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Metabolomic analysis revealed the differential responses in two pedigrees of clam *Ruditapes philippinarum* towards *Vibrio harveyi* challenge



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ABSTRACT

Manila clam *Ruditapes philippinarum* is an important marine aquaculture shellfish. This species has several pedigrees including White, Zebra, Liangdao Red and Marine Red distributing in the coastal areas in North China. In this work, we studied the metabolic differences induced by *Vibrio harveyi* in hepatopancreas from White and Zebra clams using NMR-based metabolomics. Metabolic responses (e.g., amino acids, glucose, glycogen, ATP and succinate) and altered mRNA expression levels of related genes (ATP synthase, heat shock protein 90, defensin and lysozyme) suggested that *V. harveyi* induced clear disruption in energy metabolism and immune stresses in both White and Zebra clam hepatopancreas. However, *V. harveyi* caused obvious osmotic stress in Zebra clam hepatopancreas, which was not observed in *V. harveyi*-challenged White clams samples. In addition, *V. harveyi* challenge induced more severe disruption in energy metabolism and immune stress in White clams than in Zebra clams. Overall, our results indicated that the biological differences between different pedigrees of *R. philippinarum* should be considered in immunity studies.

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1. Introduction

Vibrios, such as *Vibrio anguillarum*, *Vibrio splendidus*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio furnissii*, *Vibrio tapetis* and *Vibrio harveyi*, are main causative pathogens of vibriosis in both fish and shellfish [1,2]. Among these vibrios, *V. harveyi* is a luminous species of bacteria and commonly isolated from marine source and has been described to be pathogenic for fish, mollusk and crustacean in warm seas [3]. Infections by *V. harveyi* can lead to massive mortalities in both marine vertebrates and invertebrates, by killing up to 80% of the estimated stock [3,4]. However, no studies have been performed on the biological responses of clam towards *V. harveyi* challenges.

Manila clam *Ruditapes philippinarum* is one of the most important economic species in shellfishery in China. Due to its good flavor, *R. philippinarum* is consumed as a type of seafood. This marine aquaculture species has a wide geographic distribution along the coast in China and high tolerance to environmental factors, such as salinity and temperature. However, there

are several pedigrees (White, Zebra, Liangdao Red and Marine Red) of *R. philippinarum* distributing in the coastal environment in China. Among these pedigrees, White and Zebra clams are the dominant pedigrees [5]. Evidences indicated that various pedigrees of clams had differential tolerances to environmental stressors [5,6]. Yan et al. reported that Zebra clam had the highest survival rate and tolerance to environmental stressors (e.g., high temperature) than other pedigrees [6]. These findings demonstrated the differential responses and corresponding mechanisms of different pedigrees of clam towards the environmental stimuli. However, few studies have considered the biological differences between various pedigrees of clams, which may introduce undesirable variation in immunity studies on Manila clam. Therefore, it is necessary to assess the differential responses of *R. philippinarum* to marine pathogens.

To better understand the responsive mechanisms of *R. philippinarum* towards *V. harveyi*, a global analysis on the biological responses and corresponding biomarkers should be carried out at molecular level (e.g., metabolite). As a well-established system biology approach, metabolomics have been widely used in multiple research areas, including drug toxicity, environmental sciences and immunology. Routinely, metabolomic studies focus on the metabolic profiles and perturbations in metabolic pathways to

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characterize the exogenous-induced responses in biological systems (e.g., cell, tissue, urine, plasma) [7,8]. With their rapid developments in analytical chemistry, both mass (MS) spectrometry and nuclear magnetic resonance (NMR) spectroscopy are widely applied in metabolomics [7,9]. Specifically, high resolution proton nuclear magnetic resonance (HR- ^1H NMR) spectroscopy is suited to detect a large range of endogenous low molecular weight metabolites in an organism since this technique allows metabolites to be analyzed simultaneously [10]. To date, however, very few studies have been carried out to compare the immunity responses of marine aquaculture shellfish, *R. philippinarum*, towards marine bacterial pathogen challenges using NMR-based metabolomics.

In this study, the metabolomic responses were compared in two pedigrees (White and Zebra) of Manila clam *R. philippinarum* challenged by *V. harveyi*. The tissue of hepatopancreas from *R. philippinarum* was used for metabolomic analysis, since this organ is an important digestive and immune organ in bivalves and can accumulate a large number of bacteria due to the filter-feeding habit of *R. philippinarum*. The aim of this work was to illustrate the differential effects and mechanisms induced by *V. harveyi* in two pedigrees (White and Zebra) of *R. philippinarum* using NMR-based metabolomics.

