Fish and Shellfish Immunology 72 (2018) 679–689

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Hybridization improved bacteria resistance in abalone: Evidence from physiological and molecular responses

Shuang Liang[a,b,c,d], Xuan Luo[a,b,c,*], Weiwei You[a,b,c], Caihuan Ke[a,b,c,*]

[a] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361102, China
[b] College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, China
[c] Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Xiamen 361102, China
[d] Tianjin Key Laboratory of Aqua-Ecology and Aquaculture, College of Fisheries, Tianjin Agricultural University, Tianjin 300384, China

ARTICLE INFO

Keywords:
Hybridization
Abalone
Immune response
HSP70
Ferritin
CSDP

ABSTRACT

Hybridization is an effective way of improving germplasm in abalone, as it often generates benign traits in the hybrids. The hybrids of *Haliotis discus hannai* and *H. gigantea* have shown heterosis in terms of disease resistance than one or both parental species. In the present study, to elucidate the physiological and molecular mechanism of this heterosis, we analyzed the dynamic changes of several immune indexes including survival rate, total circulating haemocyte count (THC), phagocytic activity, reactive oxygen species level (ROS) and phenoloxidase activity (PO) in two parental species, *H. discus hannai* (DD) and *H. gigantea* (GG), and their reciprocal hybrids *H. discus hannai* ♀ × *H. gigantea* ♂ (DG), *H. gigantea* ♀ × *H. discus hannai* ♂ (GD) challenged with a mixture of *Vibrio harveyi*, *V. alginolyticus* and *V. parahaemolyticus* (which have been demonstrated to be pathogenic to abalone). Besides, we cloned and analyzed three important immune genes: heat shock protein 70 (*hsp70*), *ferritin* and cold shock domain protein (*csdp*) in *H. discus hannai* and *H. gigantea*, then further investigated their mRNA level changes in the four abalone genotypes after bacterial challenge. Results showed that these physiological and molecular parameters were significantly induced by bacterial exposure, and their changing patterns were obviously different between the four genotypes: (1) Survival rates of the two hybrids were higher than both parental species after bacterial exposure; (2) DG had higher THC than the other three genotypes; (3) Phagocytosis responded slower in the hybrids than in the parental species; (4) DD's ROS level was lower than the other three genotypes at 48 h post infection; (5) Phenoloxidase activity was lower in DD during the infection compared to the other genotypes; (6) mRNA levels of *hsp70* and *csdp*, were always lower in at least one parental species (DD) than in the hybrids after the bacterial exposure. Results from this study indicate that the hybrids are more active or efficient in immune system function, hence they could effectively defense against a bacterial invasion, leading to higher survival rates after challenge. This study provides physiological and molecular evidences for interpreting the disease resistant heterosis in this abalone hybrid system, which could help us in a better understanding and utilization of heterosis in abalone aquaculture.

1. Introduction

Abalone is a high economically valued marine species around the world because of its high nutritive value and exceptional delicacy. It is an important economical maricultural species cultured worldwide, and China is the leading abalone producing country [1,2]. Based on the food and agriculture organization (FAO) statistical data, the abalone aquaculture production of China reached 127,967 tons in 2015, which accounted for 90.20% of the world's production (FAO FishStat J 2015) [3]. Although abalone aquaculture is a huge industry in China, problems still exist, one of them is a decrease in abalone genetic diversity brought by repeated inbreeding, which would lead to abalone's germplasm decline. Several disease outbreaks happened in China abalone industry during the past few years, resulting in severe economic losses [4,5]. To solve this problem, scientists and abalone producers were prompted to investigate the methods of abalone genetic improvement, hybridization breeding has been proven to be one effective approach.

Heterosis, or hybrid vigour, refers to a biological phenomenon that hybrids often show advantages in growth, fitness, or yield relative to their parental species [6]. Heterosis is a widespread phenomenon in plants and animals [6,7], it has been utilized in agriculture and aquaculture for several years [7–10]. Due to abalone's external reproductive

* Corresponding authors. State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361102, China.
E-mail addresses: xluo@xmu.edu.cn (X. Luo), chke@xmu.edu.cn (C. Ke).

https://doi.org/10.1016/j.fsi.2017.11.009
Received 14 May 2017; Received in revised form 6 November 2017; Accepted 6 November 2017
Available online 07 November 2017
1050-4648/

strategy, hybridization between abalone species naturally occurs in the ocean [11]. Artificial hybridization between abalone species started 50 years ago [12], intraspecific hybrids (such as hybrids from different ecotypes of *Haliotis diverscolor* [13,14] and *H. discus hannai* [15]), and interspecific hybrids (such as hybrids of *H. gigantea* and *H. discus hannai* [16], *H. discus hannai* and *H. fulgens* [17]) were produced. Some of these abalone hybrids showed advantages towards their parental species in growth, disease resistance, or other market-favored traits (color, taste, amino acid content, etc) [18]. In our practice, we introduced *H. gigantea*, which was reported to be more resistant to thermal stress and disease than *H. discus hannai* [19], into China, next we did hybridization between *H. gigantea* and *H. discus hannai*, to improve the latter's seed quality [20]. A better disease resistant ability in the hybrids was observed and proved by our field trial and *in vitro* bacterial challenge experiments [20–22]. After 19 months' growing under the same conditions in abalone farm (from 7, April, 2007 to 8, November, 2008), the survival rates of DD, GG, DG and GD were separately 51.83%, 88.45%, 90.33% and 92.34% [20]. When injected or immersed with pathogenic bacteria, DG and GD also had significant higher survival rates than DD [21; This study]. While the hybridization technique has been successfully utilized in abalone breeding, the physiological and molecular mechanisms underlying abalone heterosis remains unclear.

Resistance to pathogens is one of the most important traits in abalone aquaculture as it determines abalone's survival rate during production and directly influences the final yield [23]. Like other invertebrates, abalone only has innate immune function [24], which is composed of cellular and humoral immune functions [25]. Cellular immune response is mainly conducted by haemocytes through a series of processes including chemotaxis, recognition, attachment, phagocytosis, and respiratory burst to eliminate foreign particles [26]. While humoral immune function is mainly conducted by various immune-related enzymes located in haemocytes and hemolymph including superoxide dismutase (SOD), phenoloxidase (PO), lysozyme, etc [27,28]. Total haemocyte count (THC), phagocytic activity, reactive oxygen species level (ROS), SOD and PO activities are widely used immune-related parameters, they could reflect invertebrate's resistant ability against pathogens, and their level changes have been proven to be closely related to the genetic characteristics and physiological status of organisms, also to the external environmental factors [29–33].

Underlying the invertebrate's physiological immune functions are various regulatory molecules, they are synthesized from, or controlled by a wide range of immune-related genes [25], among which heat shock protein 70 (*hsp70*), *ferritin*, and cold-shock protein (*csdp*) have been proven to be important genes. The participation of *hsp70* in invertebrate immune responses has been observed in various invertebrate species [34–40], *hsp70* is regarded as an activator of the invertebrate innate immune systems [34–36]. *ferritin* levels vary during or after pathogen challenges in different marine invertebrates such as ark shell [41], abalone [42,43], shrimp [44], clam [45], crab [46], and scallop [47,48], thus suggesting its role in the invertebrate innate immune system. It is suggested that *ferritin* regulates the free iron level in the host to defend bacteria, as iron is the essential element for most bacteria to maintain their normal physiological functions [49]. *csdp*, featured by its biological function in the cold adaption, its involvement in mollusk's response to cold stress has been studied in two scallop species [50,51]. Recently, *csdp* has been proven to affect the immune functions of organisms as well, it is speculated to play a role in inflammation phase and neuroinflammation [52–54]. *csdp* functions as a molecular chaperone in organisms and regulates the transcription and translation of various genes [55], including those related to immune responses. However, the role of *csdp* in invertebrate immune system remains unknown.

To better understand the physiological and molecular mechanisms underlying the resistance heterosis in abalone, in this study, we investigated the reactions of the *H. gigantea, H. discus hannai* and their reciprocal hybrids to the exposure to a mixture of pathogenic *Vibrio* spp, their survival rates post infection, changes in physiological immune parameters levels, and transcription patterns of the above-mentioned genes were identified.

2. Materials and methods

2.1. Abalones

Two species of abalone, *H. discus hannai* (DD), *H. gigantea* (GG), and their reciprocal hybrids *H. discus hannai* ♀ × *H. gigantean* ♂ (DG), *H. gigantean* ♀ × *H. discus hannai* ♂ (GD), were used in this study, they were at the same age (3 years) and similar in size (shell length 7.19 ± 0.43). For each genotype, 100 individuals were used. Abalones were collected from the Fuda Abalone Company (Jinjiang, China) and acclimated under the same culture conditions (24 ± 1 °C, 33‰ salinity, and 7.8 pH) for 14 days prior to experimentation. Abalones were fed with fresh red alga (*Gracilaria lemaneiformis*), the seawater was replaced every day.

2.2. Tissues sampling for hsp70, ferritin, and csdp cloning

For cDNA cloning of *hsp70* and *ferritin* in *H. gigantea*, and *csdp* in *H. discus hannai* (*hsp70* and *ferritin* have been cloned in *H. discus hannai* before [35,42], *csdp* has not been cloned in abalone species yet), five tissues (gill, mantle, muscle, hemolymph and visceral mass) of the two species were collected, snap-frozen in liquid nitrogen, and then stored at −80 °C.

2.3. Bacterial challenge, survival rate observation and tissue sample collection

Three species of vibrios: *Vibrio harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* were used in the bacterial challenge, which were previously isolated from moribund abalone and demonstrated to be virulent to abalone [56,57].

