5–15 min and 15 min, respectively. However, [Ca^{2+}]i at 50–60 min, and [cAMP]i at 30–60 min returned to saline control when the shrimp received OA at 10 nmol shrimp^{-1}, and at 1 and 10 nmol shrimp^{-1}, respectively. These results suggest that OA administration by injection at ≤1000 pmol shrimp^{-1} mediates transient upregulation of immunity together with the increased resistance of *P. monodon* to *Pho. damselae*, which are modulated through intracellular Ca^{2+} and cAMP second messenger pathways.

1. Introduction

Tiger shrimp, *Penaeus monodon*, native to the coasts of the Arabian peninsula and the Pacific and Indian Ocean coasts of Australia, Indonesia, south and southeast Asia, and South Africa, is one of the most common penaeid shrimp species currently being cultured in the world. Culture of this species intensified from 1988 onward in Taiwan and expanded rapidly to other countries in Asia. It is known that the intensive culture usually go along with the rapid degradation of ponds environments resulting in increased incidences of diseases. Cultured shrimp consistently encounter various environmental stressors in intensive culture ponds. Commercial penaeid shrimp farming has been severely adversely impacted by epidemics associated with viruses and *Vibrio* infections, which have caused serious economic losses worldwide.

Catecholamines (CAs), a class of biogenic amines, act as neurotransmitters, neuromodulators and neurohormones. In crustaceans, CAs have been identified and quantitatively measured in nervous systems, haemolymph and gonadal tissues [1–4], and are required for a variety of physiological and immunological functions, and behaviors. The release of CA is the primary response to physiological stress, and the subsequent induction of hyperglycemia and suppression of immunity are the secondary responses [5–7]. The relationship between neuroendocrine and immunity in shrimp is of primary concern.

Octopamine (OA) and tyramine (TA) as adrenergic transmitters are counterparts in invertebrate and vertebrate, and they are decarboxylation products of the tyrosine while TA is as the biological precursor of OA [8]. Effects of OA on immunomodulation studies in invertebrates were reported by many scientists and reviewed by Adamo [9]. OA was demonstrated to accelerate clearing of circulating bacteria in

* Corresponding author.
E-mail address: winton@mail.npust.edu.tw (W. Cheng).

https://doi.org/10.1016/j.fsi.2019.06.007
Received 4 March 2019; Received in revised form 4 June 2019; Accepted 5 June 2019
Available online 06 June 2019

haemolymph, enhance phagocytosis, modulate activities of haemocytes, and increase resistance against pathogen infection in insects [10,11]. In our previous studies, OA administration at ≤1000 pmol shrimp^{-1} for *Litopenaeus vannamei* and at ≤ 250.0 pmol prawn^{-1} for *Macrobrachium rosenbergii* caused the transient up-regulation in immunity, and promoted their resistance against pathogens [12,13]. OA exerts its effects by binding to G-protein coupled receptors (GPCR) and share the structural motif of seven transmembrane domains. The activation of OA receptors is coupled with different second messenger pathways depending on species, tissue source, and receptor type. The second messengers include adenosine 3′,5′-cyclic monophosphate (cAMP), calcium, diacylglycerol (DAG), and inositol 1,4,5-trisphosphate (IP_3). The cAMP activates protein kinase, calcium and DAG activate protein kinase C, and IP_3 mobilizes calcium from intracellular stores. OA-mediated generation of these second messengers is associated with changes in cellular response affecting animal physiology and behaviors [14].

In crustaceans, the innate immunity system are mainly defense mechanisms, that is activated when pathogen-related molecular patterns are recognized by soluble or by cell-surface pattern recognition proteins. Three types of circulating haemocytes are generally recognized as hyaline cells (HCs), semigranular cells (SGCs), and large granular cells (GCs) in crustaceans [15], which play critical roles in immunological defense functions [16,17]. The prophenoloxidase (proPO) system, which is contained in granulocytes, can be activated by several microbial polysaccharides through the non-self recognition system [18], leading to haemocyte degranulation [19], and it is involved in encapsulation, melanization, and recognition [20]. 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) [21]. Superoxide anions are the first product released from RBs and are scavenged by superoxide dismutases (SODs) [22].

The aims of the present study were to examine the effects of OA on (1) the susceptibility of *P. monodon* to *Photobaterium damselae*, and (2) the immune responses of *P. monodon*. Immune parameters including total haemocyte count (THC), differential haemocyte count (DHC), PO activity, RBs, SOD activity, phagocytic activity, and the clearance efficiency of shrimp to *Pho. dameselae* were used as indicators. Furthermore, intracellular Ca^{2+} responses and cAMP levels in haemocytes were detected to evaluate the signaling pathway of OA.

2. Materials and methods

2.1. Penaeus monodon

Tiger shrimp, *P. monodon*, (11–14 g) 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 27 ± 1 °C and a salinity of 20‰ for 12 days before experimentation. Only healthy shrimp in the intermolt stage (stage C) were used for this study. The molt stage was determined by examining the uropoda, in which partial retraction of the epidermis could be distinguished [23].

Five studies were conducted. To determine the susceptibility of shrimp to *Pho. damselae*, test and control groups were comprised of 10 shrimp each in triplicate. For the experiment of immune parameter assays, 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 analyse Ca^{2+} responses or cAMP levels of haemocytes *in vivo*. For the 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 27 ± 1 °C and pH at 7.6–8.4.

2.2. Photobacterium damselae

For the study, a known pathogenic strain, *Pho. damselae*, was isolated from diseased *P. monodon*, displaying symptoms of anorexia, poor growth, rough shell, milky musculature, and pale and atrophic hepatopancreas [24]. 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 studies of phagocytic activity and clearance efficiency in shrimp, respectively.

2.3. Effect of octopamine on the susceptibility of tiger shrimp to Pho. damselae

OA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in sterile saline (0.85% NaCl) to concentrations of 5×10^{-5} and 5×10^{-6} mol L^{-1}, respectively, were conducted for the studies following the previous studies [12,13]. *P. monodon* (11.2 ± 0.5 g) that was injected with 5×10^{-5} and 5×10^{-6} mol L^{-1} OA solution (around 20 μL) into the ventral sinus of the cephalothorax individually to reach respective doses of 1000 and 100 pmol shrimp^{-1} in the initial stage. After 2 h of 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 receiving 20 μL of sterile saline and then were injected with *Pho. damselae* at 2×10^5 cfu shrimp^{-1} served as the saline-challenged controls, and those receiving OA at 1000 pmol shrimp^{-1} and then were injected with 20 μL of sterile saline served as the unchallenged control (Table 1). Experimental and

Table 1
Effect of octopamine on the susceptibility of *Penaeus monodon* challenged with *Photobacterium damselae*.