2. Materials and methods

2.1. Chemicals

Sodium dihydrogen phosphate (Na_2HPO_4) and disodium hydrogen phosphate (NaH_2PO_4) (all in analytical grade) were purchased from Guoyao Chemical Co. Ltd. (Shanghai, China). Extraction solvents, methanol and chloroform (HPLC grade), were purchased from Guoyao Chemical Co. Ltd. (Shanghai, China). Deuterium oxide (D_2O , 99.9% in D) and sodium 3-trimethylsilyl [2,2,3,3-D4] propionate (TSP) were purchased from Cambridge Isotope Laboratories (Miami, FL).

2.2. Experimental animals and conditions

Thirty two adult clams *R. philippinarum* (shell length: 3.4–3.8 cm, $n = 16$ from White and Zebra pedigrees, respectively) were purchased from local culturing farm in Yantai, China. After transported to the culture laboratory, the clams were allowed to acclimate in aerated seawater (25 °C, 33 psu, collected from pristine environment) in the laboratory for 1 week and fed with the *Chlorella vulgaris* Beij at a ration of 2% tissue dry weight daily. After acclimation, the clams were randomly divided into four tanks (one control and one *V. harveyi* challenge for White and Zebra clams, respectively) containing eight individual clams in 20 L aerated seawater.

2.3. Challenge experiment

The bacterium, *V. harveyi*, was kindly provided by Prof. Baozhong Liu (Institute of Oceanology, CAS). The bacteria were cultured in liquid 2216E broth (Tryptone 5 g L^{-1} , yeast extract 1 g L^{-1} , $\text{C}_6\text{H}_5\text{Fe} \cdot 5\text{H}_2\text{O}$ 0.1 g L^{-1} , pH 7.6) at 29 °C and centrifuged at 3000 g for 5 min to harvest the bacteria. For the challenge experiment, live *V. harveyi* bacteria were re-suspended in filtered sea water (FSW) and adjusted to a concentration of 5×10^8 CFU mL^{-1} (1 unit OD_{550} corresponds to 5×10^8 CFU mL^{-1}). For both bacterial challenged groups, the clams were challenged with high density of *V. harveyi* with final concentrations of 1×10^7 CFU mL^{-1} for both White and Zebra clams. The groups without any treatment were used as control groups. After exposure for 24 h, the hepatopancreas tissue of each clam was dissected quickly and flash-frozen in liquid

nitrogen, and then stored at -80 °C before RNA and metabolite extraction.

2.4. Metabolite extraction

The polar metabolites in clam hepatopancreas tissues ($n = 8$ for each treatment) were extracted by the modified extraction protocol as described previously [11,12]. Briefly, the hepatopancreas tissue (ca. 100 mg wet weight) was homogenized and extracted in 4 mL g^{-1} of methanol, 5.25 mL g^{-1} of water and 2 mL g^{-1} of chloroform. The methanol/water layer with polar metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The extracts of hepatopancreas tissue were then re-suspended in 600 μl phosphate buffer (100 mM Na_2HPO_4 and NaH_2PO_4 , including 0.5 mM TSP, pH 7.0) in D_2O . The mixture was vortexed and then centrifuged at 3000 g for 5 min at 4 °C. The supernatant substance (550 μl) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

2.5. RNA extraction and quantitation of gene expressions

The mRNA expression of the housekeeping genes (Table 1) in *R. philippinarum* hepatopancreas was determined by qRT-PCR. The data were analyzed with geNorm to calculate the expression stability (M values) and the optimal number of reference genes required for accurate normalization (V values) [13]. GeNorm identified 40s ribosomal protein s20 as the most stable gene, which was lower than the expression stability threshold of 1.5, then were followed by glyceraldehyde-3-phosphate dehydrogenase, ubiquitin-conjugating enzyme e2d3, elongation factor 1- α , 18S rRNA and β -actin to determine the number of genes required for optimal data normalization. The results showed that the V2/3 value 0.139 is less than the proposed geNorm cutoff value of 0.15, which meant that the gene of 40s ribosomal protein s20 was the most stable gene and was then used as the internal control for gene expression normalization.

Total RNA from clam hepatopancreas was extracted following the manufacturer's directions (Invitrogen, LifeTechnologies, Carlsbad, CA, USA), and the first-strand cDNA was synthesized according to M-MLV RT Usage information (Promega, Madison, WI, USA). Gene-specific primers employed for quantification of mRNA expression including ATP synthase, heat shock protein 90 (HSP 90), defensin and lysozyme were listed in Table 1. The fluorescent real-time quantitative PCR amplifications were carried out in triplicate in a total volume of 50 μl containing 25 μl of $2 \times$ SYBR Premix Ex TaqTM (TaKaRa), 1.0 μl of $50 \times$ ROX Reference DYE II, 12.0 μl DEPC-treated H_2O , 1.0 μl of each primer, 10.0 μl of 1:20 diluted cDNA. The fluorescent real-time quantitative PCR program was as following: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 45 s, 72 °C for 30 s. Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with the ABI 7500 SDS software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative CT method ($2^{-\Delta\Delta\text{CT}}$ method) was used to analyze the relative expression level of the genes [14].