30 abalones from each genotype were used to assess survival rate post bacterial challenge. Abalones were immersed with seawater containing a mixture of the three vibrios (in the same proportion) at a concentration of 1×10^9 CFU/mL and then reared under 24 °C. At 0 h (control), 6 h, 12 h, 24 h, and 48 h after exposure, survival rates of the four genotypes were recorded, abalones reared in seawater without bacteria addition were used as the control group.

Another 70 abalones from each genotype were used for immune parameters determination and immune gene transcriptional analysis. Abalones were immersed with the same vibrios at a concentration of 1×10^7 CFU/mL (which had been demonstrated to be the sub-lethal concentration in our previous unpublished experiment), and then reared under 24 °C. At 0 h (control), 12 h, 24 h, and 48 h after exposure, 6 abalones from each genotype were sampled for their hemolymph for cellular and humoral immune parameters determination, the hemolymph was placed on ice. At 0 h (control), 6 h, 12 h, 24 h, and 48 h after exposure, another 6 abalones from each genotype were sampled for their gill and hemolymph. The gill was snap-frozen in liquid nitrogen and stored at −80 °C, the hemolymph was centrifuged at 800 g for 5 min to collect circulating haemocytes, the supernatant was discarded, and the remaining was snap-frozen in liquid nitrogen and stored at −80 °C.

2.4. Total circulating haemocytes count (THC) determination

THC was determined using SYBR green I (Sigma-Aldrich, USA), a fluorescent dye that binds to double-stranded DNA. 200 μL of fresh hemolymph was fixed with an equal volume of 3% formalin solution. Fixed circulating haemocytes were incubated with 1,000x SYBR green I for 120 min in the dark at room temperature before running in a flow cytometer. The circulating haemocytes stained with SYBR green I were differentiated from other particles in the hemolymph based on green fluorescence (FL-1 detector) of flow cytometer [58].

2.5. Phagocytosis assay

200 μL of fresh hemolymph was centrifuged at 780 g for 10 min.

Table 1
Primers used in our experiments.

Primer	Sequence (5′-3′)	Amplification efficiency
deg*hsp70*F	GGKTCCACDCGTATTCCAAAG	
deg*hsp70*R	ATCRACCTCCTCRATGGTTGG	
degFerritinF	ATGGCCCAAACTCAACCC	
degFerritinR	TTCATGATYCTYTCCATGTTDGC	
degCSDPF	ACHGTSAARTGGTTYAAY	
degCSDPR	TTYVCCVWCHCCVACACT	
gsp*hsp70*F	CGAGTATGTTGTGAAGGTCTGGGTCTGT	
gsp*hsp70*R	ACAGACCCAGACCTTCACAACATACTCGG	
gspFerritinF	CCTCCTCGGATGCCTTCT	
gspFerritinR	TTCCCTGGAGAAGAACGTCA	
gspCSDPF	CACTTCTAAGGTATTTCTTTGGGTTGTT	
gspCSDPR	TAAGAGCGGATATGGCTTCAT	
rt*hsp70*F	AGGAGGAGATAGAGCGTAT	102.270%
rt*hsp70*R	TCGGTGATGGTCTTCTTG	
rtFerritinF	GAACTTCCACTGCGAGAG	104.233%
rtFerritinR	CGGATGCCTTCTTGAAATAC	
rtCSDPF	GAGCGGATATGGCTTCAT	106.336%
rtCSDPR	TCTTTGGGTTGTTCTTCACT	
β-actinF	GGTATCCTCACCCTCAAGT	103.711%
β-actinR	GGGTCATCTTTTCACGGTTG	

Pelleted circulating haemocytes were resuspended in filtered sterile seawater (FSSW). Then, 30 μL of fluorescent beads (Polysciences Fluoresbrite YG Microspheres, 2.00 μm, final concentration: 0.3% of the commercial suspension) were added to each tube. After 120 min incubation at 18 °C, 230 μL of 3% formalin solution in FSSW was added into the tube to stop the reaction. The percentage of phagocytic cells equals to the percentage of circulating haemocytes that had engulfed more than three beads revealed by flow cytometer analysis based on FITC fluorescence [59]. 200 μL of FSSW mixed with 30 μL fluorescent beads was used as reagent control.

2.6. Reactive oxygen species level

Reactive oxygen species (ROS) production of circulating haemocytes was measured according to a previous study [60] using 2′7′-dichlorofluorescein diacetate (DCFH-DA). 400 μL of fresh hemolymph was mixed with 4 μL of DCFH-DA (final concentration: 0.01 mM) and then incubated at 18 °C for 120 min. When diffused into the cells, DCFH-DA was hydrolyzed to 2′7′-dichlorofluorescein (DCFH), thereby trapped within the cells. Then, the intracellular DCFH was oxidized to highly fluorescent 2′7′-dichlorofluorescein by mostly H_2O_2 and related superoxide species. After 1 h of incubation, DCF fluorescence, quantitatively related to the ROS production of haemocytes, was measured at 500–530 nm by flow cytometry. DCF fluorescence was expressed in arbitrary units (A.U.).

2.7. Phenoloxidase (PO) activity

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from l-dihydroxyphenylalanine (L-DOPA; Sigma-Aldrich, USA) [61]. The assay was conducted in 96-well microplate, 100 μL fresh hemolymph was mixed with 50 μL L-DOPA (0.5 mg/mL in PBS) and incubated for 30 min at 26 °C, followed by measurement of absorbance at 490 nm. The phenoloxidase activity was expressed as the OD 490 value representing dopachrome formation.

2.8. ESTs of hsp70, ferritin, csdp, rapid amplification of cDNA ends (RACE) and cDNA sequence analysis

Total RNA was extracted from *H. discus hannai* and *H. gigantea* tissues using the TRIpure reagent (Invitrogen, USA) in an RNase-free environment. RNA was extracted from 50 mg of the gill, mantle, muscle, hemolymph and visceral mass separately, then equally mixed for RACE use. Paired degenerate primers for *hsp70*, *ferritin*, and *csdp* (Table 1, designated as deg*hsp70*, deg*ferritin*, and deg*csdp*) were designed to obtain ESTs of these genes. Based on the ESTs obtained, gene-specific primers (Table 1, named as gsp*hsp70*, gsp*ferritin*, and gsp*csdp*) were designed to amplify their cDNA ends, then RACE was conducted using a SMART RACE cDNA synthesis Kit (Clontech, USA), according to the manufacturer's recommendations.

Homologous analysis of sequence was conducted using online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), open reading frames analysis was conducted using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi), amino acid sequence analysis was conducted using Pfam (http://pfam.janelia.org), multiple sequence alignment was completed using CLUSTALX (http://www.clustal.org/clustal2/), and phylogenetic trees were constructed using the neighbor-joining method as provided in MEGA (http://www.megasoftware.net).

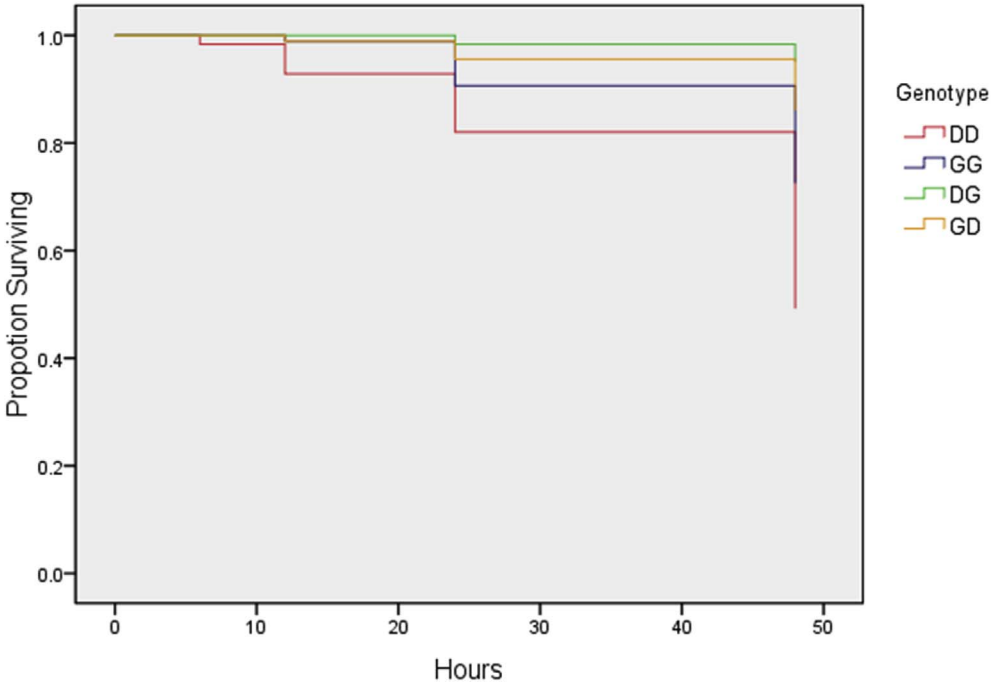

Fig. 1. Kaplan–Meier cumulative survival curves of the four abalone genotypes during 48 h bacterial exposure (DD: *H. discus hannai*, GG: *H. gigantea*, DG: *H. discus hannai*♀ × *H. gigantea*♂, GD: *H. gigantea*♀ × *H. discus hannai*♂).

Table 2
Overall comparisons of survival curves of the four genotypes.

	Chi-square	df	P
Log Rank (Mantel-cox)	32.200	3	< 0.001
Breslow (Generalized Wilcoxon)	29.220	3	< 0.001
Tarone-Ware	31.804	3	< 0.001

dF = degrees of freedom, P = probability of significance; α = 0.05.