Octopamine (pmol shrimp^{-1})	Bacterial dose (cfu shrimp^{-1})	No. of shrimp	Survival rate after challenge (hrs)					
			6	24	48	72	120	168
1000	saline	30	100	100	100	100	100	100
saline	2×10^5	30	100.0 ± 0.0^a	83.3 ± 5.8^b	63.3 ± 5.8^b	43.3 ± 5.8^b	43.3 ± 5.8^b	43.3 ± 5.8^b
100	2×10^5	30	100.0 ± 0.0^a	90.0 ± 0.0^{ab}	80.0 ± 10.0^a	66.7 ± 5.8^a	66.7 ± 5.8^a	66.7 ± 5.8^a
1000	2×10^5	30	100.0 ± 0.0^a	93.3 ± 5.8^a	80.0 ± 0.0^a	73.3 ± 5.8^a	73.3 ± 5.8^a	73.3 ± 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).

control shrimp (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 P. monodon

Tiger shrimp (12.5 ± 0.6 g) was individually injected with an OA solution as those described above. Some shrimp received 20 μL of saline, which served as the saline control. There were three treatments (saline (0), 100, and 1000 pmol shrimp^{-1}) with five sampling times (2, 4, 8, 16 and 24 h) for immune parameter determination. Six shrimp from each treatment and time were collected for the studies. In addition, another six shrimp with no treatment were used as the initial group. Therefore, 96 shrimp (tanks) ((3 treatments × 5 sampling time × 6 shrimp) + 6 shrimp at 0 h) were used for the test, and six independent experiments (tanks) in each treatment at each sampling time were conducted.

At the beginning (0 h) and after injecting for 2, 4, 8, 16 and 24 h, 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 following the instruction, and haemocytes in both top and bottom fields of the haemocytometer were counted (Leica DMIL, Leica Microsystems, Wetzlar, Germany). For DHC determination, haemocyte categories were counted in 200 haemocyte investigated under random scale of the stained haemocyte smear. One milliliter of the haemolymph mixture was centrifuged at 500 × *g* and 4 °C for 20 min, and 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 remaining portion of the haemolymph mixture was used for SOD activity assay.

PO activity of 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 [25] and Hernández-López et al. [26]. The L-DOPA and trypsin were served as a substrate and an elicitor, respectively. The details of measurements was described previously [27]. 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 in haemocytes were quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) formation as described previously [22,28]. 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 [29]. 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 [30]. 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 [31] using a Bio-Rad protein assay kit (no. 500-0006, Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as the standard.

2.5. Phagocytic activity and clearance efficiency of tiger shrimp to Pho. damselae

Penaeus monodon receiving saline or OA were the same as those described above. Tests were carried out on six shrimp (replicates) at each treatment and sampling time. For phagocytic activity and the clearance efficiency analysis, 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 after receiving saline or OA for 2 h. After injection of the bacterial suspension, the shrimp were kept for 1 h in a separate tank containing 40 L of water at 27.0 ± 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 sub-samples: 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. [32], and the procedure was formerly described in detail [12]. 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 [33]. 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 12 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 for 2, 4, 8, 16 and 24 h were expressed as the test groups. The clearance efficiency toward *Pho. damselae*, 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 intracellular Ca^{2+} signaling

For the estimation of intracellular Ca^{2+} signaling in haemocytes, shrimp received saline or OA at 1 or 10 nmol shrimp^{-1} for 10 min, and then the haemolymph was individually collected and diluted as those described above. The Ca^{2+} signaling in haemocytes were measured using a Fluo-4 Direct™ calcium assay Kits (F10471, Invitrogen, CA, USA). Briefly, the diluted haemolymph was deposited in 96-well microplates (previously coated with poly-L-lysine) resulting in 2×10^5 cells well^{-1}, and then was centrifuged at 400 × *g* for 3 min. Plasma was removed and the haemocytes were washed three times with 200 μL Hanks' balanced salt solution (HBSS), then 100 μL HBSS was added, followed by 100 μL 5 mM Fluo-4 Direct™ calcium reagent solution added. The fluorescence was measured using a spectraMax Gemini-XPS microplate reader (Molecular Devices, Sunnyvale, CA, USA) setting appropriate wavelengths for excitation at 494 nm and emission at 516 nm. The related fluorescence units were recorded in real time at 30-s interval for 60 min. Tests were carried out on six shrimp (replicates) at each treatment. Therefore, there were 18 shrimp used for this studies. The determined data were selected at 10, 20, 30, 40, 50, and 60 min for statistical analysis.

2.7. Haemocyte cAMP assays

Shrimp (11.8 ± 0.7 g) received saline or OA at 1or 10 nmole shrimp^{-1} and then haemolymph were collected the same as those described above except sampling times were at 5, 15, 30 and 60 min. The haemolymph mixture was centrifuged at 400 × *g* and 4 °C for 15 min, and the haemocyte pellet was washed twice with HBSS. The resultant haemocyte pellet was then suspended in a HBSS at concentration of 2×10^6 cells mL^{-1}. A 500 μL haemocyte suspension was used for cAMP assay. The cAMP level was determined using a cAMP ELISA Kit (Item No. 581001, Cayman Chem. Comp., MI, USA) by following the

manufacturer's instructions. The OD at 420 nm was measured by an ELISA plate reader, and the cAMP concentration was calculated from a known cAMP concentration standard curve. Tests were carried out on six shrimp (replicates) at each treatment and sampling tine. In addition, another six shrimp with no treatment were used as the initial group. Therefore, there were 78 shrimp were used for this study.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyse 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 arcsine-transformation before analysis. A statistically significant difference required that $p < 0.05$.

3. Results

3.1. Effects of OA on the susceptibility of P. monodon to Pho. damselae

The survival rates of shrimp receiving different concentration of OA in *Pho. damselae* challenge tests are shown in Table 1. All of the unchallenged shrimp receiving OA at 1000 pmol shrimp^{-1} then injected with saline survived. Among the test groups, survival rates of shrimp receiving OA at 100 and 1000 pmol shrimp^{-1} were significantly higher than those of saline-challenged shrimp at 48–168 h. After challenging for 168 h, survival rates of shrimp receiving OA at 100 and 1000 pmol shrimp^{-1} were 23.4% and 30.0% higher than those of the saline-challenged control, respectively. However, no significant difference was observed in survival rate between shrimp receiving OA at 100 and 1000 pmol shrimp^{-1} (Table 1).