2.6. ^1H NMR spectroscopy

Metabolite extracts of hepatopancreas from clams were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 25 °C) as described previously [15]. All ^1H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker).

Table 1

The list of primers used for the determination of internal control and quantification of gene expressions by qRT-PCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Reference genes		
β -actin	ACACCTATGTACGTCCGATCC	GAAGCGTAAAGTGACAGGACAGC
18S ribosomal RNA gene	ATGACTTCCGCCCGTGT	CTCAGATTTCTGAACAGGTGTCTG
Elongation factor 1-alpha	CATTGGGGAGGTTTGCTGTC	TCAACCTGTCCCACAGGCAT
Ubiquitin-conjugating enzyme e2d 3	TTACTAGATGAATGGAACCGTCCT	GTGAGCATATAGCCAGCAAAT
Glyceraldehyde-3-phosphate dehydrogenase	CTTATACTGCCACCCAGAAGGT	TCAGGGGTTGGTACACGGAAT
40s ribosomal protein s20	CTGGGATAGATTTAGATGCGT	CAAGTTCAAGGGGGGATAATCT
Tested genes		
ATP synthase	TATCTGCTTACATCCAACT	TGACCACAACCTACAT
HSP 90	GGGCATTGAGGTCATTAC	CTGTCCAGGATTTCTTTG
Defensin	GTTGCCCTGAAGATGAA	ATTGCGTGTGGTGCTGT
Lysozyme	AAATGCCTCTCTGATG	TAGGGTTGCTTATCTGG

2.7. Spectral pre-processing and multivariate analysis

All one dimensional ^1H NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab version 7.0 (The MathsWorks, Natick, MA) [16]. Each spectrum was segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. The bins of residual water peak between 4.70 and 5.20 ppm were excluded from all the NMR spectra. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed (glog) with a transformation parameter $\lambda = 2.0 \times 10^{-8}$ to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks [16]. Data were mean-centered before multivariate data analysis.

The supervised multivariate data analysis methods, partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structure with discriminant analysis (O-PLS-DA), were sequentially used to uncover and extract the statistically significant metabolite variations between control White and control Zebra clam samples and between control and *V. harveyi*-challenged groups. The results were visualized in terms of scores plots to show the classifications and corresponding loadings plots to show the NMR spectral variables contributing to the classifications. The model coefficients were calculated from the coefficients incorporating the weight of the variables in order to enhance interpretability of the model. Then metabolic differences responsible for the classifications between control and *V. harveyi*-challenged groups could be detected in the coefficient-coded loadings plots. The coefficient plots were generated by using MATLAB (V7.0, the Mathworks Inc., Natick, USA) with an in-house developed program and were color-coded with absolute value of coefficients (r). A hot color (i.e., red) corresponds to the metabolites with highly positive/negative significances in discriminating between groups, while a cool color (i.e., blue) corresponds to no significance. The correlation coefficient was determined according to the test for the significance of the Pearson's product-moment correlation coefficient. The validation of each model was conducted using 10-fold cross validation and the cross-validation parameter Q^2 was calculated, and an additional validation method, permutation test (permutation number = 200), was also conducted in order to evaluate the validity of the PLS-DA models. The R^2 in the permuted plot described how well the data fit the derived model, whereas Q^2 describes the predictive ability of the derived model and provides a measure of the model quality. If the maximum value of Q^2 max from the permutation test was smaller than or equal to the Q^2 of the real model, the model was regarded as a predictable model. Similarly, the R^2 value and difference between the R^2 and Q^2 were used to evaluate the possibility of over-fitted models [17,18]. Metabolites were

assigned following the tabulated chemical shifts [19] and quantified by using the software, Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada). The metabolite concentrations were normalized to the mass of hepatopancreas tissue by calculating the concentrations of metabolites in each NMR tube.

2.8. Statistical analysis

Metabolite concentrations were tested for normal distribution (Ryan-Joiner's test) and homogeneity of variances (Bartlett's test). All metabolite concentrations were expressed as means \pm standard deviation. One way analysis of variance (ANOVA) with Tukey's test was performed on metabolite concentrations between control White and control Zebra clam samples and between control and *V. harveyi*-challenged groups, respectively. A P value less than 0.05 was considered statistically significant. The Minitab software (Version 15, Minitab Inc., USA) was used for the statistical analysis.