2.9. Real-time PCR analysis of three genes in the four genotypes of abalones after infection

The EST sequences obtained were almost the same in the four genotypes, based on the region where the four genotypes shared 100% same sequence (no SNPs), paired real-time PCR primers (Table 1, named as rt*hsp70*, rt*ferritin*, and rt*csdp*) were designed using Beacon Designer (http://www.premierbiosoft.com/molecular_beacons); β-actin was used as an internal control as described previously [62]. The real-time PCR experiment was conducted in a 7500 fast qPCR system (ABI, USA). The reaction system contained 1 mL of cDNA (10-times diluted), 1 mL of each primer (10 pmol/L) and 10 mL of 2 × DyNAmo ColorFlash Master Mix (Thermo, USA). The cycling parameters used were: 95 °C for 7 min, 35 cycles at 95 °C for 20 s, and 60 °C for 1 min. The fluorescent signal intensities were recorded at the end of each cycle. Melting curve analysis was performed from 60 °C to 95 °C with continuous fluorescence reading every 0.5 °C to ensure the uniformity of the amplification. The relative mRNA level of target genes was calculated based on the Ct values of this gene and β-actin normalized to that of the cDNA standard.

2.10. Statistical analysis

Results were expressed as the mean ± SD. Analysis of all data was performed using SPSS 16.0 (http://www.ibm.com/analytics/us/en/technology/spss). For the bacteria-survival experiment, Kaplan-Meier survival analysis with statistical estimators (log-rank, breslow and tarone-ware) was conducted to compare the statistical differences among survival rates of different genotypes. Two-way ANOVA was used to analyze the general impacts of infection length (0 h, 6 h, 12 h, 24 h, and 48 h), genotype (DD, GG, DG, and GD) and their interaction on immune parameter levels and immune gene transcription. Then, within a certain time point (0 h, 6 h, 12 h, 24 h or 48 h), or within a certain genotype (DD, GG, DG or GD), one-way ANOVA followed by least significant difference (LSD) analysis was used to compare the immune index level or gene mRNA level differences among different genotypes or among different exposure lengths.

3. Results

cDNA sequences of *hsp70*, *ferritin*, *csdp*, and their sequence analysis were provided in the supplement information.

3.1. Kaplan–Meier survival curves of abalones after bacterial exposure

During the 48 h after exposure, no dead abalone was found in the control group, while significant deaths were observed in bacteria challenged abalones. Kaplan-Meier test showed that there were significant differences in survival rates between different genotypes (Fig. 1, Table 2): DD's survival rate was significantly lower than the other three genotypes, GG's survival rate was significantly lower than DG but not GD, while DG and GD were not significantly different in survival rate. Specifically, at 6 h, no death occurred except in DD genotype. As exposure time increased, all the four genotypes started to die at different degrees. At the end of the exposure experiment (48 h), DD showed the lowest survival rate, 60%, while survival rates of GG, DG and GD were respectively 80%, 96.67% and 90%.

Table 3
Summary of two-way ANOVA results: Effects of exposure length, genotype and their interaction on abalone (A) immune parameters and (B) genes expression.

(A)

Source of variation	Dependent Variable	df	MS	F	P
Exposure length	THC	3	332832.863	51.082	< 0.001
	Phagocytic ability	3	0.071	76.841	< 0.001
	ROS	3	27838.780	19.226	< 0.001
	PO	3	0.009	151.612	< 0.001
Genotype	THC	3	947898.768	145.481	0.008
	Phagocytic ability	3	0.004	4.264	< 0.001
	ROS	3	10147.964	7.008	< 0.001
	PO	3	0.010	177.382	< 0.001
Genotype × Length	THC	9	677684.020	104.009	< 0.001
	Phagocytic ability	9	0.016	17.122	< 0.001
	ROS	9	7012.309	4.843	< 0.001
	PO	9	0.001	10.694	< 0.001
Error	THC	62	6515.610		
	Phagocytic ability	62	0.001		
	ROS	62	1448.012		
	PO	62	5.852E-5		
Total	THC	78			
	Phagocytic ability	78			
	ROS	78			
	PO	78			

(B)

Source of variation	Dependent Variable	df	MS	F	P
Exposure length	Haemocytes-*hsp70*	4	12.806	32.428	< 0.001
	Haemocytes-*ferritin*	4	6.814	22.861	< 0.001
	Haemocytes-*csdp*	4	0.597	9.018	< 0.001
	Gill-*hsp70*	4	10.573	22.781	< 0.001
	Gill-*ferritin*	4	45.136	15.427	< 0.001
	Gill-*csdp*	4	50.137	42.620	< 0.001
Genotype	Haemocytes-*hsp70*	3	0.943	2.387	0.087
	Haemocytes-*ferritin*	3	0.201	0.673	0.575
	Haemocytes-*csdp*	3	0.826	12.476	< 0.001
	Gill-*hsp70*	3	5.953	12.827	< 0.001
	Gill-*ferritin*	3	6.157	2.104	0.119
	Gill-*csdp*	3	4.363	3.709	0.021
Genotype × Length	Haemocytes-*hsp70*	12	0.833	2.111	0.046
	Haemocytes-*ferritin*	12	0.562	1.884	0.076
	Haemocytes-*csdp*	12	0.398	6.008	< 0.001
	Gill-*hsp70*	12	0.909	1.959	0.064
	Gill-*ferritin*	12	3.824	1.307	0.262
	Gill-*csdp*	12	3.731	3.171	0.004
Error	Haemocytes-*hsp70*	32	0.395		
	Haemocytes-*ferritin*	32	0.298		
	Haemocytes-*csdp*	32	0.066		
	Gill-*hsp70*	32	0.464		
	Gill-*ferritin*	32	2.926		
	Gill-*csdp*	32	1.176		
Total	Haemocytes-*hsp70*	52			
	Haemocytes-*ferritin*	52			
	Haemocytes-*csdp*	52			
	Gill-*hsp70*	52			
	Gill-*ferritin*	52			
	Gill-*csdp*	52			

dF = degrees of freedom, MS = mean square, F = MS factor/MS error and P = probability of significance; α = 0.05.

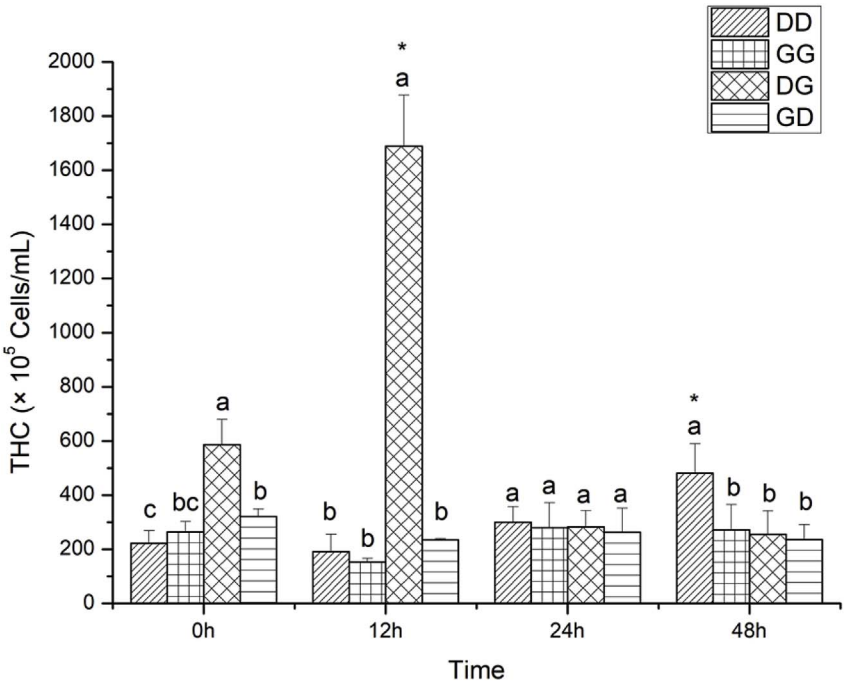

Fig. 2. Total haemocyte counts of the four genotypes during 48 h bacterial exposure. Different fill pattern represents different genotype (DD: *H. discus hannai*, GG: *H. gigantea*, DG: *H. discus hannai*♀ × *H. gigantea*♂, GD: *H. gigantea*♀ × *H. discus hannai*♂). According to LSD post-hoc test results: (1) Letters (a, b and c) are used to indicate significant differences ($p < 0.05$) in THC between different genotypes (DD, GG, DG and GD) within a same time point (0, 12, 24 or 48 h); (2) If a certain genotype's THC significantly changed ($p < 0.05$) along the exposure time, asterisk (*) is used to mark the time point when its THC reached the highest level (LSD comparison between its THC at this time point and its THC at any other time point shows significant difference, $p < 0.05$).

3.2. Cellular and humoral parameters

No abalone was dead during the sub-lethal bacterial exposure. While two-way ANOVA analysis showed significant differences in the immune parameters levels between different genotypes or exposure lengths (Table 3, Figs. 2–5).

3.2.1. THC

The THC variation patterns in the four genotypes are shown in Fig. 2. At 0 h, THC of DG was significantly higher than that of the other three genotypes, while DD had the lowest THC. At 12 h after exposure, DG got a dramatic increase in THC, compared to the other three genotypes. At 24 h and 48 h, DG's THC decreased significantly, while DD's THC increased steadily and peaked at 48 h. THC of GG and GD were relatively steady during the whole exposure period.

3.2.2. Phagocytic ability

Haemocytes' 2-hours phagocytosis rate of fluorescent beads were used to represent haemocytes' phagocytic ability. Changes of phagocytosis ability in the four genotypes during the 48 h are shown in Fig. 3. Circulating haemocytes' phagocytic ability of all the four genotypes started to increase as the exposure started. In DD and GG genotypes, haemocytes' phagocytic ability were highest at 12 h and then started to decrease in the next 36 h, while in DG and GD genotypes, haemocytes' phagocytic ability kept increasing for 24 h, and then fell back to the initial value at 48 h.

