VERSITA

Cent. Eur. J. Biol. • 9(11) • 2014 • 1048-1057
DOI: 10.2478/s11535-014-0317-y

Central European Journal of **Biology**

Immunotoxic Effect of Benzo[α]Pyrene and Chrysene in Juvenile White Shrimp *Litopenaeus vannamei*

Research Article

Xianyun Ren, Luqing Pan*, Lin Wang

Department of Aquaculture,
The Key Laboratory of Mariculture of Ministry of Education,
Ocean University of China, Qingdao 266003, P. R. China

Received 30 September 2013; Accepted 11 April 2014

Abstract: The effects of benzo(a)pyrene (Bap) (0.03, 0.3 and 3 μg L^{-1}) and chrysene (CHR) (0.3, 2.1 and 14.7 μg L^{-1}) on the function of the immune system of juvenile white shrimp *Litopenaeus vannamei* were determined under laboratory conditions. This included the total hemocyte count (THC) in the hemolymph,phagocytic activityand pro-phenoloxidase (pro-PO) activity of the hemocyte, phenoloxidase (PO) activity, α_2-macroglobulin (α_2-M) activity, bacteriolytic activity and antibacterial activity in the hemolymph. The results showed that BaP and CHR could inhibit the immune function of *L. vannamei* significantly under high concentration BaP and CHR exposure. The results of this study indicated that the immunotoxicity of PAHs in a descending order was BaP > CHR. Moreover, the results indicated the THC in hemolymph, pro-PO activity and phagocytic activity of hemocyte, and bacteriolytic activity in hemolymphcould be used as potentially suitable biomarkersfor early warning indication of PAHs toxicity, this could provide useful information for toxic risk assessment of environmental pollutants.

Keywords: *Litopenaeus vannamei* • *Benzo[α]pyrene* • *Chrysene* • *Immune system*

1. Introduction

In the past two decades, industrial development has resulted in continuous discharge of various organic and inorganic materials into aquatic environments. These chemical substances accumulate within the bodies of aquatic organisms, causing different toxicological responses [1]. There are 16 polycyclic aromatic hydrocarbons (PAHs) on the the US EPA priority pollutant list [2], including benzo(a)Pyrene (BaP) and chrysene (CHR). These chemicals have highly mutagenic, carcinogenic and toxic features and are distributed widely in the global environment. In 2011, according to the most recent exposure assessment results, the European Food Safety Authority revised standards on food in PAHs, proposing the use of total levels of benzo[a] pyrene, benzo[a]fluoranthene, benzo[b]fluoranthene and chrysene as PAHs pollution index evaluation of food. According to statistical data, the highest concentration of PAHs in coastal water and sediment can reach up to 551.2 ng L^{-1} and 4567 ng g^{-1}, respectively [3].

Once entering the body system of an organism, PAHs can exert their toxic effect by either the original compound or the resulting production of reactive oxygen species (ROS) during metabolism [1,4], causing lysosomal membrane disruption [5]. It has been demonstrated that the antioxidant system of aerobic organisms is important for eliminating ROS, and the induction of antioxidant enzymes can be considered as a self-defense process of the body to adapt unsafe environment and to reduce toxicity. However, an imbalance between formation and neutralization of these reactive species can induce immunosuppression [6].

Due to the complexity of invertebrates' immune systems, their immune systems are more sensitive to environmental contaminants [7]. There is considerable evidence that the immune systems of these organisms are vulnerable to PAHs. An effective immune response is important in themaintenance of the health, disease and survival of an organism [8]. PAHs can alter cellular function (viability and immune function) in various invertebrate species, such as bivalves [9-11] and

* *E-mail: panlq@ouc.edu.cn*

crustaceans [12,13]. Therefore, immune function is important in assessing sublethal effect of contaminant exposure [14].

The white shrimp *L. vannamei* has become the most important cultivated shrimp species in China owing to its fast growth rate and strong adaptability [15]; however, the knowledge about biological effects of PAHs exposure in *L. vannamei* is severely lacking. The previous study suggested exposure to contaminants could comprise immune function and progressively led to infectious diseases. In the present study, we examined the effects of the 4-ringed PAH (CHR) and the 5-ringed PAH (BaP) as representatives of PAHs on the parameters of immune system in juvenile *L. vannamei*, including total hemocyte count (THC) in the hemolymph, phagocytic activity of the hemocyte, and pro-phenoloxidase (pro-PO) activity, phenoloxidase (PO) activity, α_2-macroglobulin (α_2-M) activity, bacteriolytic activity and antibacterial activity in the hemolymph. These results will offer scientific information for the selection of proper immune parameters to assess the environmental contamination risk on *L. vannamei* and to better understand the regulatory mechanism of the immune system.

2. Materials and methods

2.1 Animals

Healthy *L. vannamei*, averaging 9.5 ± 0.5 cm in body length, were obtained from a commercial farm in Nanshan, Qingdao, China. The shrimps were acclimated in tanks containing aerated seawater (salinity 31‰, pH 8.2) at 25 ± 0.5°C for one week prior to experimentation. During the acclimation period, one third to half of the water in each tank was replaced twice daily, and the shrimps were fed with a formulated shrimp diet three times per day. Only apparently healthy animals at inter-molt stage were used for the study. The molt stage was decided by the examination of uropoda in which partial retraction of the epidermis could be distinguished.

2.2 Experimental setup

After acclimatization, the shrimps were exposed to different BaP concentrations (0.03, 0.3 and 3 μg L^{-1}) and chrysene concentrations (0.3, 2.1 and 14.7 μg L^{-1}). There were 60 shrimps in each tank, and three replicate tanks were randomly selected for each level including the control group (supposed 0 level). Seawater prepared with the same concentrations of BaP and CHR was added into corresponding tanks to maintain the concentrations of BaP and CHR during the exposure experiment. BaP and CHR were first dissolved in acetone, and the final acetone concentration was 0.001% in all tanks (the acetone test has been done in a preliminary experiment with the result that there was no influence on shrimp). The exposure concentrations of BaP and CHR were based on the concentrations of BaP and CHR in the coastal seawater, surface sediments in China, as well as their solubility (22°C-25°C). Shrimps were sampled at day 1, 3, 6, 10, 15 and 21, and eight shrimps were sampled for each sampling time and each concentration.