3. Results and discussion

3.1. Metabolic differences in hepatopancreas between White and Zebra clams

A representative ^1H NMR spectrum of hepatopancreas tissue extracts from a White clam is shown in original (Fig. 1(A)) and generalized log (glog) transformed (Fig. 1(B)) forms in Fig. 1. Several metabolite classes were identified, including amino acids (branched chain amino acids: valine, leucine and isoleucine, aspartate, glutamate, glutamine, phenylalanine, etc.), energy storage compounds (ATP, glucose and glycogen), intermediates in Krebs cycle (succinate and fumarate) and osmolytes (betaine, taurine, glycine and homarine). As observed, the original NMR spectrum (Fig. 1(A)) is dominated by an organic osmolyte, betaine (3.27 and 3.90 ppm), which is the result of a two-step reaction of choline: $\text{choline} \rightarrow \text{betaine aldehyde} + \text{NAD}^+ \rightarrow \text{betaine} + \text{NADH}$ [20]. Additionally, other organic osmolytes, taurine (3.27 and 3.45 ppm) and glycine (3.56 ppm), were of relatively high concentrations in clam hepatopancreas as well (Fig. 1). These osmolytes play important roles in osmotic regulation in clam towards environmental salinity changes and therefore were observed in clam hepatopancreas with high concentrations [20].

The supervised pattern recognition method, O-PLS-DA, was initially performed on the NMR spectral datasets of hepatopancreas extracts from control White and control Zebra clam samples to detect the metabolic differences. Apparently, O-PLS-DA resulted in a clear separation between White and Zebra clam samples with a reliable Q^2 value greater than 0.4 (Fig. 2(A)). The significant metabolic differences between the metabolic profiles from two pedigrees were identified and labeled in the loading plot (Fig. 2(B)).

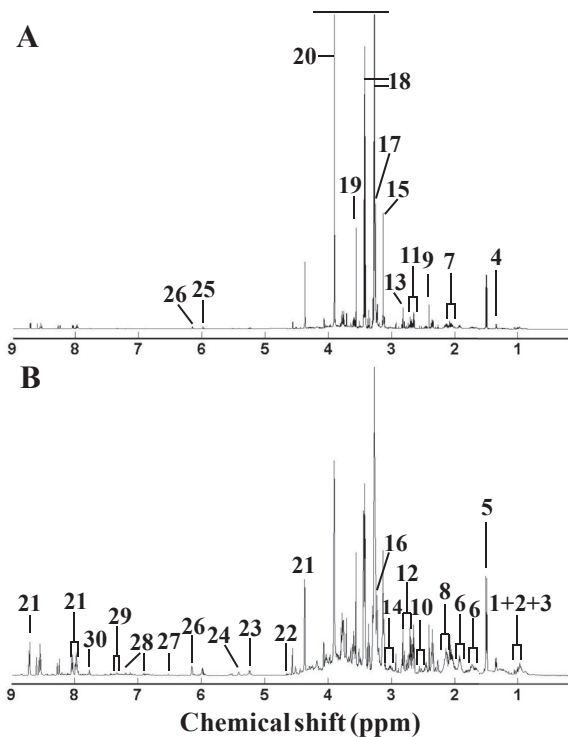


Fig. 1. A representative 1-dimensional 500 MHz ^1H NMR spectrum of hepatopancreas extracts from a White clam of control group in original (A) and generalized log transformed ($\lambda = 2.0 \times 10^{-8}$) (B) forms. **Keys:** (1) leucine, (2) isoleucine, (3) valine, (4) threonine, (5) alanine, (6) arginine, (7) glutamate, (8) glutamine, (9) succinate, (10) β -alanine, (11) hypotaurine, (12) aspartate, (13) dimethylglycine, (14) lysine, (15) malonate, (16) choline, (17) phosphocholine, (18) taurine, (19) glycine, (20) betaine, (21) homarine, (22) β -glucose, (23) α -glucose, (24) glycogen, (25) unknown 1 (5.98 ppm), (26) ATP, (27) fumarate, (28) tyrosine, (29) phenylalanine and (30) unknown 2 (7.68 ppm).

Basically, there were a couple of abundant metabolites including alanine and aspartate in hepatopancreas from White clams (Fig. 2(B), Table 2). The metabolic profiles of Zebra clam hepatopancreas showed high levels of branched chain amino acids, arginine and glycogen compared with those of White clam samples. Since both White and Zebra clams belong to the species *R. philippinarum* with the similar *genotypic milieu*, the phenotypic differences (e.g., metabolic differences) might be generated from the differential gene expressions related to various metabolisms. These metabolic differences between White and Zebra pedigrees implied the potential differentiations between these two pedigrees responding to bacterial challenges.