3.2. Effect of OA on the immunological parameters of P. monodon

THC, HCs, SGCs, and GCs of shrimp receiving OA at 100 and 1000 pmol shrimp^{-1} were significantly higher at 2–4 h, 2 h, 2–4 h and 2–4 h, respectively, than those of shrimp in the saline control. When shrimp received OA at 100 and 1000 pmol shrimp^{-1} for 2 h, THC, HCs, SGCs, and GCs were significantly higher by 77.7% and 104.4%, 43.9% and 74.5%, 103.4% and 121.7%,187.6% and 242.0%, respectively, than those receiving saline. After 8 h of the injection, THC, HCs and GCs of shrimp receiving OA at 1000 pmol shrimp^{-1} were significantly lower than those of shrimp receiving saline and OA at 100 pmol shrimp^{-1}. However, no significant difference was observed in THC, HCs, SGCs or GCs among the three treatments at 16–24 h (Fig. 1Ã D).

PO activities in haemocytes per 50 μL of haemolymph of shrimp receiving OA at 100 and 1000 pmol shrimp^{-1} were significantly higher than those of shrimp receiving saline after 2–4 h of injection with a dose-dependent tendency, and significantly increased by 19.8% and 33.1% at 2 h, respectively, compared to those of saline-injected shrimp. On the contrary, PO activities per granulocyte (SGCs + GCs) of shrimp receiving 100 and 1000 pmol shrimp^{-1} were significantly lower than those of shrimp receiving saline for 2–4 h, and significantly decreased by 51.4% and 53.9% at 2 h, respectively, compared to shrimp in the saline control. After 8 h of injection, PO activities per granulocyte of shrimp receiving saline and OA at 100 pmol shrimp^{-1} were significantly lower than those of shrimp receiving OA at 1000 pmol shrimp^{-1}. However, no significant difference was observed among the three treatments in PO activity per 50 μL of haemolymph at 8–24 h or in per granulocyte at 16–24 h, and between the two OA-injected treatments in per granulocyte at 2–4 h (Fig. 2A and B).

For shrimp receiving OA at 100 and 1000 pmol shrimp^{-1}, RBs in haemocytes per 10 μL of haemolymph and per haemocyte, and SOD activities in haemocytes were significantly higher than those of shrimp in the saline control at 2–4 h. After 2 h of injection, there was a

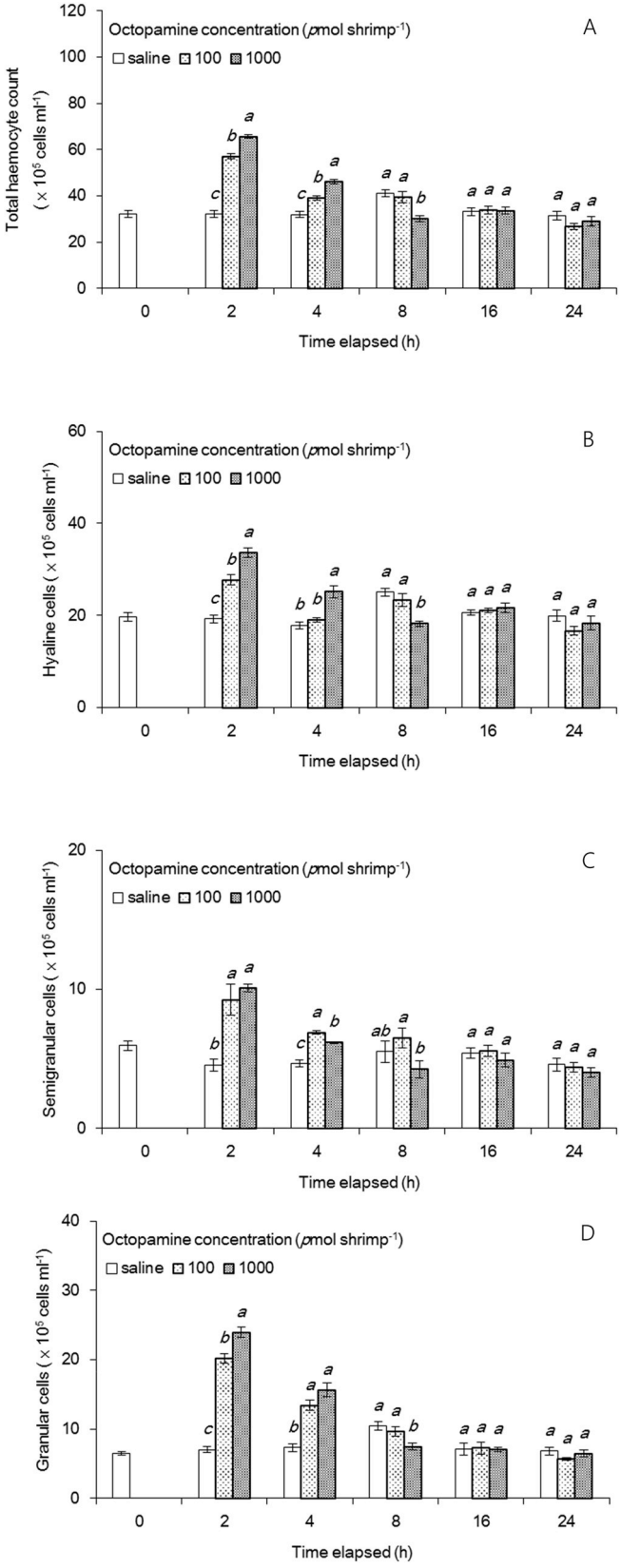

Fig. 1. Total haemocyte count (A), hyaline cells (B), semigranular cells (C) and granular cells (D) of *Penaeus monodon* receiving saline or octopamine at 100 or 1000 *p*mol shrimp^{-1} for 0, 2, 4, 8, 16 and 24 h. Each bar represents the mean value from 6 samples with the standard error. Bars with different letters significantly differ ($p < 0.05$) among treatments at the same sampling time.