2.3 Tissue sample preparation

Hemolymph (0.3 mL) was withdrawn from the cardiocoelom at the posterior margin of carapace of the shrimp into a 1mL sterile syringe (25 gauge needle) containing 0.3 mL improved precooled anticoagulant for *L. vannamei* (0.34 M NaCl, 0.01 M KCl, 0.01 M EDTA-Na_2 and 0.01 M HEPES, pH 7.45 and 780 mOsm kg^{-1}). 1.0 mL of hemolymph was pipetted in Eppendorf tube and centrifuged at 800×g for 10 min under 4°C, and then gathered the blue supernatant into a new tube and stored at −80°C as a hemolymph sample [16]. After the hemolymph sample was prepared, the cells precipitated at the bottom of the tube were suspended and rinsed gently by 150 μL shrimp salt solution (SSS, 450 mmol L^{-1} NaCl, 10 mmol L^{-1} KCl, 10 mmol L^{-1} HEPES, pH 7.3) and then centrifuged at 800×g for 10min under 4°C. The resulting supernatant was discarded, and repeated the wash process again. Afterwards, 150 μL SSS was added into the tube and then the cells were broken into pieces for 1min by Ultrasonic Cell Disruption System with output power at 20W and duty cycle at 30% in the ice-bath. Finally, the sample was centrifuged at 15, 000×g for 20min under 4°C and the supernatant was pipetted into a new tube and stored at −80°C as the HLS sample [17].

2.4 Immune parameters assay

2.4.1 Total Hemocyte Counts (THC)

A drop of each diluted hemolymph sample was placed in a hemocytometer and observed under a light microscope to determine its total hemocyte counts.

2.4.2 Phagocytic activity

Phagocytic activity of the haemocyte was measured using *V. anguillarum* using previously published methods (Yue *et al.* [18]). 100 μL of haemocyte suspension and equal volume of bacterial suspension (1×10^7 cells mL^{-1}) were pipetted into plasticmicroplate. Then the mixture was incubated in a moist chamber for 30min at 37°C. After incubation, the microplate was placed at room temperature (25°C) for 15min. One drop of the mixturewas pipetted onto a glass slide and then dried at room temperature, fixed in methanol, stained

with Giemsa stain, decolorized in MilliQ water, air-dried and observed using Olympus light microscope (10×ocular,100×oil immersion objective). The number of phagocytic haemocytes among random 200 haemocytes was counted. Phagocytic activity defined as phagocytic rate (PR) was calculated as:

$$PR\% = \frac{\text{number of phagocytic haemocytes}}{200\ \text{haemocytes}} \times 100\%$$

2.4.3 Pro-PO activity assay

Spectrophotometrical methods were employed to determine the pro-PO activity of HLS with L-DOPA as the substrate [19]. Firstly, 100 μL HLS sample and 100 μL trypsin (25 mg mL^{-1}) were added into a 96-well ELISA plate, and then the plate was put into microplate reader (MDC, spectra Max190, America) and shaken for several seconds, and then the plate was incubated in the microplate reader darkly at 25°C for 25 min. Afterwards, the plate was taken out and 100 μL L-DOPA (3 mg mL^{-1}) was added into the sample well, and then the plate was shaken for several seconds, and incubated in the dark for 10 min. After that, the optical density (OD) value at the length of 490 nm was recorded every 110 s; a total of 15 times during incubation. The absorbance of blank control was assayed with 100 μL SSS instead of 100 μL HLS sample. One unit of pro-PO activity was defined by the increase of 0.001 in OD_{490nm} per minute.

2.4.4 PO activity assay

PO activity was assayed spectrophotometrically on the formation of dopachrome from L-DOPA as previously described by Ashida [20]. 100 μL hemolymph sample and 100 μL L-DOPA (3 mg mL^{-1}) were successively added into a 96-well ELISA plate. The plate was put into microplate reader and shaken for several seconds, and then OD_{490nm} was recorded at a interval of 110 s 15 times altogether. The absorbance of blank control was assayed with 100 μL SSS instead of 100 μL hemolymph sample. One unit of PO activity was defined by the increase of 0.001 in OD_{490nm} per minute per mL hemolymph.

2.4.5 α_2-Macroglobulin activity assay

Analysis of α_2-macroglobulin (α_2-M) activity was determined by the method of trypsin hydrolyzing the substrate BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) [21,22]. Briefly, 100 μL of bovine pancreatic trypsin solution (1mg in 1 mL 0.1 M Tris–HCl buffer, pH 8.0) was incubated with 100 μL plasma for 10 min at 37°C. After incubation, 20 μL (40 μg) soybean trypsininhibitor (SBTI, Sigma) was added and incubated for 10 min at 37°C. Finally, 500 μL of 1 mg L^{-1} BAPNA (Sigma) in 0.1 M Tris–HCl buffer (pH 8.0), was added and incubated for 5 min at 37°C, then thereaction was stopped by the addition of 2 mL of 30% acetic acid. As acontrol, shrimp plasma was substituted by Tris–HCl buffer. Optical density at 405 nm was recorded and activity was calculated as micrograms of trypsin trapped by α_2-M in 1 mL plasma using commercial trypsin (Sigma) as reference.

2.4.6 Antibacterial and bacteriolytic activities assay

Antibacterial activity and bacteriolytic activity in hemolymph were measured using *V. anguillarum* and *Micrococcus lysodeikticus* (Sigma) respectively, following the modified methods of Hultmark [23] and shrimp pathogenic bacteria *Vibro harveyi* were used for antibacterial activity, and the bacteria *Micrococcus lysoleikticus* for bacteriolytic activity. Firstly, the cryopreserved liquid *Vibro harveyi* strains were thawed and inoculated and spread on the solid tryptone soy broth (TSB) medium containing 1.5% tryptone, 0.5% soy peptone and 2% NaCl with 1.5% agar and pH 7.2±0.2, afterwards the bacteria were incubated at 37°C for 24 h and next the bacteria were washed down gently from the plate into a new sterile tube by sterile 0.1 M phosphate buffer solution (PSB, pH 6.4), and finally prepared into a suspension with a certainconcentration (OD_{570nm}=0.3-0.5). The same process was repeated with *Micrococcus lysoleikticus*, except that it was not incubated on nutrient medium.