3.2. Differential metabolic responses in hepatopancreas from White and Zebra clams challenged by *V. harveyi*

O-PLS-DA and one way ANOVA were applied on the ^1H NMR spectral data and quantified metabolite concentrations to detect the metabolic biomarkers in *V. harveyi*-challenged White and Zebra clams, respectively (Fig. 3). Fig. 3(A) and (C) indicated that O-PLS-DA resulted in clear classifications between control and *V. harveyi*-challenged groups, respectively, with reliable Q^2 values (>0.4). Apparently, the concentrations of amino acids (valine, leucine, isoleucine and threonine), glucose and glycogen were significantly ($P < 0.05$) increased in *V. harveyi*-challenged White clam hepatopancreas, as shown in the loading plot of O-PLS-DA (Fig. 3(B) and Table 2). The levels of succinate and ATP were significantly ($P < 0.05$) decreased. In marine mollusks, animals can use high

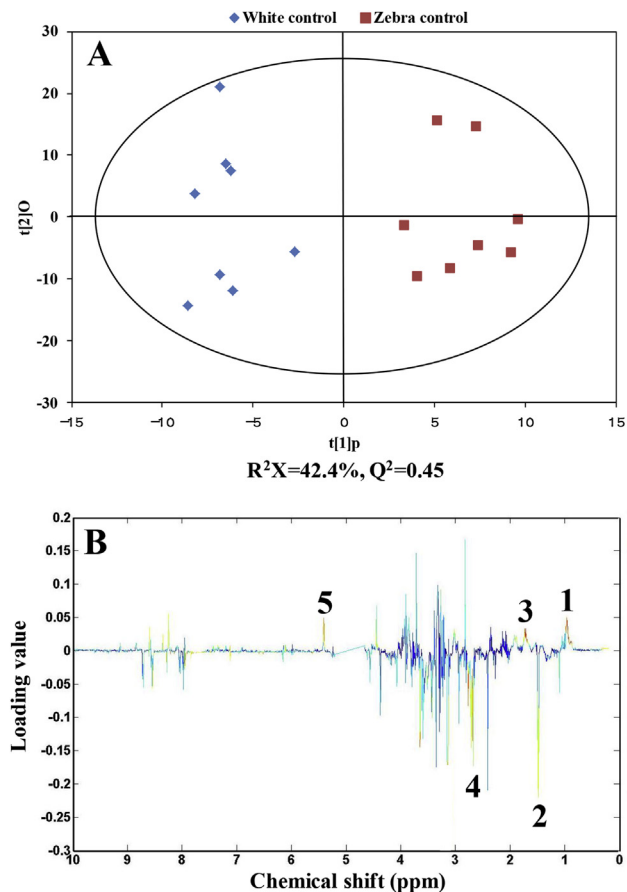


Fig. 2. O-PLS-DA scores plot derived from ^1H NMR spectra of hepatopancreas extracts of *R. philippinarum* from control White (\blacklozenge) and control Zebra (\blacksquare) clam groups (A) and corresponding coefficient plot (B). The color map shows the significance of metabolite variations between the two classes (control White and control Zebra). Peaks in the positive direction indicate metabolites that are more abundant in the control Zebra group. Consequently, metabolites that are more abundant in the control White group are presented as peaks in the negative direction. **Keys:** (1) branched chain amino acids: leucine, isoleucine and valine, (2) alanine, (3) arginine, (4) aspartate and (5) glycogen.

concentrations of amino acids to regulate their intracellular osmolarity with their environment [21]. However, no significant alteration in osmolytes (e.g., betaine, homarine, taurine, glycine, and hypotaurine) was observed. In other metabolic pathways, the large pools of oxidizable amino acids can be used extensively in cellular energy metabolism [21,22]. Therefore, the elevated amino acids implied the depleted energy demand in *V. harveyi*-challenged White clam samples, which was consistent with the elevated glucose and glycogen, and depleted ATP and succinate, an intermediate of Krebs cycle. In the metabolic pathways related to innate immunity, however, the branched chain amino acids (BCAA) including valine, leucine and isoleucine, have availability on the immune system to function by incorporating BCAA into proteins [23]. Upon pathogenic infection, there is a remarkable increase in demand for BCAA for substrates by the immune system [23]. These BCAA then provide energy and are used as the precursors for the biosynthesis of new protective molecules [23]. In our case, therefore, the branched chain amino acids could be accumulated to deal with the infection of *V. harveyi* in clam immune organ, hepatopancreas. In the previous study, *V. anguillarum* challenge induced a similar profile of amino acids in gills of mussel *Mytilus galloprovincialis*, which was related to the disturbances in both osmotic regulation and energy metabolism due to the correlated alteration of osmolytes, homarine and betaine [24].