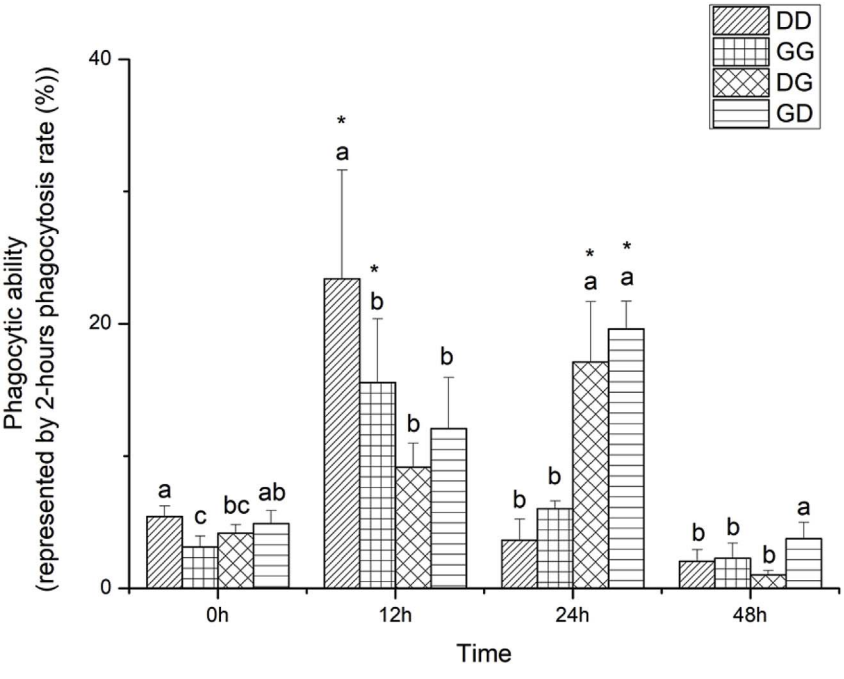

Fig. 3. Haemocytes' phagocytosis abilities in the four genotypes during 48 h bacterial exposure. Different fill pattern represents different genotype (DD: *H. discus hannai*, GG: *H. gigantea*, DG: *H. discus hannai*♀ × *H. gigantea*♂, GD: *H. gigantea*♀ × *H. discus hannai*♂). According to LSD post-hoc test results: (1) Letters (a, b and c) are used to indicate significant differences ($p < 0.05$) in phagocytic ability between different genotypes (DD, GG, DG and GD) within a same time point (0, 12, 24 or 48 h); (2) If a certain genotype's phagocytic ability significantly changed ($p < 0.05$) along the exposure time, asterisk (*) is used to mark the time point when its phagocytic ability reached the highest value (LSD comparison between its phagocytic activity at this time point and its phagocytic activity at any other time point shows significant difference, $p < 0.05$).

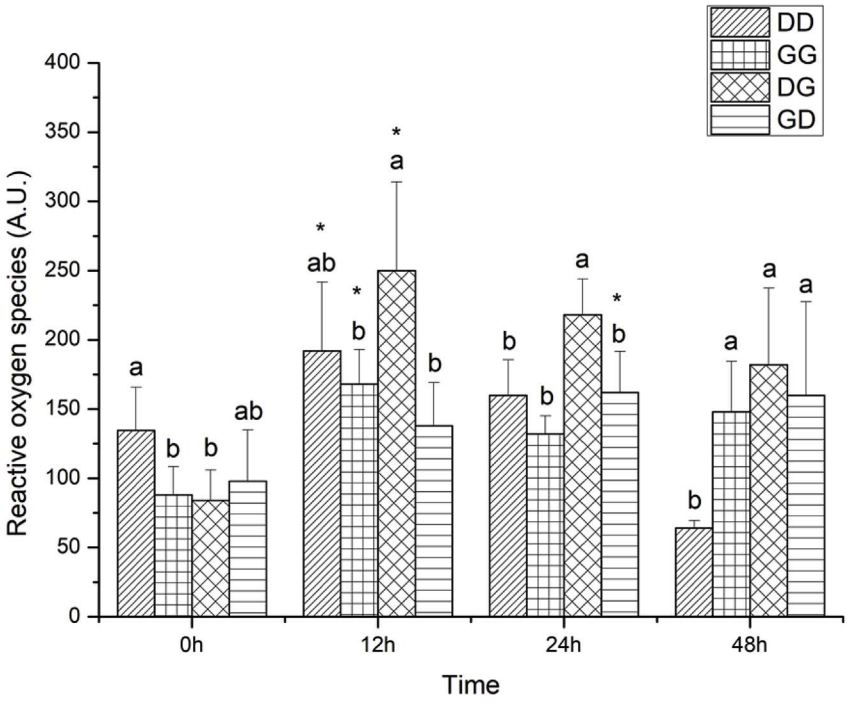

Fig. 4. ROS level of the four genotypes during 48 h bacterial exposure. Different fill pattern represents different genotype (DD: *H. discus hannai*, GG: *H. gigantea*, DG: *H. discus hannai*♀ × *H. gigantea*♂, GD: *H. gigantea*♀ × *H. discus hannai*♂). According to LSD post-hoc test results, letters (a, b and c) are used to indicate significant differences ($p < 0.05$) in ROS level between different genotypes (DD, GG, DG and GD) within a same time point (0, 12, 24 or 48 h) (2) If a certain genotype's ROS level significantly changed ($p < 0.05$) along the exposure time, asterisk (*) is used to mark the time point when its ROS got was the highest level (LSD comparison between its ROS level at this time point and its ROS level at any other time point shows significant difference, $p < 0.05$).

3.2.3. ROS level

ROS level changes in the four genotypes during the 48 h are shown in Fig. 4. Rise in ROS level was observed in all genotypes. DD, GG and DG's ROS levels peaked at 12 h, while GD's ROS level peaked at 24 h. At 48 h, DD had lower ROS level than the other three genotypes.

3.2.4. PO activity

Changes in the four genotypes' PO activity during the 48 h are shown in Fig. 5. Rise in PO activity was observed in GG and DG, but not in DD and GD genotypes. Between the four genotypes, DD's PO activity was always lower than that of the other three genotypes at all time points. At 48 h, DD got the lowest PO level, while the other three genotypes' PO level ranked as: DG > GG > GD.

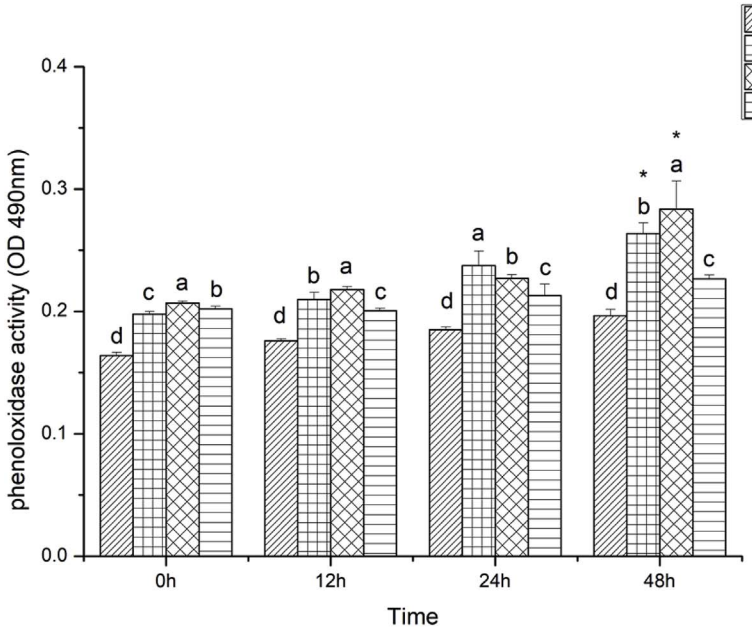

Fig. 5. PO activity of the four genotypes during 48 h bacterial exposure. Different fill pattern represents different genotype (DD: *H. discus hannai*, GG: *H. gigantea*, DG: *H. discus hannai*♀ × *H. gigantea*♂, GD: *H. gigantea*♀ × *H. discus hannai*♂). According to LSD post-hoc test results: (1) Letters (a, b and c) are used to indicate significant differences ($p < 0.05$) in PO activity between different genotypes (DD, GG, DG and GD) within a same time point (0, 12, 24 or 48 h); (2) If a certain genotype's PO activity significantly changed ($p < 0.05$) along the exposure time, asterisk (*) is used to mark the time point when its PO activity reached the highest level (LSD comparison between its PO activity at this time point and its PO activity at any other time point shows significant difference, $p < 0.05$).