Antibacterial and bacteriolytic activity were determined as follows: 300 μL bacterial suspension and 10 μL hemolymph sample were pipetted into 96-well ELISA plate and the plate was put into microplate reader and shaken for a little while, and then OD_{570nm} was read and recorded as A_0. Then the plate was was incubated in the microplate reader in dark at 37°C for 30 min and OD_{570nm} was recorded (A).The antibacterial (*Ua*) and bacteriolytic (*UL*) activity were calculated from the following equations respectively:

$$U_a = \sqrt{(A_0 - A)/A},$$

$$U_L = (A_0 - A)/A$$

(In order to eliminate the interference of hemocyanin, the absorbance of blank hemolymph control was assayed with 10 μL 0.1 M PSB (pH 6.4) instead of 10 μL hemolymph sample to revise A_0 and A.)

2.5 Statistical analysis

All statistics were performed using SPSS 11.5 software (SPSS,Chicago, USA). Data were analyzed using one-way ANOVA after checking tests of normality and homogeneity of variance. Arcsine transformation was

used for phagocytic percent. Significant differences were considered at $P<0.05$. When significant differences were found, Tukey's test was used to identify the differences amongthe four experimental groups.

3 Results

3.1 Effects of BaP and CHR exposure on total hemocyte counts of *L. vannamei*

The number of circulating haemocyte in the hemolymph ofshrimp was affected by both treatment group, and exposure time with a significant interaction between these two factors (Figure 1). No significant difference in THC was observed between the 0.03 μg L^{-1} BaP group and the control. With exposure to 0.3 and 3 μg L^{-1} BaP, the total hemocyte counts (THC) peaked at 3d and 6d, after that the THC recovered. The 0.3 μg L^{-1} group returned to the control, and the 3 μg L^{-1} group was significantly different from control group until the end of the experiment ($P<0.05$). With exposure to 0.3, 2.1 and 14.7 μg L^{-1} CHR, the 14.7 μg L^{-1} CHR treatment showed the same trend with 3 μg L^{-1} BaP group, the THC reached the minimum at 10d, then increased and tended to be stable until the end of the experiment and had significant difference compared with the control ($P<0.05$). The THC in the 0.3 and 2.1 μg L^{-1} CHR treatments reached the minimum at 6d and 10d respectively, afterwards, the THC of 0.3 CHR group returned to the initial level, while the THC in 2.1 μg L^{-1} CHR treatment remained stable up to the 21d exposure and had significant difference compared with the control ($P<0.05$).

3.2 Effect of BaP and CHR exposure on pro-PO, PO and α_2-M activities in hemolymph of *L. vannamei*

There were significant effects of BaP and CHR on pro-PO, PO and α_2-M activities ($P<0.05$).

Figure 2 shows that BaP and CHR exposure had notable effects on pro-PO activity of white shrimp *L. vannamei*. The pro-PO activity of allgroups reached to the minimum at 6d ($P<0.05$). Afterwards the pro-PO activity of all groups showed a recover trend. The 0.03 μg L^{-1} BaP and 0.3 μg L^{-1} CHR groups returned to the control level at 10d and 15d, respectively. However, the other four exposure groups remained stable at 15d up to the end of the experiment and had significant difference compared with the control ($P<0.05$).

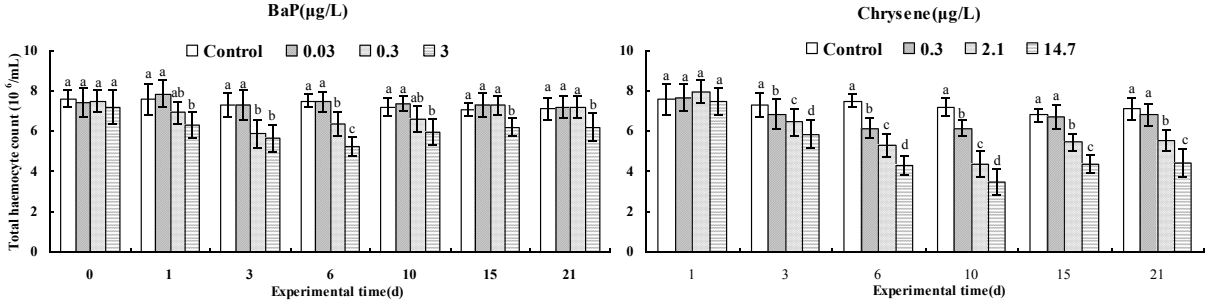

Figure 1. Total haemocyte count in hemolymph of *L. vannamei* exposed to different concentrations of BaP and CHR. Each bar represents the mean value from four replicate tanks with the standard error. Means (± S.E.) with different letters are significantly different ($P<0.05$).

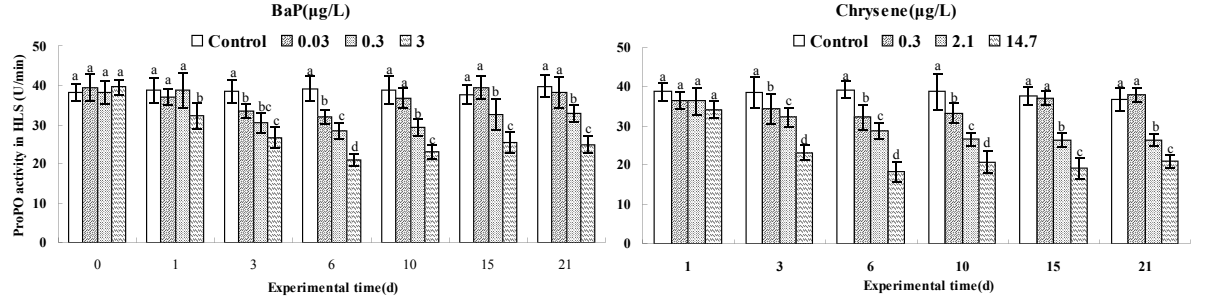

Figure 2. Pro-PO activity in hemolymph of *L. vannamei* exposed to different concentrations of BaP and CHR. Each bar represents the mean value from four replicate tanks with the standard error. Means (± S.E.) with different letters are significantly different ($P<0.05$).