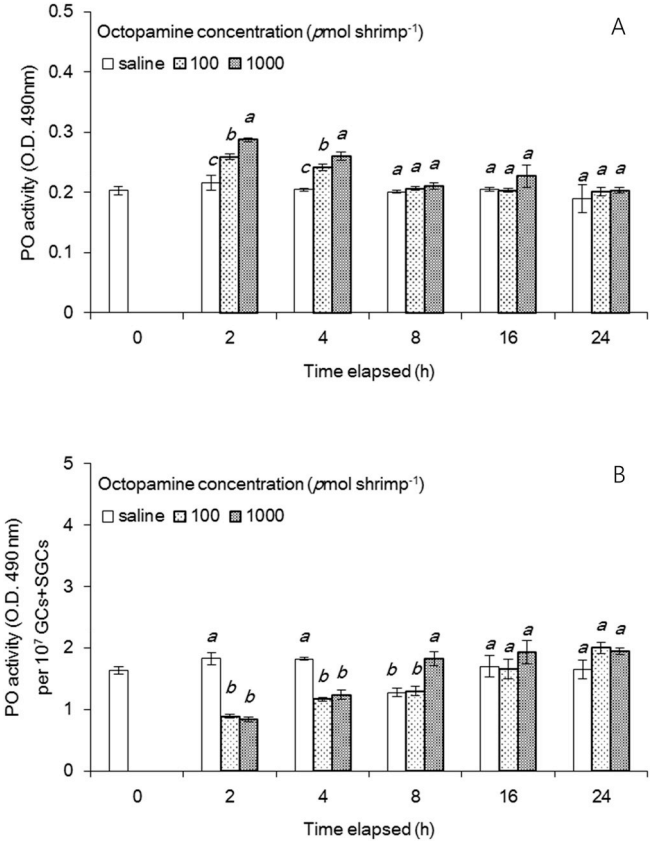

Fig. 2. Phenoloxidase (PO) activity in haemocytes per 50 μL of haemolymph (A) and in per 10^7 granulocytes (granular cells (GCs) + semigranular cells (SGCs)) (B) of *Penaeus monodon* receiving saline or octopamine at 100 or 1000 *p*mol shrimp^{-1} for 0, 2, 4, 8, 16 and 24 h. Statistical descriptions are the same as those in Fig. 1.

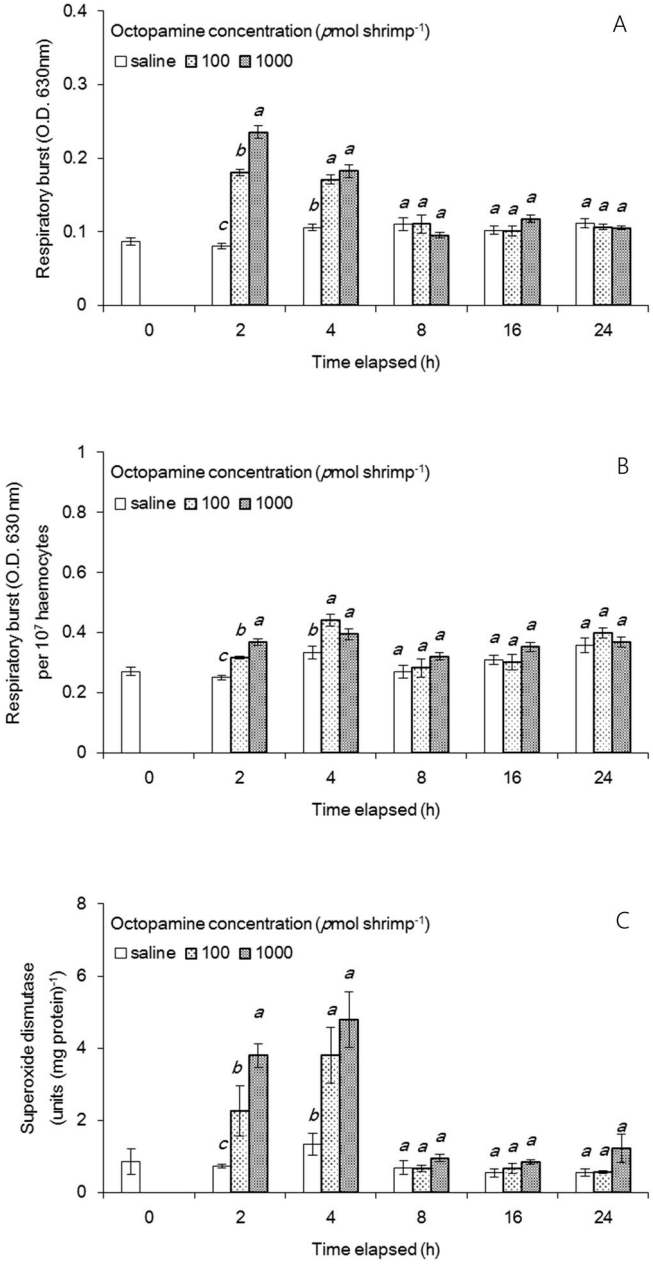

Fig. 3. Respiratory burst in haemocytes per 10 μL of haemolymph (A) and in per 10^7 haemocytes (B), and superoxide dismutase activity (C) of *Penaeus monodon* receiving saline or octopamine at 100 or 1000 *p*mol shrimp^{-1} for 0, 2, 4, 8, 16 and 24 h. Statistical descriptions are the same as those in Fig. 1.

significantly increased in RBs per 10 μL of haemolymph by 125.0% and 193.2%, in RBs per haemocyte by 26.1% and 46.7%, and in SOD activities by 208.6% and 419.3% in shrimp receiving OA at 100 and 1000 pmol shrimp^{-1}, respectively, compared to saline-injected shrimp. However, no significant difference in RBs per 10 μL of haemolymph and per haemocyte, and SOD activities were observed among the three treatments after injecting for 8–24 h (Fig. 3A, B, C).

Phagocytic activity and clearance efficiency of shrimp receiving OA at 100 and 1000 pmol shrimp^{-1} were significantly higher than those of shrimp in the saline control at 2–4, and significantly increased by 34.3% and 34.9%, and 66.6% and 66.7% at 2 h, respectively, compared to those of shrimp in the saline control. However, no significant difference in phagocytic activity or clearance efficiency was observed among the three treatments after injecting for 8–24 (Fig. 4A and B).

3.3. Effects of OA on intracellular Ca^{2+} and cAMP in haemocytes

The haemocytes of shrimp receiving saline or OA at 1 or 10 nmol shrimp^{-1} for 10 min were collected to determine Ca^{2+} responses. The significantly increased Ca^{2+} responses were observed after assessing for 30 min in OA treatments, compared to the saline control, and however, the retained high level only detected in 1 nmol shrimp^{-1} group after 60 min. No significant difference in Ca^{2+} responses were observed between the saline control and 10 nmol shrimp^{-1} group within 50–60 min (Fig. 5A and B).

cAMP in haemocytes of shrimp receiving OA at 1 nmol shrimp^{-1} was significantly higher than that of shrimp receiving saline and OA at 10 nmol shrimp^{-1} for 5 min. However, no significant difference in cAMP level was observed among the three treatments after 15–60 min of injection or between shrimp receiving saline and OA at 10 nmol shrimp^{-1} for 5 min (Fig. 6).

