Developmental and Comparative Immunology 61 (2016) 236–241

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Short communication

Metabolic product response profiles of *Cherax quadricarinatus* towards white spot syndrome virus infection

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ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form
5 April 2016
Accepted 6 April 2016
Available online 8 April 2016

Keywords:
Cherax quadricarinatus
WSSV
Metabolomics
Gill
Hepatopancreas

ABSTRACT

White spot syndrome virus (WSSV) is one of the most devastating viral pathogens in both shrimp and crayfish farms, which often causes disease outbreak and leads to massive moralities with significant economic losses of aquaculture. However, limited research has been carried out on the intrinsic mechanisms toward WSSV challenge at the metabolic level. To gain comprehensive insight into metabolic responses induced by WSSV, we applied an NMR approach to investigate metabolic changes of crayfish gill and hepatopancreas infected by WSSV for 1, 6 and 12 h. In gill, an enhanced energy metabolism was observed in WSSV-challenged crayfish samples at 1 h, as marked by increased glucose, alanine, methionine, glutamate and uracil. Afterwards, energy metabolism, lipid metabolism as well as osmoregulation were markedly increased at 6 hpi, as shown by elevated glucose, alanine, methionine, fumarate, tyrosine, tryptophan, histidine, phosphorylcholine, betaine and uracil, whereas no obvious metabolites change was detected at 12 hpi. As for hepatopancreas, disturbed lipid metabolism and induced osmotic regulation was found at 6 hpi based on the metabolic biomarkers such as branched chain amino acids, threonine, alanine, methionine, glutamate, glutamine, tyrosine, phenylalanine, lactate and lipid. However, no obvious metabolic change was shown in hepatopancreas at both 1 hpi and 12 hpi. Taken together, our present results provided essential metabolic information about host-pathogen interactions in crayfish, which shed new light on our understanding of WSSV infection at metabolic level.

1. Introduction

White spot syndrome virus (WSSV), initially appeared in Southeast Asia at the beginning of the 1990s, has provoked diminished shrimp production in commercial shrimp farms (Liu et al., 2009). Because of its wide host range of marine and brackish water crustaceans, including shrimp, crab, lobster and crayfish, this virus often leads to serious economic losses to aquaculture worldwide (Liu et al., 2011; Paz, 2010). To date, there is no effective treatment available for WSSV infection. Hence, it is necessary to make a thorough inquiry into the host molecular response to WSSV infection. Research related to the changes post WSSV infection in shrimp and crayfish has made great progress with the application of genomics and proteomics (Chai et al., 2010; Jeswin et al., 2016; Li et al., 2013b, 2014; Liu et al., 2011; Wang et al., 2007; Zhao et al., 2007). On the other hand, the metabolic analysis can indicate the biochemical state of the cells, organs and tissues, and manifests the correlation among the biological metabolic networks, thus helping people to understand the influence of virus invasion into the host. It is worthwhile noting that there are limited researches concentrated on the metabolic study post WSSV infection. Chen et al. detected that WSSV infection had significant effects on the levels of glucose and lactate in shrimp plasma at 12 hpi, associating with other experiment results they put forward: WSSV infection could induce the mammalian Warburg effect (Chen et al., 2011). Further research on the metabolic changes in WSSV-challenged shrimp hemocytes confirmed this Warburg effect and

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http://dx.doi.org/10.1016/j.dci.2016.04.006
0145-305X/

also proposed that this Warburg effect in shrimp hemocytes benefited the virus to meet its requirements during genome replication (Su et al., 2014).

Previous research mainly concentrated on the metabolic changes of hemocytes after WSSV stimulation, studies about the metabolic changes of other tissues were scarcely investigated in WSSV infected shrimp. It is generally known that gill is the respiratory organ of crustaceans, viruses in the hemolymph are absorbed mainly by gill and then induce changes in the physiological function in this organ (Wang et al., 2012), which is a target organ of WSSV infection that plays an important role in immune response (Ren et al., 2015). Besides, the hepatopancreas of crustaceans also have various functions, such as energy and lipid metabolism and indicator of nutritional status (Wang et al., 2008). The gill and hepatopancreas are thus ideal organs to investigate metabolic changes towards WSSV infection. It has been reported that progeny WSSV virions have been clearly detected in the nucleus of infected Hpt cells *in vivo* from crayfish *Procambarus clarkii* as early as 12 hpi (Wu et al., 2015). Also, the red claw crayfish *Cherax quadricarinatus* Hpt cells *in vitro* were responsive to WSSV infection both in gene and protein level at 1 hpi and 12 hpi as the early and late infection stages, respectively, in which the relative increase of nucleoside and the enhanced amino acid metabolites were detected at 12 hpi, implying that the DNA replication machinery might be sequestered to produce viral DNA and protein synthesis, therefore 12 hpi was defined as late infection stage (Liu et al., 2011; Jeswin et al., 2016), which was similar to shrimp (Li et al., 2015). Hence, the time intervals of 1, 6 and 12 hpi for the early, middle and late stage of WSSV infection accordingly were selected in our present study. Additionally, understanding of the metabolic response in these tissues during different infection stages would be helpful for future WSSV disease control.