The PO activity in shrimp haemolymph decreased after 0.3 μg L^{-1} BaP, 3 μg L^{-1} Bap, 2.1 μg L^{-1} CHR and 14.7 μg L^{-1} CHR exposure (Figure 3), and the PO activity of the four treatments reached the minimum at 6d (P<0.05), afterwards the PO activity of the four groups showed a recover trend, and they remained stable at 15d up to the end of the experiment and had significant difference compared with the control (P<0.05). In contrast, the 0.03 μg L^{-1} BaP and 0.3 μg L^{-1} CHR groups did not show significant variation with the control group.

Bap and CHR exposure had remarkable effects on α_2-M activity in haemolymph (Figure 4). The α_2-M activity of 0.03 μg L^{-1} BaP and 3 μg L^{-1} CHR exposure group had no significant difference compared with the control. The other exposure groups, the α_2-M activity decreased at the first 6d-stress (P<0.05). Afterwards the α_2-M activity showed a recovery. The 0.3 μg L^{-1} BaP and 2.1 μg L^{-1} CHR groups, the α_2-M activity gradually recovered to the control level at 15 and 10d, respectively. However the 3 μg L^{-1} Bap and 14.7 μg L^{-1} CHR groups remained at low level and significantly different from control values until the end of the experiment (P<0.05).

3.3 Effect of BaP and CHR exposure on phagocytosis, antibacterial and bacteriolytic activities in hemolymph of *L. vannamei*

Figure 5 shows that BaP and CHR exposure had notable effects on phagocytic activity of *L. vannamei*. The phagocytic activity of all Bap and CHR groups reached to the minimum at 6d. Afterwards, they all remained stable up to the end of the experiment and had significant difference compared with the control (P<0.05), except for the 0. 03 μg L^{-1} BaP and 0.3μg L^{-1} CHR groups, they both returned to the control at 15d.

The effects of BaP and CHR on the bacteriolytic activity of *L. vannamei* were illustrated in Figure 6. In comparison with the control, the bacteriolytic activity of 0.03 μg L^{-1} Bap and 0.3 μg L^{-1} CHR exposure groups decreased significantly at day 3 and day 6 respectively, afterwards they both recovered to the control level. On the other hand, the treatments of the other groups, the bacteriolytic activity were inhibited significantly at the whole exposure time (P<0.05).

The antibacterial activityin haemolymph of 0.03 μg L^{-1} Bap and 0.3 μg L^{-1} CHR exposure groups had no

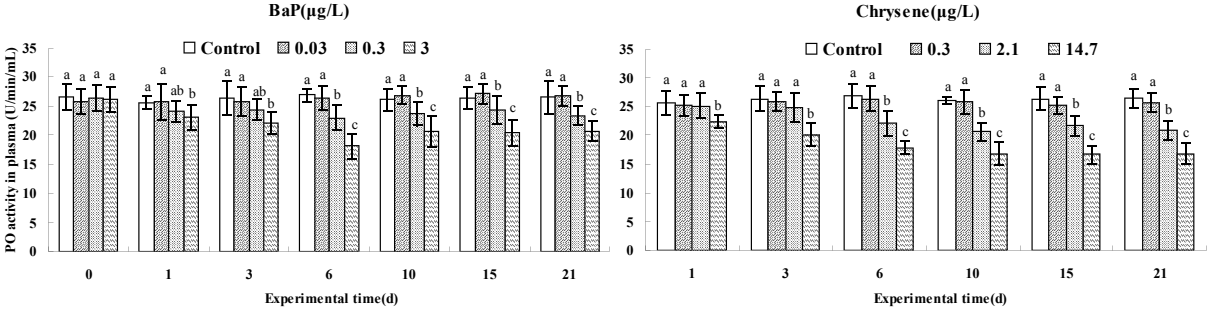

Figure 3. PO activity in hemolymph of *L. vannamei* exposed to different concentrations of BaP and CHR. Each bar represents the mean value from four replicate tanks with the standard error. Means (± S.E.) with different letters are significantly different (P<0.05).

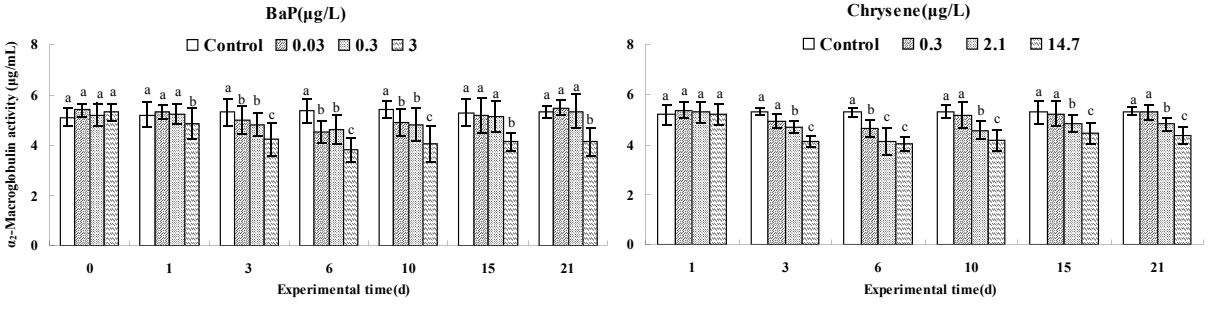

Figure 4. α_2-Macroglobulin activity in hemolymph of *L. vannamei* exposed to different concentrations of BaP and CHR. Each bar represents the mean value from four replicate tanks with the standard error. Means (± S.E.) with different letters are significantly different (P<0.05).

significant difference compared with the control. With exposure to the other exposure groups, the antibacterial activity was decreased significantly and reached to the minimum at 6d, and then it increased. The 0.3 μg L^{-1} Bap group recovered to the control, and the 0.3 μg L^{-1} BaP, 2.1 and 14.7 μg L^{-1} CHR groups tended to be stable after 10d-stress, then remained stable, significantly lower than the control (P<0.05) (Figure 7).