Table 2

Metabolite concentrations ($\mu\text{mol g}^{-1}$ wet tissue) in hepatopancreas from White and Zebra clams challenged by *Vibrio harveyi*. Values are presented as mean \pm standard deviation.

Metabolites	Chemical shift (ppm, multiplicity) ^a	White clam		Zebra clam	
		Control	<i>V. harveyi</i>	Control	<i>V. harveyi</i>
Valine	1.05 (d)	0.10 \pm 0.02	0.15 \pm 0.04^b	0.14 \pm 0.02[†]	0.10 \pm 0.03^b
Isoleucine	1.00 (d)	0.07 \pm 0.02	0.10 \pm 0.02^b	0.10 \pm 0.01[†]	0.07 \pm 0.03^b
Leucine	0.94 (t)	0.11 \pm 0.02	0.15 \pm 0.02^c	0.15 \pm 0.02[†]	0.12 \pm 0.02^b
Threonine	1.34 (d)	0.19 \pm 0.04	0.27 \pm 0.05^b	0.22 \pm 0.03	0.29 \pm 0.05^b
Alanine	1.48 (d)	3.86 \pm 0.62	3.61 \pm 0.94	2.77 \pm 0.58[†]	3.84 \pm 0.64^b
Arginine	1.70 (m)	1.08 \pm 0.15	1.27 \pm 0.31	1.46 \pm 0.27[†]	1.32 \pm 0.28
Glutamate	2.05 (m)	1.72 \pm 0.29	1.63 \pm 0.30	1.82 \pm 0.25	2.02 \pm 0.38
Glutamine	2.14 (m)	0.53 \pm 0.15	0.64 \pm 0.18	0.61 \pm 0.26	0.56 \pm 0.14
Succinate	2.41 (s)	0.57 \pm 0.15	0.30 \pm 0.07^b	0.62 \pm 0.25	0.56 \pm 0.29
β -alanine	2.54 (t)	0.14 \pm 0.06	0.12 \pm 0.04	0.16 \pm 0.05	0.13 \pm 0.04
Hypotaurine	2.66 (t)	2.07 \pm 0.32	1.98 \pm 0.30	2.24 \pm 0.28	1.72 \pm 0.25^c
Aspartate	2.68 (ABX)	1.92 \pm 0.35	1.76 \pm 0.29	1.31 \pm 0.32[†]	1.23 \pm 0.22
Dimethylglycine	2.91 (s)	0.13 \pm 0.02	0.12 \pm 0.03	0.15 \pm 0.04	0.13 \pm 0.04
Lysine	3.02 (t)	0.44 \pm 0.08	0.47 \pm 0.10	0.50 \pm 0.09	0.34 \pm 0.08^c
Choline	3.19 (s)	0.21 \pm 0.04	0.23 \pm 0.06	0.18 \pm 0.05	0.27 \pm 0.06
Phosphocholine	3.21 (s)	0.63 \pm 0.14	0.53 \pm 0.16	0.67 \pm 0.19	0.38 \pm 0.13^b
Taurine	3.27 (t), 3.45 (t)	34.72 \pm 1.88	35.59 \pm 1.79	33.27 \pm 2.04	37.56 \pm 2.32^c
Betaine	3.27 (s)	32.34 \pm 1.68	31.84 \pm 1.59	31.12 \pm 1.29	33.87 \pm 1.62^b
Glycine	3.56 (s)	4.51 \pm 1.17	4.23 \pm 1.36	3.45 \pm 0.74	4.97 \pm 0.82^c
glucose	4.64 (d), 5.23 (d)	2.38 \pm 0.62	3.92 \pm 1.04^b	2.42 \pm 0.48	3.87 \pm 1.12^b
Homarine	4.37 (s)	8.85 \pm 1.87	8.43 \pm 1.76	8.29 \pm 1.56	5.42 \pm 1.07^c
ATP	6.14 (d)	0.56 \pm 0.13	0.29 \pm 0.07^c	0.58 \pm 0.08	0.44 \pm 0.07^c
Fumarate	6.52 (s)	0.011 \pm 0.002	0.013 \pm 0.004	0.010 \pm 0.003	0.012 \pm 0.003
Tyrosine	6.91 (d)	0.09 \pm 0.03	0.09 \pm 0.02	0.07 \pm 0.02	0.09 \pm 0.04
Phenylalanine	7.10 (d)	0.15 \pm 0.02	0.16 \pm 0.02	0.18 \pm 0.03	0.13 \pm 0.03^b

^as = singlet, d = doublet, t = triplet, m = multiplet, ABX = complex multiplet involving 2 protons (A and B) and a heavy atom (X).

^{b,c}Statistical significances ($P < 0.05^b$, $P < 0.01^c$) between control and *Vibrio harveyi*-challenged *R. philippinarum* samples were determined by one-way ANOVA.