Till now, metabonomics has been applied to identify and quantify the variation of endogenous metabolites. NMR-based metabonomics is one of the main techniques for metabolomics study with advantage of outstanding reproducibility, simple pretreatment, high throughput and nondestructive testing. In addition, it has been well-illustrated that ^{1}H NMR metabolic method provides a legible and convenient way to learn the metabolic changes induced by xenobiotic, especially by virus in animal tissues (Azmi et al., 2005; Huang et al., 2013). To obtain the biological response induced by WSSV infection, we explored the metabolic changes of crayfish gill and hepatopancreas post WSSV infection by using ^{1}H NMR in the present study.

2. Materials and methods

2.1. Purification of intact WSSV viral particles

Healthy *Procambarus clarkii* was collected and used for amplification of WSSV. The infection, proliferation and purification of virus were performed according to Xie et al. (2005). Briefly, all the tissues of infected crayfish excluding hepatopancreas were collected and homogenized in TNE buffer (50 mM Tris-HCl, 400 mM NaCl, 5 mM EDTA, pH 8.5), then centrifuged at 3500 *g* for 5 min at 4 °C to remove the remaining tissues. After filtration by nylon net (400 mesh), the supernatant was centrifuged at 30,000 *g* for 30 min at 4 °C. Then, the upper loose pellet was rinsed out carefully and the lower white pellet was suspended in 2 mL TN buffer (50 mM Tris-HCl, 10 mM NaCl, pH 7.4). After centrifugation at 3500 *g* for 5 min, the supernatant was centrifuged again at 30,000 *g* for 20 min at 4 °C to deposit virus particles, and then resuspended and kept them in 1 mL TN buffer. The purity degree of isolated virus was evaluated by negative-staining transmission electron microscopy (TEM) and the concentrated virus was quantified by the competitive PCR described by Xu et al. (2001).

2.2. Crayfish infected by WSSV and tissue samples harvested for metabolic analysis

Sixty healthy adult male intermolting red claw crayfish *C. quadricarinatus* (15–18 cm in length and 90–110 g in weight) were purchased from Hangzhou, Zhejiang Province, China. After acclimation, they were infected with live WSSV (10^5 copy of virions/crayfish) by injection via the base of the fourth walking leg and served as experimental group. Crayfish injected with crayfish saline solution (CFS; 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 6.8) were used as a mock infection and considered as control group. Gill and hepatopancreas were dissected from individuals at 1, 6 and 12 h post infection and quickly frozen by liquid nitrogen before metabolite extraction. Ten replicates were performed for each experimental sample from different time points as well as controls.

2.3. Metabolite extraction

Aqueous metabolites of *C. quadricarinatus* (n = 10 for each treatment) gill and hepatopancreas were extracted according to the protocol described by Ye et al. (2014). In brief, each sample (approximately 400 mg wet weight) was homogenized with 600 μL of ice-cold extract solution (methanol: milli Q = 2:1, *v*/*v*) for 30 s at 5000 rpm for 5 times. Supernatant was collected respectively after centrifugation (12,000 rpm, 10 min, 4 °C). The remaining solid residues were extracted once again as above. Then the mixed two supernatants with aqueous metabolites were dried in a centrifugal concentrator to remove methanol. The extracts were then dissolved in 600 μL of Na^+-K^+ phosphate buffer (0.15 M NaH_2PO_4/K_2HPO_4, pH 7.4, containing 30% D_2O and 0.001% TSP) (Xiao et al., 2009). Following centrifugation (12,000 rpm, 10 min, 4 °C), a total of 550 μL supernatant was transferred into 5 mm NMR tubes (Norell, ST500-7, USA) for NMR detection.