3.3. Transcription patterns of hsp70, ferritin and csdp in circulating haemocytes and gills of the four genotypes after bacterial infection

3.3.1. hsp70

Transcriptions of *hsp70* in circulating haemocytes in the four genotypes after infection are shown in Fig. 6A. Two-way ANOVA showed significant effects of exposure length, genotype and their interaction on *hsp70* mRNA levels (Table 3). Post challenge transcription patterns of *hsp70* were similar among the evaluated genotypes: the *hsp70* level reached the highest level at 12 h, followed by a decrease to a lower level. In total, DD had the lowest overall *hsp70* mRNA levels, while the other three genotypes showed no significant differences in overall *hsp70* mRNA levels. In gills (Fig. 6B), two-way ANOVA also showed significant effects of exposure length, genotype and their interaction on *hsp70* mRNA levels (Table 2). Transcription patterns of *hsp70* in the

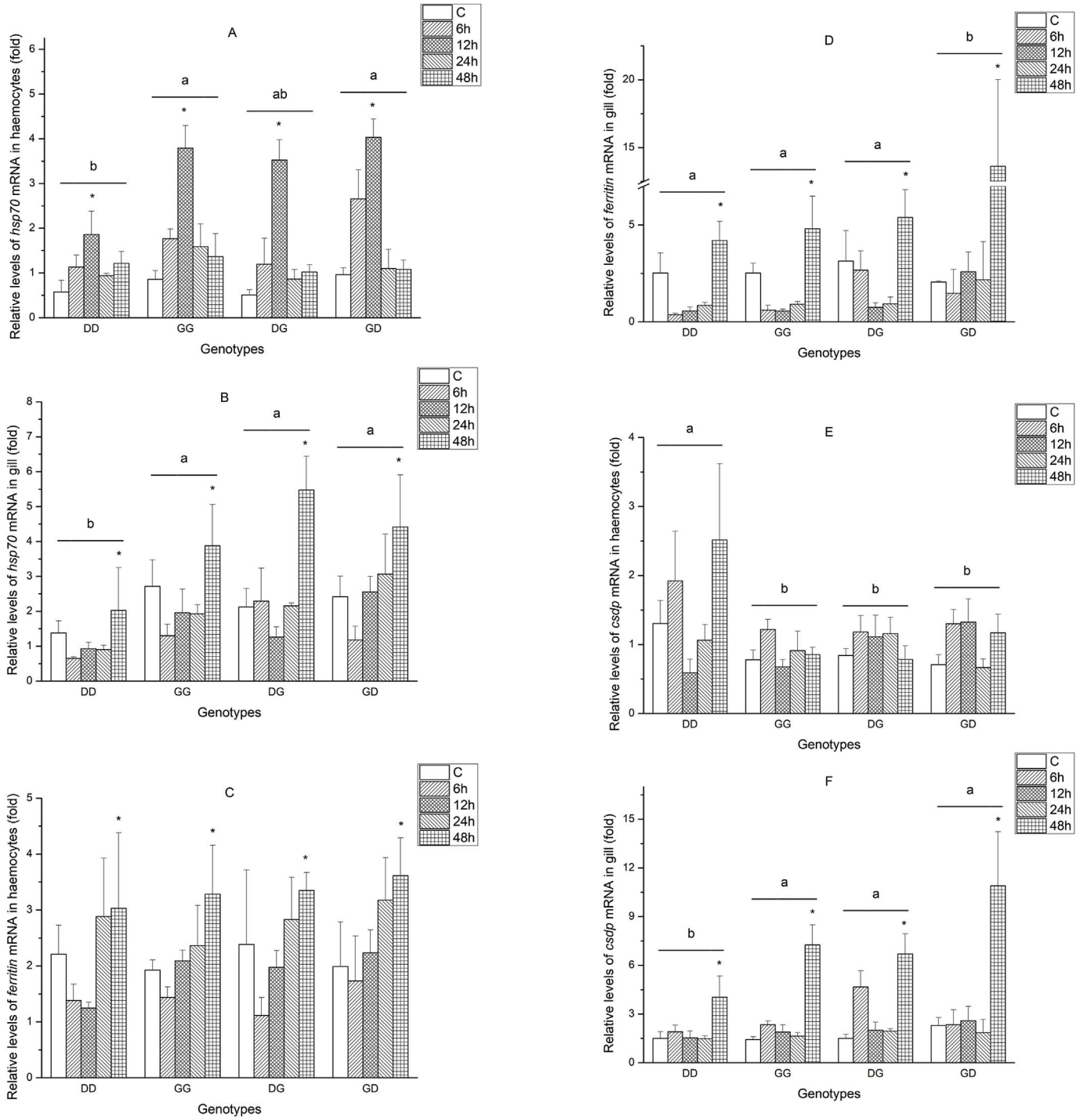

Fig. 6. Relative mRNA levels of *hsp70*, *ferritin* and *csdp* in haemocytes and gills of the four genotypes during the 48 h after bacterial infection. Different fill pattern represents different time point post infection (DD: *H. discus hannai*, GG: *H. gigantea*, DG: *H. discus hannai*♀ × *H. gigantea*♂, GD: *H. gigantea*♀ × *H. discus hannai*♂). According to LSD post-hoc test results: (1) Letters (a, b and c) are used to indicate significant differences ($p < 0.05$) in overall gene transcription level (during the exposure) among different genotypes (DD, GG, DG and GD); (2) If a certain genotype's gene transcription significantly changed ($p < 0.05$) along the exposure time, asterisk (*) is used to mark the time point when its gene transcription reached the highest level (LSD comparison between its gene transcription level at this time point and its gene transcription level at any other time point shows significant difference, $p < 0.05$).

evaluated genotypes were similar: the *hsp70* level reached the highest level at 48 h. Among the four genotypes, DD showed significantly lower overall *hsp70* mRNA levels post infection than the other three genotypes, while GG, DG, GD were not significantly different in overall *hsp70* mRNA levels.

3.3.2. *ferritin*

Transcriptions of *ferritin* in circulating haemocytes in the four genotypes after infection are shown in Fig. 6C. Two-way ANOVA showed significant effects of exposure length and genotype × exposure length interaction on *ferritin* mRNA levels (Table 3). At 48 h, ferritin mRNA levels reached the highest in all evaluated genotypes. In gills (Fig. 6D), two-way ANOVA showed significant effects of exposure length, genotype and their interaction on *ferritin* mRNA levels (Table 3). Similarly, *ferritin* mRNA reached the highest levels in the four evaluated genotypes at 48 h. Among the four genotypes, overall *ferritin* mRNA

levels in GD significantly exceeded that of the other three genotypes, while GG, DG, GD were not significantly different in overall *ferritin* mRNA levels.

3.3.3. csdp

Transcriptions of *csdp* in circulating haemocytes of the four genotypes after infection are presented in Fig. 6E. Two-way ANOVA showed significant effects of exposure length, genotype and genotype × exposure length interaction on *csdp* mRNA levels (Table 3). DD showed higher overall *csdp* mRNA levels than GG and the hybrids. In gills (Fig. 6F), two-way ANOVA also showed significant effects of exposure length, genotype and their interaction on *csdp* mRNA levels (Table 3). *csdp* mRNA levels of all the evaluated genotypes significantly rose at 48 h. Among the four genotypes, DD showed significantly lower overall *csdp* mRNA levels post infection than the other three genotypes, whereas GG, DG, and GD showed no significant differences in overall *csdp* mRNA levels.

4. Discussion

Disease resistance is one of the most important traits of aquaculture species as it is linked directly to the final yield [63]. This trait had been proven to be improved through hybridization in many aquatic species [63–65]. For example, compared to their parents, the hybrids of *H. gigantea* and *H. discus hannai* were more superior in survival rate (in natural or artificial cultural habitat), or improved their resistance to a certain disease. A significantly higher antibacterial activity was also found in the hybrid of greenlip abalone *H. laevigate* and blacklip abalone *H. rubra* [66], however, the hybrid of *H. rufescens* and *H. discus hannai* did not show heterosis for non-susceptibility to the intracellular rickettsial-like bacterium “Candidatus Xenohaliotis californiensis” (WS-RLOs) that causes Withering Syndrome [67]. Hence, hybridization between abalone species does not always bring disease resistance heterosis into the hybrids, it may depend on the genetic characteristics of the parental species or the cross combinations.

In our previous investigation, the reciprocal hybrids of *H. discus hannai* and *H. gigantea* had a significant improvement in farm-growing survival rate, compared to their parental species [20]. Our laboratory test also found the hybrids to be more resistant to pathogenic bacterial (*V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*) injection than the parental species [52]. In this study, we changed the method of bacterial challenge from injection to immersion, which correspond more with the natural way that abalone gets infected. The result still supported the heterosis, DG, GD got 26.67% and 20% improvement in survival rate after bacterial challenge, compared to the mid-parent value (70%). This suggests that the hybrids are more efficient in immune system responses, which may lead to a higher ability in bacteria clearance and homeostasis maintenance. To further dissect the mechanisms under this survival heterosis, we analyzed the changes of several immune parameters and molecular gene transcriptions in the four genotypes post infection.

4.1. Non-specific immune parameters

Haemocytes are the central cells in invertebrate innate immune system, amount of haemocytes is regarded as an important indicator of invertebrates' physiological immune status [25,68–76]. Studies on immune response differences between different genotypes or stocks, also gave us some information on the relationship between organisms' genetics and their haemocyte responses [72,77].

In our study, we found a close relationship of THC variation with bacteria invasion, especially a notable elevation of THC in the hybrid: *H. discus hannai* ♀ × *H. gigantean* ♂ (DG). Elevation of THC upon biotic stress was observed in invertebrates challenged with LPS [71,78], virus [73], protozoa [79], this response could be caused by pathogens-induced cellular proliferation, or rapid cellular differentiation in response to antigenic challenge [71]. As the main defense cells in invertebrate innate immune system, the increased circulating haemocytes will then participate in defense against pathogenic microbes [80]. In our study, we found DG to be different in THC from the other three genotypes, it had a higher basal THC value, and its THC was more sensitive to bacterial challenge, indicating that DG may be more active in haemocyte-mediated immune response, this was supported by the survival rate data (where DG got the highest survival rate during 48 h of pathogenetic bacterial exposure). To the best of our knowledge, no previous study has been done on the THC variations upon biotic challenge between parental species and hybrids. However, THC variations between varied species or genotypes were analyzed, between two shrimp species *Fenneropenaeus chinensis* and *Marsupenaeus japonicas* [72], which were susceptible and resistant to the white spot syndrome virus separately. *M. japonicas* was found to have more haemocytes than *F. chinensis* after virus injection, and correspondingly, survived a longer time after the injection. Similarly, between two hard clam stocks, the QPX (parasite) resistant stock NY was higher in basal THC than the susceptible stock FL [76]. Hence, in invertebrates, concentration of circulating haemocytes could be a positive indicator of organisms' resistant ability to pathogens.