[†]Meant the metabolite levels were significantly different ($P < 0.05$) between control White and control Zebra clam samples.

The metabolic responses of *V. harveyi*-challenged Zebra clam hepatopancreas were different from those in *V. harveyi*-challenged White clam samples. Based on the corresponding loading plot of O-PLS-DA, threonine, alanine, taurine, betaine, glycine and glucose were increased in *V. harveyi*-challenged Zebra clam samples (Fig. 3(D)). BCAA, hypotaurine, lysine, phosphocholine, homarine,

ATP and phenylalanine were significantly ($P < 0.05$) decreased. The alteration of threonine, ATP and glucose was similar to those in *V. harveyi*-challenged White clam hepatopancreas tissue samples. The elevation of osmolytes including taurine, betaine and glycine clearly indicated the osmotic stress induced by *V. harveyi* in Zebra clam hepatopancreas. Considering the osmotic stress indicated by

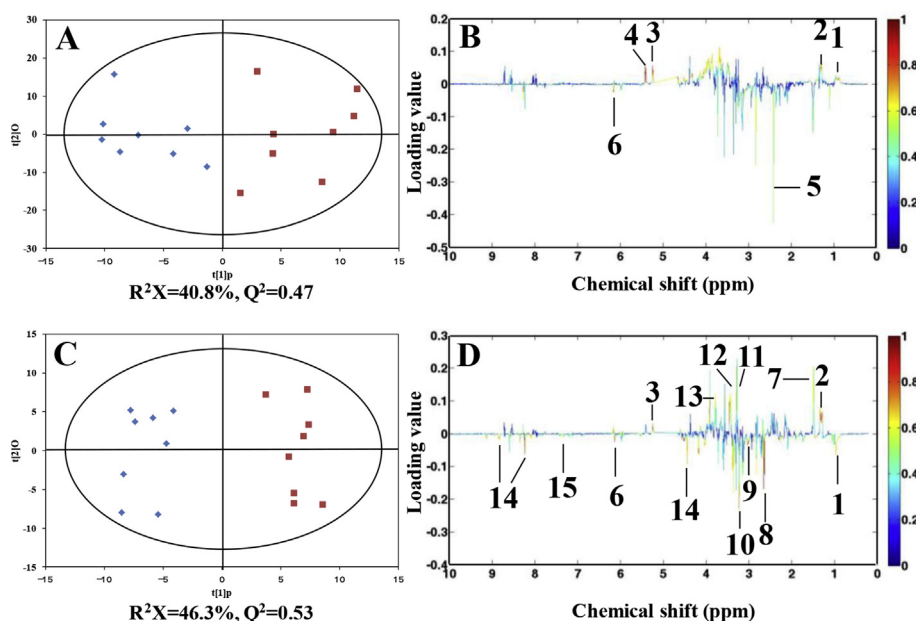


Fig. 3. OPLS-DA scores plots derived from ^1H NMR spectra of hepatopancreas tissue extracts from control (\blacklozenge) and *V. harveyi*-challenged groups (\blacksquare), (A) White clam and (C) Zebra clam and corresponding coefficient plots (B) and (D). The color map shows the significance of metabolite variations between the two classes (control and *V. harveyi* challenge). Peaks in the positive direction indicate metabolites that are more abundant in the *V. harveyi*-challenged groups. Consequently, metabolites that are more abundant in the control group are presented as peaks in the negative direction. **Keys:** (1) branched chain amino acids: leucine, isoleucine and valine, (2) threonine, (3) glucose, (4) glycogen, (5) succinate, (6) ATP, (7) alanine, (8) hypotaurine, (9) lysine, (10) phosphocholine, (11) betaine, (12) taurine, (13) glycine (14) homarine and (15) phenylalanine.