4. Discussion

The effects of stress-induced hormones/neurohormones on immunity are complex in invertebrates [9]. In insects, acute stress leads to a transient increase of haemolymph OA up to 10-fold over baseline levels [34,35], and OA is considered with a biphasic effects on immune function. The survival rates of cockroaches, *Periplaneta americana*, coinjected with *Staphylococcus aureus* and OA at 0.1 mM or clonidine, an OA agonist, were significantly higher than those of cockroache that treated with saline control [36]. 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 [12]. *M. rosenbergii* and *L. vannamei* receiving OA at 25.0 or

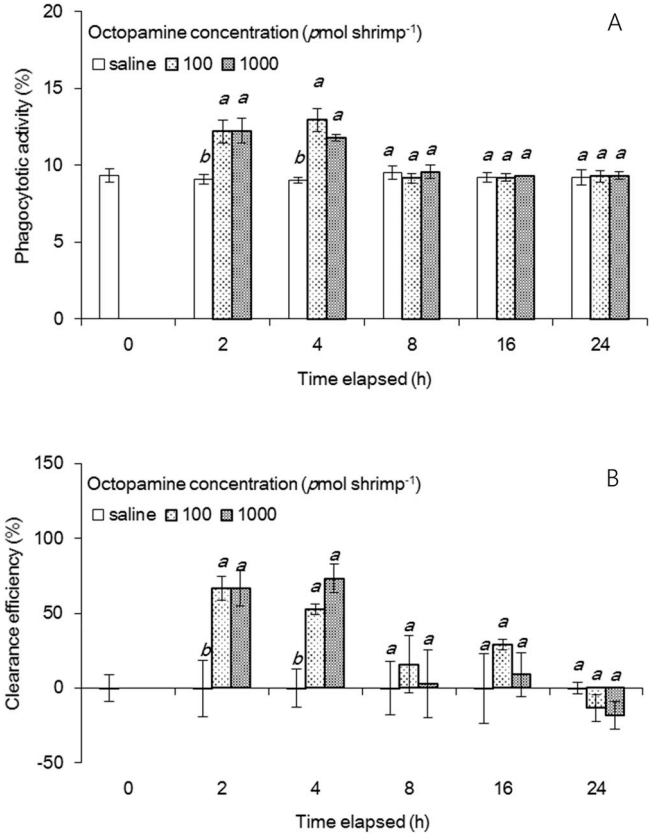

Fig. 4. Phagocytic activity (A) and clearance efficiency (B) of *Penaeus monodon* receiving saline or octopamine at 100 or 1000 *p*mol shrimp^{-1} for 0, 2, 4, 8, 16 and 24 h. Statistical descriptions are the same as those in Fig. 1.

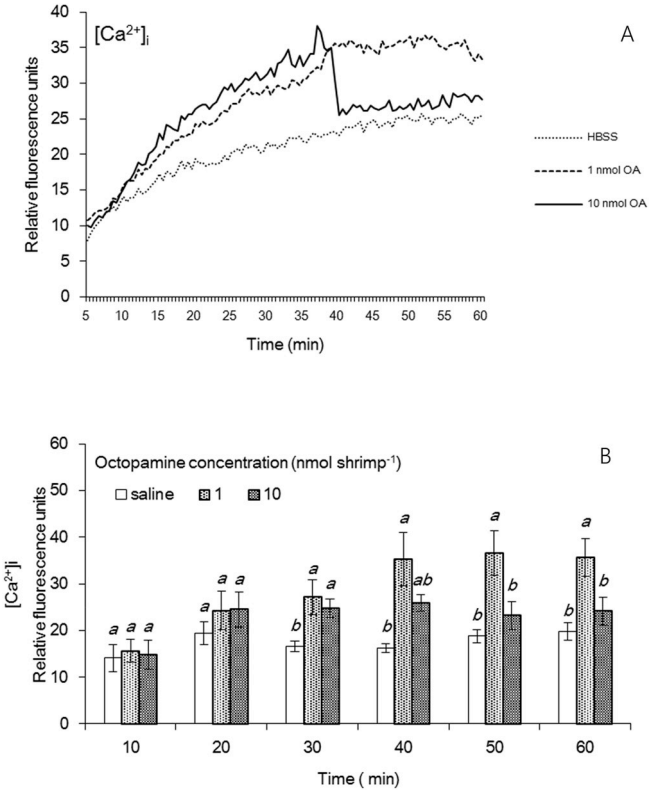

Fig. 5. Intracellular Ca^{2+} concentration ([Ca^{2+}]i) of *Penaeus monodon* received saline or octopamine at 1 or 10 nmol shrimp^{-1} for 10 min. The related fluorescence units recorded in real time at 30 s interval for 60 min (A), and those determined data selected at 10, 20, 30, 40, 50, and 60 min were used for statistical analysis (B). Tests were carried out on six shrimp (replicates) at each treatment. Statistical descriptions are the same as those in Fig. 1.

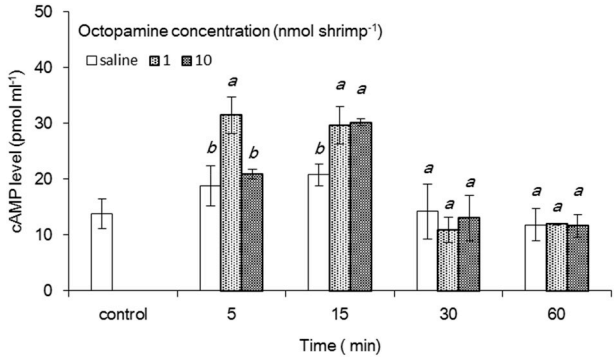

Fig. 6. cAMP level of *Penaeus monodon* receiving saline or octopamine at 1or 10 nmole shrimp^{-1} for 0, 5, 15, 30 and 60 min. Statistical descriptions are the same as those in Fig. 1.

250.0 pmol prawn^{-1}, and at 100 or 1000 pmol shrimp^{-1}, respectively, showed the increased resistance to pathogens infection [12,13]. In the present study, *P. monodon* injected with OA at 100 or 1000 pmol shrimp^{-1} significantly increased the resistance to *Pho. damselae*. This fact suggests that OA may promote the resistance abilities of *M. rosenbergii*, *L. vannamei* and *P. monodon*, when the shrimp had received exogenous OA at a lower dose than the physiological baseline by injection. Further study is needed to verify biphasic effects of OA on immune resistance function of shrimp.