3.4 Comparison of the immunotoxicity between BaP and CHR in the same concentration

With the same concentration BaP and CHR (0.3 μg L^{-1}) exposure, the THC reached the minimum at 3d and 6d, respectively. The THC decreased by19.6% and 18.3%; relative to the control group. As the exposure time progressed to 15d, the THC returned to levels similar to shrimps from the control group (Figure 1). And the pro-

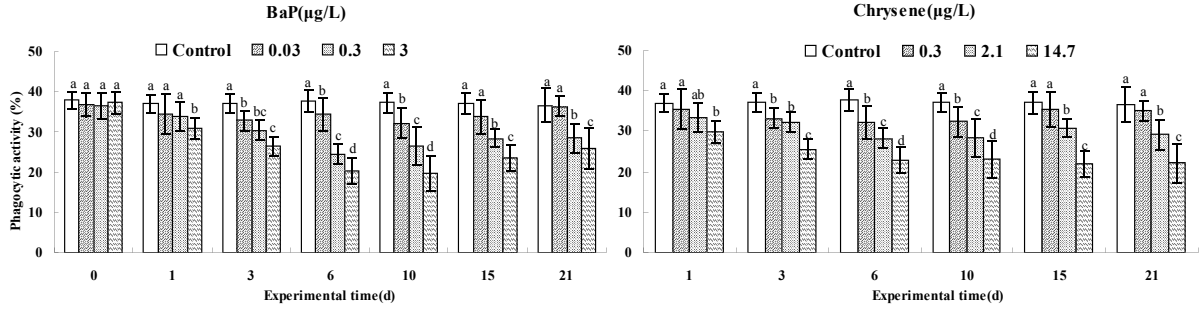

Figure 5. Phagocytic activity of haemocyte in *L. vannamei* exposed to different concentrations of BaP and CHR. Each bar represents the mean value from four replicate tanks with the standard error. Means (± S.E.) with different letters are significantly different (P<0.05).

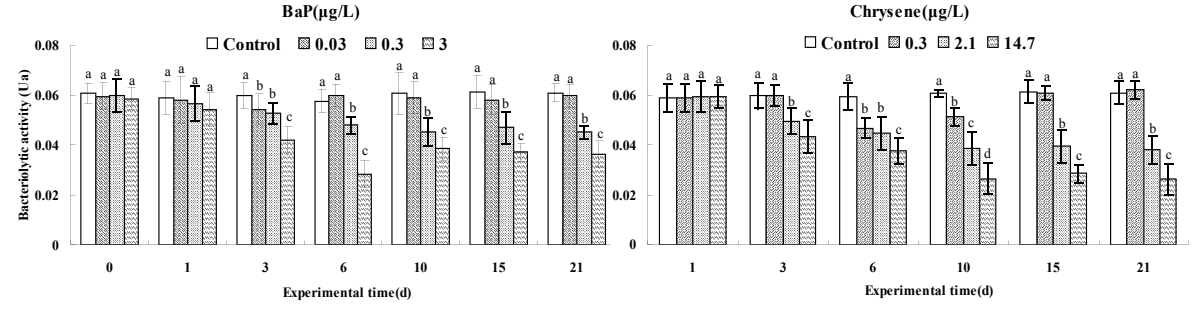

Figure 6. Bacteriolytic activity in hemolymph of *L. vannamei* exposed to different concentrations of BaP and CHR. Each bar represents the mean value from four replicate tanks with the standard error. Means (± S.E.) with different letters are significantly different (P<0.05).

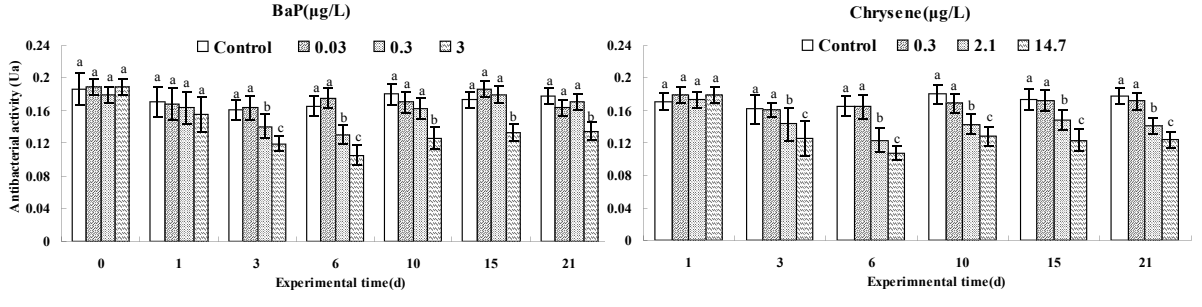

Figure 7. Antibacterial activity in hemolymph of *L. vannamei* exposed to different concentrations of BaP and CHR. Each bar represents the mean value from four replicate tanks with the standard error. Means (± S.E.) with different letters are significantly different (P<0.05).