In this study, induction of phagocytic ability and reactive oxygen species production in abalone was observed after bacterial challenge, this was consistent with studies in other invertebrates [75,77,81,85]. Phagocytosis, together with the process of reactive oxygen species (superoxide anion, hydrogen peroxide, hydroxide ions and singlet oxygen) production, played significant roles in invertebrate cellular immune system [25]. They determine the organism's efficiency in eliminating foreign microorganisms. In our experiment, we found different changing patterns of phagocytic activity and ROS level among the four genotypes, phagocytic activity of the two hybrids responded slower than both parental species. Similarly, when the two species of shrimps *F. chinensis* and *M. japonicas* were challenged with virus, their phagocytic activity also showed a different changing pattern, the less resistant species *F. chinensis* responded earlier (peaked at 42 h) in phagocytosis process than *M. japonicas* (peaked at 78 h), hence, we could infer that the delayed phagocytic activity in the two hybrids may contribute to a more thorough clearance of the invading pathogens, but this hypothesis needs more evidences to support. ROS production pattern of *H. discus hannai* (DD) also showed difference with the other three genotypes, resulting in higher overall ROS levels in *H. gigantea* (GG) and the two hybrids than DD, as ROS level was always positively related to anti-pathogen ability in invertebrates [72,73,76], GG and the two hybrids could be stronger in their anti-bacteria abilities. To sum up, upon bacterial challenge, slower response of phagocytic activity, and higher ROS levels (48 h) in the two hybrids, could contributed to their higher bacterial clearance rates, resulting in higher survival rates.

Phenoloxidase, as the terminal enzyme in proPO system, acts in invertebrate immune system by stimulating several cellular responses including phagocytosis, nodule formation and respiratory burst [72]. Phenoloxidase activity could be activated by various pathogens, regarded as an indicator of organism infectious state [72,74,76,81,82]. In our study, we observed a lower PO level in DD compared to the other three genotypes (Fig. 4). PO activity comparison was also conducted in a shrimp hybrid system [83], in that study, two parental species from different geographic position showed distinct PO level, while one hybrid had a PO level close to mid-parent value, the other hybrid's PO level exceed the mid-parent value, suggesting that genetic variations could reflect in PO level differences. However, that study did not investigate correlation between PO level and the shrimps' susceptibility to biotic stress. From our result, we could infer that the two hybrids and GG were more efficient in PO-related immune responses compared to DD.

From our results, we could conclude that when challenged with bacterial stress, the hybrid DG could be more active in producing or invoking circulating haemocytes to organize immune reactions, and the

two hybrids could be more efficient in clearance of bacteria with slower phagocytic responses and higher ROS levels, and more active in proPO system mediated immune responses.

4.2. Immune genes transcriptions

We observed a quick response of *hsp70* after bacterial challenge in circulating haemocytes and gills in the four abalone genotypes, which agrees with the phenomena observed in other invertebrates. The oyster *Crassostrea gigas* challenged with vibrios and LPS elevated its *hsp70* mRNA levels [84], similar increasing of *hsp70* transcription was also observed in the hard clam *Meretrix meretrix* [85]. Besides, we observed a faster transcription of *hsp70* in heamocytes than in gills, this could be explained by the time that these circulating haemocytes need to infiltrate the gill, as tissue-infiltrated heamocytes are known to be another important immunologic function in invertebrates [86,87]. Between the four genotypes, the overall *hsp70* mRNA levels were always higher in GG and the two hybrids than in DD. As higher mRNA level of *hsp70* indicate higher ability to initiate and regulate immune functions, combined with the survival rate experiment, we could infer that the GG and the two hybrids are more active in immune responses compared to DD, showing heterosis.

The vital role of *ferritin* in invertebrate immune systems have been highlighted in several studies, it is regarded as an acute phase reaction protein that participates in organisms' immune responses [88,89]. A similar *ferritin* transcription pattern was observed in the four genotypes, the increase of gill *ferritin* at 48 h could be the result of iron competition between host and bacteria, as iron is a necessary element for the basic survival of most bacterial pathogens. Therefore, contending for iron has been regarded as the hosts' strategy to defend against invading pathogens [90,91]. No much ferritin transcription difference existed between the parents and hybrids except for a notable higher ferritin mRNA level in the gill of *H. gigantea*♀ × *H. discus hannai*♂(GD), indicating that GD may be more efficient in *ferritin*-related immune responses than the other genotypes.

The relationship between *csdp*/YB-1 proteins and organism's immune functions are gaining increasing attentions in recent years. YB-1 protein could suppress transcription of the important immune gene MHC, which is involved in the γ-interferon pathway [92]. In turbot *Scophthalmus maximus*, *csdp* was found to change its mRNA level after virus and bacterial challenges [54]. As a RNA chaperone, *csdp* acts in regulating the transcription and translation of various genes [55], hence, we could infer that the involvement of *csdp* in an aquatic organism's immune responses could be the result of its gene-regulating function. In this study, *csdp* mRNA levels elevated in the gill of abalones challenged with vibrios, and the mRNA level differences between the four genotypes could be correlated with the differences in their resistant ability: DD got the lowest *csdp* mRNA level, indicating that the other three genotypes could secrete more *csdp* during infection to regulate the immune-related genes, further to obtain a greater ability to combat bacteria. However, in haemocytes, no large variations in *csdp* mRNA level were observed, hence, we could infer that *csdp* is not a direct immune related gene in mollusks, it acts more like a regulating molecule in mollusks.

5. Conclusions

In this study, we analyzed and compared the cellular responses and molecular responses of *H. discus hannai*, *H. gigantea* and their hybrids to bacterial challenge, hoping to get insights into the physiological and molecular basis of the bacteria resistance heterosis observed in this hybrid system. Different response patterns between parents and hybrids were found. The hybrids were more vigorous in expression of immune initiating gene *hsp70,* and more active in immune-regulating enzyme phenoloxidase. Enhanced transcription or expression of these molecules could then influence the downstream physiological cellular responses in the hybrids, resulting in hybrids' higher survival rate after bacterial stress. However, the molecular basis of heterosis is complex, heterosis is considered to originate from genetic code difference between parental and hybrid genotypes [6,7], next-generation sequencings of genome, transcriptome and epigenetics are powerful techniques that would facilitate our comprehensive understanding of heterosis phenomena and should be applied into the future work of abalone heterosis study.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (U1605213), National Natural Science Foundation of China (No. 41106120), National Natural Science Foundation of China (No. 31472277), The earmarked fund for Modern Agro-industry Technology Research System (No. CARS-49), The Major Scientific and Technological Project of Fujian (No. 2016NZ0001-4; No. 2016N5012), Fundamental Research Funds for the Central Universities (No. 20720150077), National Marine Economic Development Demonstration Project in Xiamen (No. 16CZB023SF12), EUR-cooperative Foundation of Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources (No. FJMBIO1504).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.11.009.

References

[1] H.R. Gordon, P.A. Cook, World abalone fisheries and aquaculture update: supply and market dynamics, J. Shellfish Res. 23 (2004) 935–940.
[2] H.R. Gordon, P.A. Cook, World abalone supply, markets, and pricing: 2011 update, J. Shellfish Res. 32 (2013) 5–7.
[3] Food and Agriculture Organization of the United Nations (FAO), Rome, 2015. FishStatJ. www.fao.org/fishery/statistics/en.
[4] X. Guo, Use and exchange of genetic resources in molluscan aquaculture, Rev. Aquacult. 1 (2009) 251–259.
[5] Z.Q. Nie, S.P. Wang, The status of abalone culture in China, J. Shellfish Res. 23 (2004) 941–945.
[6] Z.J. Chen, Genomic and epigenetic insights into the molecular bases of heterosis, Nat. Rev. Genet. 14 (2013) 471–482.
[7] S.A. Goff, A unifying theory for general multigenic heterosis: energy efficiency, protein metabolism, and implications for molecular breeding, New Phytol. 189 (2011) 923–937.
[8] P.C. McKeown, A. Fort, D. Duszynska, R. Sulpice, C. Spillane, Emerging molecular mechanisms for biotechnological harnessing of heterosis in crops, Trends Biotechnol. 31 (2013) 549–551.
[9] R.A. Veitia, D. Vaiman, Exploring the mechanistic bases of heterosis from the perspective of macromolecular complexes, Faseb J. 25 (2011) 476–482.
[10] V.K. Baranwal, V. Mikkilineni, U.B. Zehr, A.K. Tyagi, S. Kapoor, Heterosis: emerging ideas about hybrid vigour, J. Exp. Bot. 63 (2012) 6309–6314.
[11] D.J. McClary, M.A. Sewell, Hybridization in the sea: gametic and developmental constraints on fertilization in sympatric species of Pseudechinus (Echinodermata: Echinoidea), J. Exp. Mar. Biol. Ecol. 284 (2003) 51–70.
[12] F. Lafarga De La Cruz, C. Gallardo-Escárate, Intraspecies and interspecies hybrids in Haliotis: natural and experimental evidence and its impact on abalone aquaculture, Rev. Aquacult. 3 (2011) 74–99.
[13] W. You, C. Ke, X. Luo, D. Wang, Growth and survival of three small abalone Haliotis diversicolor populations and their reciprocal crosses, Aquac. Res. 40 (2009) 1474–1480.
[14] W. You, Preliminary studies on hybridization between Japanese stock and Taiwan stock of Haliotis diversicolor, J. Xiamen Univ. 44 (2005) 701 [In Chinese with an English abstract].
[15] G. Zhang, H. Que, X. Liu, H. Xu, Abalone mariculture in China, J. Shellfish Res. 23 (2004) 947–950.
[16] X. Luo, C. Ke, W. You, D. Wang, F. Chen, Molecular identification of interspecific hybrids between *Haliotis discus hannai* Ino and *Haliotis gigantea* Gmelin using amplified fragment-length polymorphism and microsatellite markers, Aquac. Res. 41 (2010) 1827–1834.
[17] W. You, Q. Guo, F. Fan, P. Ren, X. Luo, C. Ke, Experimental hybridization and genetic identification of Pacific abalone *Haliotis discus hannai* and green abalone *H. fulgens*, Aquaculture 448 (2015) 243–249.
[18] N.G. Elliott, Genetic improvement programmes in abalone: what is the future? Aquac. Res. 31 (2000) 51–59.
[19] X.S. Gao, Q. Wang, R.B. Wang, Abalone, Liaoning Science and Technology Press,