In crustaceans, haemocytes played crucial roles the physiological and immunological responses, and can be regulated by stress-induced biogenic amines. Kim and Kim [37] showed that OA and 5-hydroxytryptamine (5-HT) through injection induce a significant increase in the THC of haemolymph, and mediate a rapid increase of circulating haemocyte population in response to bacterial challenge via Rac1 signal in *Spodoptera exigua*. OA also affects haemocyte behavior to modulate haemocyte mobilization in response to stress in *Galleria mellonella* [38]. Söderhäll [39] indicated that the stimulation with microbial polysaccharides or repeated bleeding induces a need for new haemocytes, thereby stimulating cell division in the haematopoietic tissue (HPT) and the release of immature and mature haemocytes in crustacean. In crayfish, *Pacifastacus leniusculus*, serotonin (5-HT) had no direct effect on HPT cell proliferation, but participated in haematopoiesis through stimulating haemocytes to release astakine 1 (Ast 1) that reduced the activity of transglutaminase on the HPT leading to the stimulation of HPT cell migrating out of the tissue, which causes an increase in the number of circulating haemocyte [40]. In the present study, OA-injected *P. monodon* exhibited significant increases in THC, HCs, SGCs, and GCs after 2 and/or 4 h of injection, compared to the saline-injected and initial control. The similar tendency was also observed in OA-injected *M. rosenbergii* and *L. vannamei* except HCs of *L. vannamei* maintained unchanged [12,13] and in TA-injected *L. vannamei* [41]. These results suggest that OA and TA enhanced mobilization of fixed haemocytes and regenerated haemocytes releasing into the circulatory system, which resulted in the elevation of THC and DHC in OA- and TA-injected shrimp. In addition, saline- and OA 100 pmol shrimp^{-1}-injected shrimp exhibited higher levels in THC, HCs and GCs after 8 h of injection than the initial control, and meanwhile, those in OA 1000 pmol shrimp^{-1}-injected shrimp recovered to baseline levels. The results suggest that the elevation of circulating haemocytes population responding to injecting operation was later than OA treatment [13], and OA at a reasonable concentration might promote the redistribution of haemocytes.

The proPO system is one of the critical innate immune defense

mechanism in crustaceans, which exists in granular haemocytes and can be regulated by biogenic monoamine [27]; it is involved in non-self recognition, haemocyte communication, and the production of melanin. In the present study, shrimp receiving OA at 100 and 1000 pmol shrimp^{-1} significantly elevated THC and granulocytes within 2–4 h of injection, and meanwhile, the significantly increased PO activity in haemocytes per 50 μL of haemolymph and the significantly decreased PO activity in per granulocyte, compared to those in saline control, were observed. Although PO activity in haemolymph showed no significant difference among treatments after 8 h of injection, yet the obviously decreased THC and granulocytes accompanied with the significantly induced PO activity per granulocyte revealed in 1000 pmol shrimp^{-1} OA treatment. These were similar to the findings in *L. vannamei* and *M. rosenbergii* injected with saline and OA that the expression patterns of granulocytes disagreed with the PO activity per granulocyte and agreed with the PO activity in haemocytes per 50 μL haemolymph in an attempt to maintain homeostasis [12,13]. It was therefore concluded that for maintaining the homeostasis, the varied PO activity in haemolymph followed with the change of granulocytes were modulated by the inverse reaction of PO activity in per granulocyte.

The species of reactive oxygen intermediates may be produced by several different immune reactions [42], which are needed to kill invading pathogens during phagocytosis is known a key cellular defense mechanism. The clearance efficiency is an important humoral defense mechanism. Clearance from the circulation is induced by humoral factors such as agglutinins, lectins, cytotoxic factors [43], and antimicrobial factors [44]; this causes bacterial aggregation in the circulating haemolymph, and enhances the clearance rate [45]. In the present study, OA-injected *P. monodon* exhibited the increases of RBs, SOD activity, and clearance efficiency and phagocytic activity against *Pho. damselae*, which agreed with the increased resistance of *P. monodon* to *Pho. damselae*. Significant increase in RBs as well as phagocytic activity, clearance efficiency and resistance against pathogens were also observed in *L. vannamei* and *M. rosenbergii* injected with OA [12,13]. These facts suggest that OA promotes humoral and cellular immune responses leading to rapidly elevate resistance against pathogens in shrimp, and the elevation in RBs of shrimp receiving OA is correlated with the increase phagocytic activity.

Actions of biogenic amines were shown to be mediated via the activation of G-protein-coupled receptors (GPCRs) pathways, thereby inducing the generation of intracellular second mesesengers like cAMP and/or Ca^{2+} [8]. In insects, many TA and/or OA receptors have been identified such as α-adrenergic-like OA receptors (OAαRs), β-adrenergic-like OA receptors (OAβRs) and OA/TA or TA1 receptors [46,47]. Activation of OAαRs evokes an increase in intracellular Ca^{2+} concentration ([Ca^{2+}]i), whereas activation of OAβRs induces an increase in cellular cAMP concentration ([cAMP]i) but no increase in [Ca^{2+}]i [47,48]. Activation of TA1 receptors results in both a decrease of intracellular [cAMP]i and generation a [Ca^{2+}]i signal [49,50]. Huang et al. [51] indicate that at low concentration (< 1 μM), OA stimulated haemocyte spreading and phagocytosis in the larval Lepidopteran, *Chilo suppresalis*, and increased intracellular Ca^{2+}, whereas at high concentration (> 10 μM), OA inhibited haemocyte spreading and phagocytosis as well as increased both Ca^{2+} and cAMP.