PO activity showed the same trend, the both exposure groups reached the minimum at 6d (P<0.05), decreased by 27.6% and 17.9%; after that they both recovered to the control level (Figure 2). The PO and α_2-M activities of BaP exposure group showed the same trend, the PO activity decreased by 14.5% (Figure 3) and the α_2-M activity group decreased by 14.2% (Figure 4). However, the PO and α_2-M activities of CHR exposure groups had no significant difference compare with the control. With regards tophagocytic, bacteriolyticand antibacterial activities, both exposure groups reached the minimum at 6d, and the phagocytic activity decreased by 35.1% and 14.5% (Figure 5); the bacteriolytic activity decreased by 25.6% and 21.4% (Figure 6), the antibacterial activity of BaP exposrue group decreased by 21.1% (Figure 7), while the phagocytic activity of CHR exposure group had no change, relative to the control group. As the exposure time progressed, the phagocytic activity of CHR group returned to the control level at 15d, had no significant difference compared with the control up to the end of the experiment.

4. Discussion

4.1 Effects of BaP and CHR on immune parameters

There is considerable evidence of vulnerability of an organism's immune system to environmental contaminants. Therefore, it is important to explore the interaction and toxicity of contaminants in organisms [24]. Like other invertebrates, shrimps lack an adaptive immune system, and entirely rely on innate immune responses to fight against invading pathogens [18]. It is generally considered that the immune mechanisms of crustaceans have two distinct pathways: cellular and humoral defence mechanisms [25]. In crustaceans, the hemocyte plays a central role in mediating immune capability *via* phagocytosis, encapsulation and nodule formation, while humoral immune factors play a very important role in the immune responses, including all kinds of antibacterial, antiviral, coagulation, cell-activating, and recognition factors, and lectin, hemolysin, lysozyme, hydrolase and other immune active substances [26,27]. Therefore, the THC in hemolymph was usually used as an important parameter to evaluate the immunotoxicity of pollutions [9]. In this study, the THC in hemolymph of the shrimps decreased significantly under 21d exposures to high concentrations of BaP and CHR, which agrees with the result of Zhang *et al.* [11]. Previous work has indicated the susceptibility of its baseline immune parameters to the PAH phenanthrene, causing alterations in phagocytosis [28]. In the present study, the phagocytic activity of hemocyte in the shrimps exposed to high concentrations of BaP and CHR decreased significantly after 6 days, and presented significant dose response and time response. There were many studies focusing on the toxic effect of contamination on phagocytosis, which showed the same changes after exposure to contamination.

There have been few studies on the toxic effects of contamination on the humoral immune response. In this study, the pro-PO activity, PO activity, α_2-M activity, antibacterial activity and bacteriolytic activity of the shrimps were significantly decreased following 6d exposure to high concentrations of BaP and CHR, and presented significant dose response and time response. These results were consistent with the studies of Liu [10] and Zhang [11], and they found that BaP and Aroclor 1254 could inhibit the humoral immune factors of scallop (*Chlamys farreri*).

4.2 Immunotoxic mechanism of BaP and CHR in *L. vannamei*

PAHs, is one of a group of highly lipophilic organic compounds, they are able to penetrate model membrane systems [29], and then bind to membrane lipids which compromise basic cellular function by altering fluidity and ionic pumps [30], which is one reason of the decrease in THC. Once the PAHs are taken up by an organism, these PAHs undergo biotransformation reactions through two ways, during the reactions, reactive oxygen species (ROS) and inermediate hydrosoluble metabolites were stimulated to produce, and thereby damage biological macromolecules and tissue structures [31], which is another reason for the decrease of THC. Moreover, the haemocyte may participate in damage repair, resulting in a decrease of count in hemolymph [11]. Such a decrease in THC has also been reported in crustaceans as a result of the exposures to PAHs [26], PCBs [32] and ammonia-N [18].

The lysosomes play an important role in the immune defense system of crustaceans, which not only take part in phagocytosis, but also can release various hydrolytic enzymes to the haemolymph to participate in the humoral response [11]. Lysosomal damage arising from the accumulation of PAhs and formation of oxyradicals causes an increase in membrane permeability and subsequently the release of acid hydrolases into the cytoplasm. This sequence of events results in cellular damage/cell death (as shown by the decrease of THC), and subsequently resulting in a reduced immune function (as shown by the decrease of phagocytic activity).

In crustaceans, the prophenoloxidase (pro-PO) systemplays a key role in immunological recognition and defense [33]. Pro-PO in the activation system of shrimps

has been widely studied. Through the combination of hemocyte and microbial polysaccharides, pro-PO release into hemolymph from hemocyte in the way of degranulation, which is followed by the activation of prophenoloxidase activating protein (ppA) into active phenoloxidase [34,35]. Many studies found that protease inhibitorin various crustaceans, which is a role similar to the vertebrate α_2-M and known as the class of α_2-M, can suppress all currently known proteases. In the pro-PO system, it can regulate pro-PO kinase through a partial inhibition of serine protease activity [36,37]. According to Enghild, in the horseshoe crab (*Limulus polyphemus*), α_2-M has the activity of protease inhibitors [38]. In the present study, the activities of pro-PO, PO and α_2-M in the shrimps of treatments with 3 μg L^{-1} BaP and 14.7 μg L^{-1} CHR exposure reached minimum values at day 6 respectively. Afterwards, they all remained stable up to the end of the experiment and were significantly different to the control. However, in the 0.03 μg L^{-1} BaP and 0.3 μg L^{-1} CHR exposuregroups, the pro-PO and PO showed different trend. The pro-PO decreased from 6 days to 10 days, however the PO activity had no change throughout the experiment time. This may explain the reason of the decrease of α_2-M activity, that then activated the serine protease activity, and subsequently triggered pro-PO system, causing a stabilization in PO activity.

The decline of THC could affect other humoral immune factors, such as pro-PO, crustin, lectin and AMPs, which are mainly expressed and stored in the haemocyte of crustacean, and usually are released into hemolymph against bacterial pathogens [39,40]. In this study the THC and the other immune parameters showed remarkable decrease and presented significant dose-response and time-response to BaP and CHR. Besides THC affecting other humoral immune factors, this study had proved that PAHs could lead to protein carbonyl. The authors speculate that the decreases of humoral immune factors were due to the protein carbonyl.