2000 [In Chinese].
[20] X. Luo, Study on Genetic Basis of Hybridization between Haliotis sieboldii Reeve and Haliotis discus hannai Ino [D], Xiamen University, 2009 [In Chinese with an English abstract].
[21] S. Liang, X. Luo, W. You, L. Luo, C. Ke, The role of hybridization in improving the immune response and thermal tolerance of abalone, Fish Shellfish Immun. 39 (2014) 69–77.
[22] X. Luo, C. Ke, W. You, D. Wang, F. Chen, Aflp analysis of populations of *Haliotis discus hannai, Haliotis gigantea*, and their hybrids, J. Shellfish Res. 29 (2010) 731–734.
[23] K.D. Lafferty, E.E. Hofmann, Marine disease impacts, diagnosis, forecasting, management and policy, Phil. Trans. R. Soc. B 371 (2016).
[24] J. Ghosh, C.M. Lun, A.J. Majeske, S. Sacchi, C.S. Schrankel, L.C.C.G. Smith, Invertebrate immune diversity, Dev. Comp. Immunol. 35 (2011).
[25] C. Hooper, R. Day, R. Slocombe, J. Handlinger, K. Benkendorff, Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models, Fish Shellfish Immun. 22 (2007) 363–379.
[26] C.M. Adema, W. Vanderknaap, T. Sminia, Molluscan haemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates, Rev. Aquat. Sci. 4 (1991) 201–223.
[27] W. Vanderknaap, C.M. Adema, T. Sminia, Invertebrate blood cells: morphological and functional aspects of the haemocytes in the pond snail *Lymnaea-stagnalis*, Comp. Haematol. Int. 3 (1993) 20–26.
[28] T. Sminia, L. Barendsen, A comparative morphological and enzyme histochemical study on blood cells of the fresh water snails *Lymnaea stagnalis, Biomphalaria glabrata*, and *Bulinus truncatus*, J. Morphol. 165 (1980) 31–39.
[29] S.C. Birrer, T.B. Reusch, O. Roth, Salinity change impairs pipefish immune defence, Fish Shellfish Immunol. 33 (2012) 1238–1248.
[30] C. Hooper, R. Day, R. Slocombe, K. Benkendorff, J. Handlinger, Effect of movement stress on immune function in farmed Australian abalone (hybrid *Haliotis laevigata* and *Haliotis rubra*), Aquaculture 315 (2011) 348–354.
[31] Y. Wang, M. Hu, P.K. Shin, S.G. Cheung, Immune responses to combined effect of hypoxia and high temperature in the green-lipped mussel *Perna viridis*, Mar. Pollut. Bull. 63 (2011) 201–208.
[32] B. Allam, K.A. Ashton-Alcox, S.E. Ford, Haemocyte parameters associated with resistance to brown ring disease in *Ruditapes spp*. clams, Dev. Comp. Immunol. 25 (2001) 365–375.
[33] E.S. Loker, C.M. Adema, S. Zhang, T.B. Kepler, Invertebrate immune systems: not homogeneous, not simple, not well understood, Immunol. Rev. 198 (2004).
[34] K.B. Brokordt, R.C. Gonzalez, W.J. Farias, F.M. Winkler, Potential response to selection of *hsp70* as a component of innate immunity in the abalone *Haliotis rufescens*, PLoS One 10 (2015).
[35] P. Cheng, X. Liu, G. Zhang, J. He, Cloning and expression analysis of a *hsp70* gene from Pacific abalone (*Haliotis discus hannai*), Fish Shellfish Immun. 22 (2007) 77–87.
[36] X. Yue, B. Liu, L. Sun, B. Tang, Cloning and characterization of a *hsp70* gene from Asiatic hard clam *Meretrix meretrix* which is involved in the immune response against bacterial infection, Fish Shellfish Immun. 30 (2011) 791–799.
[37] F. Zhang, K. Jiang, M. Sun, D. Zhang, L. Ma, Multiplex immune-related genes expression analysis response to bacterial challenge in mud crab, *Scylla paramamosain*, Fish Shellfish Immun. 34 (2013) 712–716.
[38] P.A.P.Y. Valentim-Neto, J.R. Moser, A.P.M. Fraga, M.R.F. Marques, *Hsp70* expression in shrimp *Litopenaeus vannamei* in response to IHHNV and WSSV infection, Virus Dis. 25 (2014) 437–440.
[39] H. Liu, J. He, C. Chi, J. Shao, Differential *hsp70* expression in *Mytilus coruscus* under various stressors, Gene 543 (2014) 166–173.
[40] T. Brown, D. Bourne, M. Rodriguez-Lanetty, Transcriptional activation of c3 and *hsp70* as part of the immune response of *Acropora millepora* to bacterial challenges, PLoS One 8 (2013).
[41] L. Zheng, Z. Liu, B. Wu, Y. Dong, L. Zhou, J. Tian, et al., *ferritin* has an important immune function in the ark shell *Scapharca broughtonii*, Dev. Comp. Immunol. 59 (2016) 15–24.
[42] R. Qiu, Y. Kan, D. Li, *ferritin* from the Pacific abalone *Haliotis discus hannai*: analysis of cDNA sequence, expression, and activity, Fish Shellfish Immun. 49 (2016) 315–323.
[43] M. Cardinaud, N.M. Dheilly, S. Huchette, D. Moraga, C. Paillard, The early stages of the immune response of the European abalone *Haliotis tuberculata* to a *Vibrio harveyi* infection, Dev. Comp. Immunol. 51 (2015) 287–297.
[44] T. Ye, X. Wu, W. Wu, C. Dai, J. Yuan, *ferritin* protect shrimp *Litopenaeus vannamei* from WSSV infection by inhibiting virus replication, Fish Shellfish Immun. 42 (2015) 138–143.
[45] W. Maldonado-Aguayo, F. Lafarga-De La Cruz, C. Gallardo-Escarate, Identification and expression of antioxidant and immune defense genes in the surf clam *Mesodesma donacium* challenged with *Vibrio anguillarum*, Mar. Genom. 19 (2015) 65–73.
[46] J. Cao, Z. Wang, Y. Zhang, F. Qu, L. Guo, M. Zhong, et al., Identification and characterization of the related immune-enhancing proteins in crab *Scylla paramamosain* stimulated with rhubarb polysaccharides, Mol. Immunol. 57 (2014) 263–273.
[47] Y. Sun, Y. Zhang, X. Fu, R. Zhang, J. Zou, S. Wang, et al., Identification of two secreted *ferritin* subunits involved in immune defense of Yesso scallop *Patinopecten yessoensis*, Fish Shellfish Immun. 37 (2014) 53–59.
[48] d l P.T. Coba, C.B. Cárcamo, M.I. Díaz, K.B. Brokordt, F.M. Winkler, Molecular characterization of two ferritins of the scallop *Argopecten purpuratus* and gene expressions in association with early development, immune response and growth rate, Comp. Biochem. Phys. B 198 (2016) 46.
[49] Hou-Bo W, Jin-Pei P. Virulence mechanisms of pathogenic vibrio. Acta Hydrobiol. Sin. 27:422–426.
[50] C. Yang, L. Wang, V.S. Siva, X. Shi, Q. Jiang, J. Wang, et al., A novel cold-regulated cold shock domain containing protein from scallop *Chlamys farreri* with nucleic acid-binding activity, PLoS One 7 (2) (2012) e32012.
[51] Y.J. Wang, H.P. Zheng, B. Zhang, H.L. Liu, H.J. Deng, L.H. Deng, Cloning and respond of a cold shock domain protein (cncsdp) gene to cold stress in noble scallop *Chlamys nobilis* (bivalve: pectinidae), Mol. Biol. Rep. 41 (12) (2014) 7985–7994.
[52] J.P. Idrovo, A. Jacob, W.L. Yang, Z. Wang, H.T. Yen, J. Nicastro, et al., A deficiency in cold-inducible RNA-binding protein accelerates the inflammation phase and improves wound healing, Int. J. Mol. Med. 37 (2016) 423–428.
[53] M. Zhou, W. Yang, Y. Ji, X. Qiang, P. Wang, Cold-inducible RNA-binding protein mediates neuroinflammation in cerebral ischemia, BBA-Gen Subj. 1840 (2014) 2253–2261.
[54] X. Wang, Y. Zhang, C. Yang, B. Zhang, S. Chen, Cloning, characterization and expression analysis of a cold shock domain family member YB-1 in turbot *Scophthalmus maximus*, Fish Shellfish Immun. 33 (2012) 1215–1221.
[55] M.F. Wilkinson, A.B. Shyu, Multifunctional regulatory proteins that control gene expression in both the nucleus and the cytoplasm, Bioessays 23 (2001) 775–787.
[56] L. Shi, S. Liang, X. Luo, C. Ke, J. Zhao, Microbial community of Pacific abalone (*Haliotis discus hannai*) juveniles during a disease outbreak in South China, Aquac. Res. (2015) 1080–1088.
[57] Q. Jiang, L. Shi, C. Ke, W. You, J. Zhao, Identification and characterization of *Vibrio harveyi* associated with diseased abalone *Haliotis diversicolor*, Dis. Aquat. Organ 103 (2013) 133–139.
[58] L. Donaghy, B. Kim, H. Hong, H. Park, K. Choi, Flow cytometry studies on the populations and immune parameters of the haemocytes of the Suminoe oyster, *Crassostrea ariakensis*, Fish Shellfish Immun. 27 (2009) 296–301.
[59] M. Delaporte, P. Soudant, J. Moal, C. Lambert, C. Quere, P. Miner, et al., Effect of a mono-specific algal diet on immune functions in two bivalve species–*Crassostrea gigas* and *Ruditapes philippinarum*, J. Exp. Biol. 206 (2003) 3053–3064.
[60] C. Lambert, P. Soudant, G. Choquet, C. Paillard, Measurement of *Crassostrea gigas* haemocyte oxidative metabolism by flow cytometry and the inhibiting capacity of pathogenic vibrios, Fish. Shellfish Immun. 15 (2003) 225–240.
[61] Da Cleonor Silva, Activation of prophenoloxidase and removal of bacillus subtilis from the hemolymph of *Acheta domesticus* (L.) (*Orthoptera: Gryllidae*), Neotrop. Entomol. 31 (2002) 487–491.
[62] P. Cheng, X. Liu, G. Zhang, J. He, Cloning and expression analysis of a *hsp70* gene from Pacific abalone (*Haliotis discus hannai*), Fish Shellfish Immun. 22 (2007) 77–87.
[63] X. Guo, Use and exchange of genetic resources in molluscan aquaculture, Rev. Aquacult. 1 (2009) 251–259.
[64] P. Rawson, S. Feindel, Growth and survival for genetically improved lines of Eastern oysters (*Crassostrea virginica*) and interline hybrids in Maine, USA, Aquaculture 326–329 (2012) 61–67.
[65] D. Cheney, L. Burnett, A. Christy, J. Davis, R. Elston, C. Friedman, et al., Improvement of disease resistance and understanding of summer mortality in Pacific oysters *Crassostrea gigas*, J. Shellfish Res. 23 (2004) 284–285.
[66] V.T. Dang, P. Speck, M. Doroudi, B. Smith, K. Benkendorff, Variation in the antiviral and antibacterial activity of abalone *Haliotis laevigata, H. rubra* and their hybrid in South Australia, Aquaculture 315 (3–4) (2011) 242–249.
[67] R. González, K.B. Lohrmann, J. Pizarro, K. Brokordt, Differential susceptibility to the withering syndrome agent and renal coccidia in juvenile *Haliotis rufescens, Haliotis discus hannai* and the interspecific hybrid, J. Invertebr. Pathol. 116 (2) (2014) 13–17.
[68] L. Minguez, M. Halm-Lemeille, K. Costil, R. Bureau, J. Lebel, A. Serpentini, Assessment of cytotoxic and immunomodulatory properties of four antidepressants on primary cultures of abalone haemocytes (*Haliotis tuberculata*), Aquat. Toxicol. 153 (2014) 3–11.
[69] M. Cardinaud, N.M. Dheilly, S. Huchette, D. Moraga, C. Paillard, The early stages of the immune response of the European abalone *Haliotis tuberculata* to a *Vibrio harveyi* infection, Dev. Comp. Immunol. 51 (2015) 287–297.
[70] C.J. Bayne, Phagocytosis and non-self recognition in invertebrates, Bioscience 40 (1990) 723–731.
[71] S. Aladaileh, S.V. Nair, D.A. Raftos, Induction of phenoloxidase and other immunological activities in Sydney rock oysters challenged with microbial pathogen-associate molecular patterns, Fish Shellfish Immun. 23 (2007) 1196–1208.
[72] G. Jiang, Comparison of defence ability against the white spot syndrome virus between *Fenneropenaeus chinensis* and *Marsupenaeus japonicus*, Aquac. Res. 41 (2010) 1322–1330.
[73] V.T. Dang, K. Benkendorff, S. Corbeil, L.M. Williams, J. Hoad, M.S.J. Crane, et al., Immunological changes in response to herpesvirus infection in abalone *Haliotis laevigata* and *Haliotis rubra* hybrids, Fish Shellfish Immun. 34 (2013) 688–691.
[74] Z. Zhang, S. Li, C. Xie, L. Zhou, C. Li, W. Liu, et al., Innate immune response and gene expression of *Scylla paramamosain* under *Vibrio parahaemolyticus* infection, Aquac. Res. 46 (2015) 462–471.
[75] T. Becking, A. Mrugala, C. Delaunay, J. Svoboda, M. Raimond, S. Viljamaa-Dirks, et al., Effect of experimental exposure to differently virulent *Aphanomyces astaci* strains on the immune response of the noble crayfish *Astacus astacus*, J. Invertebr. Pathol. 132 (2015) 115–124.
[76] M. Perrigault, B. Allam, Differential immune response in the hard clam (*mercenaria mercenaria*) against bacteria and the protistan pathogen QPX (quahog parasite unknown), Fish Shellfish Immun. 32 (2012) 1124–1134.
[77] P. Comesana, S.M. Casas, A. Cao, E. Abollo, I. Arzul, B. Morga, et al., Comparison of haemocytic parameters among flat oyster *Ostrea edulis* stocks with different susceptibility to bonamiosis and the Pacific oyster *Crassostrea gigas*, J. Invertebr.