A putative OA/TA receptor in ganglia of *M. rosenbergii* was cloned by Reyes-Colón et al. [52]. In *M. rosenbergii*, using a pharmacologic experiment, the up-regulated circulating haemocyte and PO activity of haemolymph via α1, α2, β1 and β2-adrenergic-like OA receptors; the phagocytic activity to pathogen via α1, α2 and β2-adrenergic-like OA receptors, and clearance efficiency to pathogen via α1 and α2-adrenergic-like OA receptors were reported by Kuo and Cheng [13]. In the present study, OA was observed to upregulate immunocompetence in *P. monodon* after receiving OA at 100 and 1000 pmol shrimp^{-1} for 2–4 h then returned to the initial level. Furthermore, [Ca^{2+}]i and [cAMP]i in haemocyte significantly increased from 30 to 60 min and from 5 to 15 min, respectively, in 1 nmol shrimp^{-1} OA-injected tiger shrimp, and in addition, in 10 nmol shrimp^{-1} OA-injected shrimp, [Ca^{2+}]i and [cAMP]i in haemocyte significantly increased at 30 min and 15 min then recovered to baseline at 50 min and 30 min, respectively. These results indicate that shrimp receiving OA at 1 nmol shrimp^{-1} might induced immunocompetence through adrenergic-like OA receptors to elevate both [Ca^{2+}]i and [cAMP]i as the intracellular signaling pathways in haemocyte, whereas the shrimp receiving OA at 10 nmol shrimp^{-1} showed an early recovery of [Ca^{2+}]i and a delayed elevation of [cAMP]i, compared to those in 1 nmol shrimp^{-1} OA-injected shrimp. Those support that the different level of exogenous OA might result in the different expressing pattern of intracellular signaling pathways to modulate immune performance through adrenergic-like OA receptors. These findings should be further clarified with the full set of molecular mechanisms responsible for the biphase response in shrimp.

In conclusion, the present study documented that *P. monodon* receiving OA at ≤ 1000 pmol shrimp^{-1} showed the upregulation of humoral and cellular immune responses then led to the increased resistance to *Pho. damselae*. These effects were regulated through both Ca^{2+} and cAMP signaling pathways.

Acknowledgements

This work was financially supported by a grant (107-2313-B-020-010-MY3) from the Ministry of Science and Technology, Taiwan, ROC.

References

[1] R. Eloffson, L. Lazmyr, E. Rosengren, C. Hansso, Identification and quantitative measurements of biogenic amines and dopa in the central nervous system and haemolymph of the crayfish *Pacifastacus lenuiusculus* (Crustacea), Comp. Biochem. Physiol., C 71 (1982) 195–201.
[2] M. Fingerman, R. Nagabhushnam, Control of the release of crustacean hormones by neuroregulators, Comp. Biochem. Physiol., C 102 (1992) 343–352.
[3] C.M. Kuo, C.R. Hsu, C.Y. Lin, Hyperglycaemic effects of dopamine in tiger shrimp, *Penaeus monodon*, Aquaculture 135 (1995) 161–172.
[4] Y. Tinikul, A.J. Mercier, P. Sobhon, Distribution of dopamine and octopamine in the central nervous system and ovary during the ovarian maturation cycle of the giant freshwater prawn. *Macrobrachium rosenbergii*, Tissue Cell 41 (2009) 430–442.
[5] C.M. Kuo, Y.H. Yang, Hyperglycaemic responses to cold shock in the freshwater giant prawn, *Macrobrachium rosenbergii*, J. Comp. Physiol. B 169 (1999) 49–54.
[6] L.U. Sneddon, A.C. Taylor, F.A. Huntingford, D.G. Watson, Agonistic behavior and biogenic amines in shore crabs *Carcinus maenas*, J. Exp. Biol. 203 (2000) 537–545.
[7] C.C. Chang, M.S. Yeh, W. Cheng, Cold shock-induced norepinephrine triggers apoptosis of haemocytes via caspase-3 in the white shrimp, *Litopenaeus vannamei*, Fish Shellfish Immunol. 27 (2009) 695–700.
[8] T. Roeder, Tyrosine and octopamine: ruling behavior and metabolism, Annu. Rev. Entomol. 50 (2005) 447–477.
[9] S.A. Adamo, Norepinephrine and octopamine: linking stress and immune function across phyla, Invertebr. Surviv. J. 5 (2008) 12–19.
[10] D. Baines, T. DeSantis, R.G.H. Downer, Octopamine and 5-hydroxytryptamine enhance the phagocytic and nodule formation activities of cockroach (*Periplaneta americana*) haemocytes, J. Insect Physiol. 38 (1992) 905–914.
[11] G.B. Dunphy, R.G.H. Downer, Octopamine, a modulator of the haemocytic nodulation response of non-immune *Galleria mellonella* larvae, J. Insect Physiol. 40 (1994) 267–272.
[12] H.W. Kuo, D.W. Lin, W. Cheng, Transient enhancement of immune resistance fumctions in *Litopenaeus vannamei* through a low-dose octopamine injection, Fish Shellfish Immunol. 84 (2019) 532–540.
[13] H.W. Kuo, W. Cheng, Octopamine enhances the immune responses of freshwater giant prawn, M*acrobrachium rosenbergii*, via octopamine receptors, Dev. Comp. Immunol. 81 (2018) 19–32.
[14] T. Farooqui, Octopamine-mediated neromodulation of insect senses, Neuochem. Res. 32 (2007) 1511–1529.
[15] A. Tsing, J.M. Arcier, M. Brehèlin, Haemocytes of penaeids and palaemonid shrimps: morphology, cytochemistry and hemograms, J. Invertebr. Pathol. 53 (1989) 64–77.
[16] N.A. Ratcliffe, A.F. Rowley, S.W. Fitzgerald, C.P. Rhodes, Invertebrate immunity: basic concepts and recent advances, Int. Rev. Cytol. 97 (1985) 183–350.
[17] G.G. Martin, J.E. Hose, S. Omori, C. Chong, T. Hoodbboy, N. Mckrell, Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod crustaceans, Comp. Biochem. Physiol. B 100 (1991) 517–522.
[18] K. Söderhäll, L. Cerenius, M.W. Johansson, The prophenoloxidase activating system in invertebrates, in: K.S. Söderhäll, G.R. Iwanaga, G.R. Vasta (Eds.), New Directions in Invertebrate Immunology, SOS Publications, Fair Haven, NJ, 1996, pp. 229–253.
[19] K. Söderhäll, L. Cerenius, M.W. Johansson, The prophenoloxidase activating system and its role invertebrate defense, Ann. N. Y. Acad. Sci. 712 (1994) 155–161.
[20] M.W. Johansson, K. Söderhäll, Cellular immunity in crustaceans and the proPO