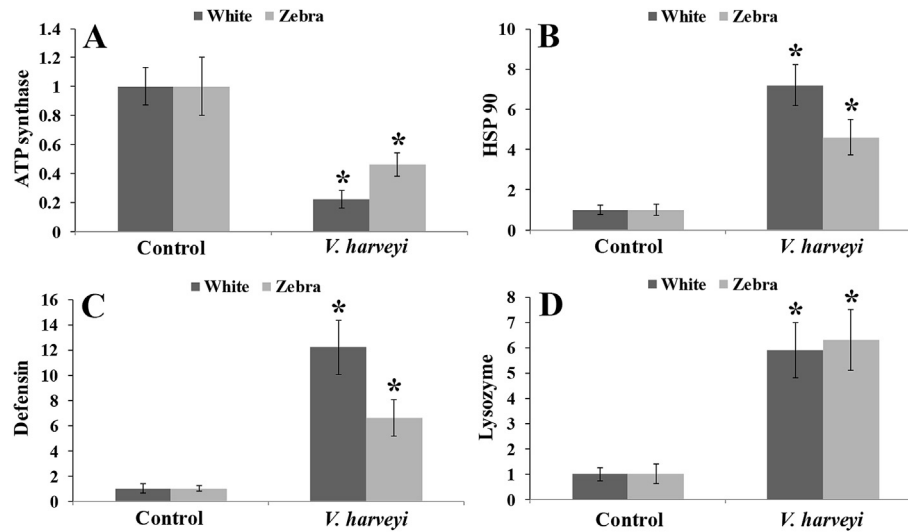


Fig. 4. Expression levels of ATP synthase, HSP 90, defensin and lysozyme mRNA relative to 40s ribosomal protein s20 measured by qRT-PCR in hepatopancreas of *R. philippinarum* from White and Zebra pedigrees after *V. harveyi* challenge for 24 h. Statistical significances ($P < 0.05^*$) between control and *V. harveyi*-challenged *R. philippinarum* samples were determined by one-way ANOVA. **Abbreviations:** HSP 90, heat shock protein 90.

osmolytes, the depletion of amino acids (BCAA, lysine and phenylalanine) and osmolytes (homarine and hypotaurine) might be used to compensate the increase of those osmolytes, taurine, betaine and glycine. Phosphocholine is synthesized in the reaction catalyzed by choline kinase, converting ATP and choline into phosphocholine and ADP. Both phosphocholine and ATP were significantly decreased, which implied the depletion of the conversion reaction of ATP to phosphocholine. The increased glucose meant the enhanced gluconeogenesis, which implied the weakened energy demand in *V. harveyi*-challenged Zebra clam samples, together with the decreased ATP.

3.3. ATP synthase, HSP 90, defensin and lysozyme expression in hepatopancreas from White and Zebra clams challenged by *V. harveyi*

In this study, four genes related to diverse functions were selected for the quantification of mRNA expression. ATP synthase is an important enzyme involved in energy metabolism through the synthesis of ATP. HSP 90, defensin and lysozyme play important roles in anti-oxidative stress and immune defense. After bacterial challenges, the expression of ATP synthase, HSP 90, defensin and lysozyme mRNA in hepatopancreas of control and *V. harveyi*-challenged clams was quantified using quantitative real-time RT-PCR technique with 40s ribosomal protein s20 as internal control (Fig. 4). The mRNA expression levels of ATP synthase were significantly ($P < 0.05$) down-regulated in *V. harveyi*-challenged White and Zebra clam hepatopancreas, respectively. In addition, the mRNA expression of ATP synthase in *V. harveyi*-challenged White clam samples were more significantly ($P < 0.05$) down-regulated than that in *V. harveyi*-challenged Zebra clam samples (Fig. 4), which was consistent with the alteration of ATP. This implied that *V. harveyi* challenge induced more severe disruption in energy metabolism in White clam hepatopancreas. The mRNA expression levels of HSP 90, defensin and lysozyme were all significantly ($P < 0.05$) up-regulated in *V. harveyi*-challenged White and Zebra clam samples, respectively. However, *V. harveyi* challenge resulted in more significant ($P < 0.05$) mRNA expression levels of HSP 90 and defensin in White clam hepatopancreas (Fig. 4). HSPs belong to a large family of molecular chaperones playing vital roles in preventing irreversible protein denaturation, aggregation and

misfolding [25,26]. They can be induced by osmotic stress, oxidative stress and pathogen infection [25]. Both defensin and lysozyme are antibacterial components that have been characterized in marine mollusks [27,28]. In this work, the significant up-regulation of mRNA expression levels of HSP 90, defensin and lysozyme were the indicators of immune stress induced by *V. harveyi* in both White and Zebra clam samples. The similar alteration in HSP 90 was observed in *V. anguillarum*-challenged scallop *Chlamys farreri* as well [25]. The more significant up-regulation of mRNA expression of HSP 90 and defensin implied that *V. harveyi* could induce more severe immune stresses in White clam than that in Zebra clam.

In summary, the differential metabolic responses induced by *V. harveyi* in two pedigrees (White and Zebra) of *R. philippinarum* were investigated using NMR-based metabolomics in hepatopancreas. Overall, *V. harveyi* induced more severe disruption in energy metabolism and immune stress in White clams than in Zebra clams, as indicated by the differential metabolic biomarkers and altered expression levels of related genes. However, *V. harveyi* caused obvious osmotic stresses in Zebra clam hepatopancreas, which was not observed in White clam samples. Our results indicated that the biological differences between different pedigrees of *R. philippinarum* should be considered in immunity studies.

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