Pathol. 109 (2012) 274–286.
[78] W. Cárdenas, J.R. Dankert, J.A. Jenkins, Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides, Fish Shellfish Immun. 17 (2004) 223.
[79] H.J. Choi, J.Y. Hwang, D.L. Choi, M.D. Huh, Y.B. Hur, N. Lee, et al., Non-specific defensive factors of the pacific oyster *Crassostrea gigas* against infection with *Marteilioides chungmuensis*: a flow-cytometric study, Korean J. Parasitol. 49 (2011) 229–234.
[80] C. Pruzzo, G. Gallo, L. Canesi, Persistence of vibrios in marine bivalves: the role of interactions with hemolymph components, Environ. Microbiol. 7 (2005) 761–772.
[81] P. Ji, C. Yao, Z. Wang, Reactive oxygen system plays an important role in shrimp *Litopenaeus vannamei* defense against *Vibrio parahaemolyticus* and WSSV infection, Dis. Aquat. Organ 96 (2011) 9–20.
[82] J. Xian, X. Zhang, H. Guo, D. Wang, A. Wang, Cellular responses of the tiger shrimp *Penaeus monodon* haemocytes after lipopolysaccharide injection, Fish Shellfish Immun. 54 (2016) 385–390.
[83] C. Yang, J. Kong, Q. Wang, Q. Liu, Y. Tian, K. Luo, Heterosis of haernolymph analytes of two geographic populations in Chinese shrimp *Fenneropenaeus chinensis*, Fish Shellfish Immun. 23 (2007) 62–70.
[84] J. Meng, L. Zhang, B. Huang, L. Li, G. Zhang, Comparative analysis of oyster (*Crassostrea gigas*) immune responses under challenge by different Vibrio strains and conditions, Molluscan Res. 35 (2015).
[85] X. Yue, B. Liu, L. Sun, B. Tang, Cloning and characterization of a *hsp70* gene from Asiatic hard clam *Meretrix meretrix* which is involved in the immune response against bacterial infection, Fish Shellfish Immun. 30 (2011) 791–799.
[86] S.E. Ford, S.A. Kanaley, D.T. Littlewood, Cellular responses of oysters infected with *Haplosporidium nelsoni*: changes in circulating and tissue-infiltrating hemocytes, J. Invertebr. Pathol. 61 (1) (1993) 49–57.
[87] M. Gonzalez, G. Arenas, Characterization of the immune response of the North scallop *Argopecten purpuratus* (Lamarck, 1819) (Mollusca: Bivalvia), Cienc 28 (28) (Mar, 2002) 247–255.
[88] E. Peatman, P. Baoprasertkul, J. Terhune, P. Xu, S. Nandi, H. Kucuktas, et al., MRNA level analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium, Dev. Comp. Immunol. 31 (2007) 1183–1196.
[89] G. Beck, T.W. Ellis, G.S. Habicht, S.F. Schluter, J.J. Marchalonis, Evolution of the acute phase response: iron release by echinoderm (*Asterias forbesi*) coelomocytes, and cloning of an echinoderm *ferritin* molecule, Dev. Comp. Immunol. 26 (2002) 11–26.
[90] Y. Sun, Y. Zhang, X. Fu, R. Zhang, J. Zou, S. Wang, et al., Identification of two secreted *ferritin* subunits involved in immune defense of Yesso scallop *Patinopecten yessoensis*, Fish Shellfish Immun. 37 (2014) 53–59.
[91] L. Zhang, W. Sun, W. Cai, Z. Zhang, Y. Gu, H. Chen, et al., Differential response of two *ferritin* subunit genes (VpFer1 and VpFer2) from *Venerupis philippinarum* following pathogen and heavy metals challenge, Fish Shellfish Immun. 35 (2013) 1658–1662.
[92] J.P. Ting, A. Painter, N.J. Zeleznik-Le, G. MacDonald, T.M. Moore, A. Brown, et al., YB-1 DNA-binding protein represses interferon gamma activation of class II major histocompatibility complex genes, J. Exp. Med. 179 (1994) 1605–1611.