system, Parasitol. Today 5 (1989) 171–176.
[21] M. Muñoz, R. Cedeño, J. Rodríguez, W.P.W. van der Knaap, E. Mialhe, E. Bachère, Measurement of reactive oxygen intermediate production in haemocyte of the penaeid shrimp, *Penaeus vannamei*, Aquaculture 191 (2000) 89–107.
[22] K.L. Bell, V.J. Smith, In vitro superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.), Dev. Comp. Immunol. 17 (1993) 211–219.
[23] L. Robertson, W. Bray, J. Leung-Trujillo, A. Lawrence, Practical molt staging of *Penaeus setiferus* and *Penaeus stylirostris*, J. World Aquac. Soc. 18 (1987) 180–185.
[24] Y.L. Song, W. Cheng, C.H. Wang, Isolation and characterization of *Vibrio damselae* infectious for culture shrimp in Taiwan, J. Invertebr. Pathol. 61 (1993) 24–31.
[25] H.S. Mason, Structures and functions of the phenolase complex, Nature 177 (1956) 79–81.
[26] J. Hernández-López, T.S. Gollas-Galván, F. Vargas-Albores, Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes), Comp. Biochem. Physiol., C 113 (1996) 61–66.
[27] C.C. Chang, P.P. Lee, W. Cheng, Norepinephrine regulates prophenoloxidase system-related parameters and gene expressions via α-and β-adrenergic receptors in *Litopenaeus vannamei*, Fish Shellfish Immunol. 33 (2012) 962–968.
[28] Y.L. Song, Y.T. Hsieh, Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species, Dev. Comp. Immunol. 18 (1994) 201–209.
[29] R. Mapanao, C.C. Chang, W. Cheng, The upregulation of immune responses in tyrosine hydroxylase (TH) silenced *Litopenaeus vannamei*, Dev. Comp. Immunol. 67 (2017) 30–42.
[30] C.H. Chiu, Y.K. Guu, C.H. Liu, T.M. Pan, W. Cheng, Immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, induced by *Lactobacillus plantarum*, Fish Shellfish Immunol. 23 (2007) 364–377.
[31] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[32] B.A. Weeks-Perkins, N. Chansue, D. Wong-Verelle, Assay of immune function in shrimp phagocytes: techniques used as indicators or pesticide exposure, in: J.S. Stolen, T.C. Fletcher, S.A. Smith, J.T. Zelikoff, S.L. Kaattari, R.S. Anderson, K. Söderhäll, B.A. Weeks-Perkins (Eds.), Technique in Fish Immunology-4, SOS Publications, Fair Haven, NJ, 1995, pp. 223–231.
[33] A. Adams, Response of penaeid shrimp to exposure to *Vibrio* species, Fish Shellfish Immunol. 1 (1991) 59–70.
[34] S.A. Adamo, C.E. Linn, R.R. Hoy, The role of neurohormonal octopamine during fight or flight behavior in the field cricket *Gryllus-bimaculatus*, J. Exp. Biol. 198 (1995) 1691–1700.
[35] A.P. Davenport, P.D. Evans, Changes in hemolymph octopamine levels associated with food-deprivation in the locust, *Schistocerca gregaria*, Physiol. Entomol. 9 (1984) 269–274.
[36] D. Baines, R.G.H. Downer, Octopamine enhances phagocytosis in cockroach hemocytes: involvement of inositol phosphate, Arch. Insect Biochem. Physiol. 26 (1994) 249–261.
[37] G.S. Kim, Y. Kim, Up-regulation of circulating hemocyte population in response to bacterial challenge is mediated by octopamine and 5-hydroxytryptamine via Rac1 signal in *Spodoptera exigua*, J. Insect Physiol. 56 (2010) 559–566.
[38] W. Diehl-Jones, C.A. Mandato, G. Whent, R.G.H. Downer, Monoaminergic regulation of hemocyte activity, J. Insect Physiol. 42 (1996) 13–19.
[39] I. Söderhäll, Crustacean hematopoiesis, Dev. Comp. Immunol. 58 (2016) 129–141.
[40] C. Noonin, Involvement of serotonin in crayfish hematopoiesis, Dev. Comp. Immunol. 86 (2018) 189–195.
[41] H.W. Kuo, C.C. Chang, W. Cheng, Tyramine's modulation of immune resistance functions in *Litopenaeus vannamei* and its signal pathway, Dev. Comp. Immunol. 95 (2019) 68–76.
[42] K. Junkulo, K. Söderhäll, I. Söderhäll, C. Noonin, Reactive oxygen species affects transglutaminase activity and regulates hematopoiesis in a crustacean, J. Biol. Chem. 291 (2016) 17593–17601.
[43] V.J. Smith, J.R.S. Chisholm, Non-cellular immunity in crustaceans, Fish Shellfish Immunol. 2 (1992) 1–31.
[44] V.J. Smith, Invertebrate immunology: phylogenetic, ecotoxicological and biomedical implication, Comp. Haematol. Int. 1 (1991) 60–76.
[45] C.B.T. van de Braak, M.H.A. Botterblom, W. Liu, N. Taverne, W.P.W. van de Knaap, J.H.W.M. Rombout, The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (*Penaeus monodon*), Fish Shellfish Immunol. 12 (2002) 253–272.
[46] P.D. Evans, B. Maqueira, Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors, Invertebr. Neurosci. 5 (2005) 111–118.
[47] B. Maqueira, H. Chatwin, P.D. Evans, Identification and characterization of a novel family of *Drosophila* β-adrenergic-like octopamine G-protein coupled receptors, J. Neuruchem. 94 (2005) 547–560.
[48] S. Balfanz, T. Strünker, S. Frings, A. Baumann, A family of octopamine receptors that specifically induce cyclic AMP production or Ca^{2+} release in *Drosophila melanogaster*, J. Neurochem. 93 (2005) 440–451.
[49] V. Reale, F. Hannan, J.M. Midgley, P.D. Evans, The expression of a cloned *Drosophila* octopamine/tyramine receptor in *Xenopus* oocytes, Brain Res. 769 (1997) 309–320.
[50] S. Robb, T.R. Cheek, F.L. Hannan, L.M. Hall, J.M. Midgley, P.D. Evans, Agonist-specific coupling of a cloned *Drosophila* octopamine/tyramine receptor to multiple second messenger systems, EMBO J. 13 (1994) 1325–1330.
[51] J. Huang, S.F. Wu, X.H. Li, A. Adamo, G.Y. Ye, The characterization of a concentration-sensitive α-adrenergic-like octopamine receptor found on insect immune cells and its possible role in mediating stress hormone effects on immune function, Brain Behav. Immun. 26 (2012) 942–950.
[52] D. Reyes-Colón, N. Vázquez-Acevedo, N.M. Rivera, S.H. Jezzini, J. Rosenthal, E.A. Ruiz-Rodríguez, D.J. Baro, A.B. Kohn, L.L. Moroz, M.A. Sosa, Cloning and distribution of a putative octopamine/tyramine receptor in the central nervous system of the freshwater prawn *Macrobrachium rosenbergii*, Brain Res. 1348 (2010) 42–54.