In some instances, low concentrations or short time of PAHs exposure can result in an immuno-stimulating effect. For a example, acute exposure of oil initially induced the stimulation of phagocytic activity in the Arctic Scallop *Chlamys islandica*. A short-term, low-dosestimulation of phagocytosis has also been reported in bivalves in response to contaminants including produced water [28], pesticides [41], and sediment [42]. Whilst the decreased activity of phagocytosis observed here in the shrimps was not associated with the former studies, the author considered that the immunotoxicity mechanism of PAHs in shrimps is still controversial and therefore requires further research.

5. Conclusion

In conclusion, the exposureof BaP and CHR could impairthe immune function of *L. vannamei*. In the same concentration of Bap and CHR (0.3 μg L^{-1}), the rank of immunotoxicity was BaP> CHR. The study also indicated that THC in hemolymph, pro-PO activity and phagocytic activity of hemocyte, and bacteriolytic activity in hemolymph could be used as potentially suitable biomarkersfor early warning indicationof PAHs toxicity. These parameters will provide useful biomarkers for studying the response of shrimp to environmental contaminants, as they were more sensitive than other immune parameters.

Acknowledgements

This research was supported by the National Marine Public Industry Research Project ”large-scale park aquaculture environment engineering ecological optimization technology integration and demonstration” (201305005).

References

[1] Wang, L., Pan, L.Q., Liu, N., Liu, D., Xu, C.Q., Miao, J.J., Biomarkers and bioaccumulation of clam Ruditapes philippinarumin response to combined cadmium and benzo[a]pyrene exposure, Food Chem. Toxicol., 2011, 49, 3407-3417.

[2] US EPA, 2009.United States Environmental Protection Agency Priority Pollutants.<http://www.epa.gov/waterscience/methods/pollutants.htm> (accessed 02.04.09).

[3] Zhou, L. M., Meng, X. H., Xiao, H., Tang, X. X., Interactive studies of three kinds of polycyclic aromatic hydrocarbonsand UV-B radiation on the growth of three speciesof red tide microalgae. J. Wuhan Univ.: Natl. Sci. Ed., 2006,52(6), 773-777 (in Chinese with English abstract).

[4] Livingstone, D.R., Organic xenobiotic metabolism in marine invertebrates. In:Gilles,R. (Ed.), Advances in Comparative and Environmental Physiology, vol 7.Springer-Verlag, Berlin, pp, 1991, 46-185.

[5] Livingstone, D.R., The fate of organic xenobiotics in aquatic ecosystems:quantitative and qualitative differences in biotransformation by invertebrates

and fish, Comp. Biochem. Physiol. C,1998, 120,43-49.

[6] Valavanidis,A., Vlahogianni, T., Dassenakis, M.,Scoullos, M., Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants, Ecotoxicol. Environ. Saf.,2006, 64,178-189.

[7] Galloway, T.S. and Depledge, M.H., Immunotoxicity in invertebrates: measurement and ecotoxicological relevance, Ecotoxicology, 2001,10, 5-23.

[8] Blaise, C., Trottier, S., Gagné, F., Lallement, C., Hansen, P.D., Immunocompetence of bivalve haemocytes as evaluated by a miniaturized phagocytosisassay. Environ. Toxicol., 2002., 17, 160-169.

[9] Hannam, M.L., Bamber, S.D., Moody, J.A., Galloway, T.S.,Jones, M.B., Effects of themodel PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop Pecten maximus,Chemosphere, 2010, 78, 779-784.

[10] Liu, J., Pan, L.Q., Zhang, L., Miao, J.J., Wang, J.,Immune responses, ROS generation and the haemocyte damage of scallop Chlamys farreri exposed to Aroclor 1254, Fish Shellfish Immun., 2009, 26, 422-428.

[11] Zhang L., Pan L.Q., Liu J., Immunotoxicity effect of Benzo[α]Pyrene on scallop Chlamys farreri,J. Ocean Univ. China, 2008,8 (1), 89-94.

[12] Dissanayake, A., Galloway, T.S., Jones, M.B., Nutritional status of Carcinus maenas (Crustacea: Decapoda) in fluences susceptibility to contaminant exposure, Aquat. Toxicol., 2008, 89, 40-46.

[13] Dissanayake, A., Galloway, T.S., Jones, M.B.,Physiological responses of juvenile and adult shore crabs Carcinus maenas(Crustacea: Decapoda) to pyrene exposure, Mar.Environ. Res., 2008,66, 445-450.

[14] Luengen, A.C., Friedman, C.S., Raimondi, P.T., Flegal, A.R., Evaluation of musselimmune responses as indicators of contamination in San Francisco Bay, Mar.Environ. Res., 2004, 57, 197-212.

[15] Fang, W.H., Li, G.L., Zhou,S., Li, X.C., Hu, L.L.,Zhou, J.F., Pharmacokinetics and Tissue Distribution of Thiamphenicol and Florfenicol in Pacific White Shrimp Litopenaeus Vannamei in Freshwater following Oral Administration, J. Aquat. Anim. Health, 2013, 25, 83-89.

[16] Liu, C.H., Chen, J.C., Effect of ammonia on the immune response of white shrimp Litopenaeus vannameiand its susceptibility toVibrio alginolyticus. Fish Shellfish Immun., 2004, 16, 321–334.

[17] Yang, L.B. and Pan, L. Q., Effects of phosphatidyl serine on immune response in the shrimp Litopenaeus vannamei, Cent. Eur. J. Biol., 2013, 8(11), 1135-1144.

[18] Yue, F., Pan, L.Q., Xie, P., Zheng, D.B., Li, J.,Immune responses and expression of immune-related genes in swimming crab Portunus trituberculatus exposed to elevated ambient ammonia-N stress, Comp. Biochem. Physiol. A, 2010, 157, 246-251.

[19] Hernández-López, J., Gollas-Galván, T., Vargas-Albores, F., Activation of the prophenoloxidase system of the brown shrimp (Penaeus californiensis Holmes),Comp. Biochem. Physiol. C, 1996, 113, 61-66.

[20] Ashida, M.,Purification and characterization of pre-phenoloxidase from hemolymph of the silkworm Bombyx mori, Arch. Biochem. Biophys., 1971,144, 749-762.

[21] Armstrong,P.B.,Quigley, J.P., Rickles, F.R., The Limulus blood cell secretes α2-macroglobulin when activated, Biol. Bull., 1990, 178, 137-143.

[22] Gollas-Galván, T., Sotelo-Mundo, R.R., Yepiz-Plascencia, G., Vargas-Requena, C., Vargas-Albores, F., Purification and characterization of alpha 2-macroglobulin from the white shrimp (Penaeus vannamei), Comp. Biochem. Physiol. C, 2003,134, 431-438.

[23] Hultmark, D., Steiner, H., Rasmuson, T., Boman, H.G., Insect immunity. purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia.Eur, J. Biochem., 1980, 106, 7-16.

[24] Chang, M., Wang, W.N., Wang, A.L., Tian, T.T., Wang, P., Zheng, Y.,Liu, Y., Effects ofcadmium on respiratory burst, intracellular Ca2+ and DNA damage in the white shrimp Litopeneaus vannamei,Comp. Biochem. Physiol. C, 2009, 149, 581-586.

[25] Hannam, M.L., Bamber, S.D., Sundt, R.C., Galloway, T.S., Immune modulationin the blue mussel Mytilus edulisexposed to North Sea produced water, Environ.Pollut., 2009, 157, 1939-1944.

[26] Dissanayake,A.,Piggott,B.,Baldwin,C.,Sloman,K .A., Elucidating cellular and behavioural effects of contaminant impact (polycyclic aromatic hydrocarbons, PAHs) in both laboratory-exposed and field-collected shore crabs, Carcinus maenas(Crustacea: Decapoda), Mar.Environ. Res.,2010, 70, 368-373.

[27] Livingstone, D.R., Martinez, P.G., Michel, X., Narbonne, J.F., Ohara, S., Ribera, D.,Winston, G.W., Oxyradical production as a pollution-

mediated mechanism of toxicity in the common mussel, Mytilus edulis(L.), and other mollusks, Funct. Ecol.,1990, 4, 415-424.

[28] Hannam, M.L., Bamber, S.D., Galloway, T.S., Moody, J.A.,Jones, M.B., Functional immune response in Pecten maximus: Combined effectsof a pathogen-associated molecular pattern and PAH exposure,Fish Shellfish Immun.,2010, 28(1), 249-252.

[29] Nelson, A.,Auffret,N.,Borlakoglu,J., Interaction of hydrophobic organic compounds with mercury adsorbed dileoylphosphatidycholine monolayers, ActaBioch. Bioph., 1990, 1021, 205-216.

[30] Camus, L., Jones, M.B., Borseth, J.F., Grosvik, B.E., Regoli, F.,Depledge, M.H., Total oxyradical scavenging capacity and cell membrane stability of haemocytes of the Arcticscallop, Chlamys islandicus, following benzo(a)pyreneexposure, Mar.Environ. Res., 2002,54, 425-430.

[31] Pan, L.Q., Ren, J.Y., and Liu, J, Responses of antioxidant systems and LPO level to benzo(a) pyreneand benzo(k)fluoranthene in the haemolymph of the scallop Chlamys Ferrari, Environ. Pollut., 2006, 141, 443-451.

[32] Smith, V.J., Johnston, P.A., Differential haemotoxic effect of PCB congeners in the common shrimp,Crangon crangon,Comp. Biochem. Physiol. C,1992, 101, 641-649.

[33] Li, F.H. and Xiang, J.H., Recent advances in researches on the innate immunityof shrimp in China, Dev. Comp. Immunol., 2003, 39, 11-26.

[34] Söderhäll, K. and Cerenius, L., Role of the prophenoloxidase-activating system in invertebrate immunity, Curr. Opin Immunol, 1998,10(1), 23-28.

[35] Cerenius, L.,Lee, B.L., and Söderhäll, K., The proPO-system: pros and cons for its role in invertebrate immunity, Trends Immunol., 2008,29(6): 263-71.

[36] Teresa, G.G., Rogerio, R., Sotelo-Mundo, Yepiz-Plascencia, Vargas-Requena, C., Vargas-Albores, F., Purification and characterization of α2-macroglobulin from the white shrimp (Penaeus vannamei), Comp. Biochem. Physiol. C, 2003, 134, 431-438.

[37] Pan, L.Q., Hu, F.W., Jing, F.T., Liu, H.J., The effect of different acclimation temperatures onthe prophenoloxidase system and other defence parameters in Litopenaeus vannamei, Fish Shellfish Immun., 2008,25, 137-142.

[38] Enghild, J.J., Thogersen, I.B., Salvesen, G., Fey, G.H., Figler, N.L., Gonias, S.L., Pizzo, S.V.,Alpha-macroglobulin from Limulus polyphemus exhibits proteinase inhibitory activity and participates in a hemolytic system. Biochemistry, 1990, 29, 10070-10080.

[39] Li, C., Zhao, J., Song, L., Mu, C., Zhang, H., Gai, Y., Qiu, L., Yu, Y., Ni, D.,Xing, K., Molecular cloning, genomic organization and functional analysis of an anti-lipopolysaccharide factor from Chinese mitten crab Eriocheir sinensis. Dev. Comp. Immunol.,2008, 32, 784-794.

[40] Imjongjirak, C., Amparyup, P., Tassanakajon, A., Sittipraneed, S., Molecular cloningand characterization of crustin from mud crab Scylla paramamosain. Mol. Biol. Rep., 2009,36, 841-850.

[41] Rickwood,C.J.,Galloway,T.S.,Acetylcholinesterase inhibition as a biomarkerof adverse effect : a study of Mytilus edulis exposed to the priority pollutant chlorfenvinphos, Aquat. Toxicol., 2004, 67, 45-56.

[42] Martín-Díaz, M.L., Blasco, J.,Sales, D., DelValls, T.A., Field validation of a battery of biomarkers to assess sedimentquality in Spanish ports,Environ. Pollut., 2008,1, 631-640.