2.4. NMR spectroscopy

All of ^{1}H NMR spectra of gill and hepatopancreas extracts were recorded at 298 K with a ^{1}H resonance frequency of 600.13 MHz by using a Bruker Avance III 600 MHz spectrometer (Bruker, Biospin, Germany) (Ding et al., 2014). For all of the extracts, we applied a nuclear overhauser effect spectroscopy (NOESY) pulse sequence (recycle delay (RD)-90°-t_1-90°-t_m-90°-acquisition) to suppress the residual water signal with RD, t_1 and mixing time (t_m) of 2 s, 4 μs and 100 ms, respectively. To assign the NMR signals, we also detected a range of two-dimensional NMR spectra for selected samples, including ^{1}H-^{1}H COSY, ^{1}H *J*-resolved, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HSQC (Aue et al., 1976a, 1976b; Braunschweiler and Ernst, 1983).

2.5. Statistical analysis

All the 1D and 2D NMR spectra were processed with corresponding software and further analyzed according to the previous method (Cloarec et al., 2005; Eriksson et al., 2008; Trygg and Wold, 2002). Multivariate data analysis was carried out on the gill and hepatopancreas of *C. quadricarinatus* between control and WSSV-infected group to test the phenotype metabolic change (Trygg et al., 2006). Following analysis by unsupervised PCA method, the metabolite alterations were detected using OPLS-DA with R^2X and Q^2, two indicators about the quality of these models. Then each model was further evaluated with the CV-ANOVA approach ($p < 0.05$). OPLS-DA loading plots, which successfully pass this test

can visually display the changed metabolites in gill and hepatopancreas extracts infected by WSSV at different time points.

3. Results and discussion

3.1. ^{1}H NMR spectra of C. quadricarinatus gill and hepatopancreas

Typical ^{1}H NMR spectra derived from gill and hepatopancreas were shown in Fig. S1 and Fig. S2, respectively. Combing these and a series of coupled information provided by 2D NMR spectra, metabolites were assigned. In gill, a total of 35 specific metabolites were identified including a range of amino acids, aliphatic organic acids, glucose, betaine, nucleotide and pyrimidine (Table 1). The detectable metabolites of hepatopancreas covered a range of amino acids, organic acids, glucose, betaine and lipid (Table 1). To acquire more details about the active WSSV-induced metabolomic changes, we conducted multivariate data analysis on the NMR data for gill and hepatopancreas extracts.

3.2. Gill metabolomic responses challenged by active WSSV

Through analyzing, only two of the OPLS-DA models passed the rigorous test of CV-ANOVA. These two models and their corresponding OPLS-DA coefficient-coded loading plots were illustrated in Fig. S3. Our results showed that infection of WSSV for 12 h had no significant effect on the gill metabolome. However, active WSSV

Table 1
NMR data of metabolites detected in crayfish gill and hepatopancreas extracts.

Key	Metabolites	Moieties	δ^{1} H (ppm) and multiplicity[a]	δ^{13} C (ppm)	Tissues
1	Isoleucine	αCH, βCH, γCH_2, $\gamma' CH_3$, δCH_3	3.67(d), 1.98(m), 1.26(m), 1.48(m), 1.01(d), 0.94(t)	62.4, 38.7, 27.0, 17.5, 14.0	G, H
2	Leucine	αCH, βCH_2, γCH, δCH_3, $\delta' CH_3$	3.74(m), 1.73(m), 1.69(m), 0.98(d), 0.96(d)	56.2, 42.6, 26.7, 24.6, 23.7	G, H
3	Valine	αCH, βCH, γCH_3, $\gamma' CH_3$	3.62(d), 2.28(m), 0.99(d), 1.04(d)	63.1, 31.7, 19.3, 20.8	G, H
4	Lactate	αCH, βCH_3, COOH	4.12(q), 1.33(d)	71.3, 22.5, 185.5	G, H
5	Threonine	αCH, βCH, γCH_3	3.58(d), 4.26(m), 1.33(d)	69.0, 63.3, 22.5	G, H
6	Alanine	αCH, CH_3, COOH	3.79(q), 1.49(d)	53.2, 19.2, 178.6	G, H
7	Acetate	CH_3, COOH	1.92(s)	26.4, 184.0	G, H
8	Methionine	αCH, βCH_2, γCH_2, S−CH_3	3.87, 2.16, 2.65(t), 2.14(s)	56.7, 32.8, 31.4, 16.7	G, H
9	Glutamate	αCH, βCH_2, γCH_2, δCO	3.77(m), 2.12(m), 2.05(m), 2.36(dt)	57.4, 29.8, 36.4, 183.9	G, H
10	Succinate	CH_2, COOH	2.41(s)	37.1, 184.3	G, H
11	Glutamine	CH_2, γCH_2	2.14(m), 2.46(m)	33.8	G, H
12	Aspartate	αCH, βCH_2, γCOOH	3.92(dd), 2.82(dd), 2.68(dd)	55.1, 39.7, 180.1	G
13	Asparagine	αCH, CH_2, $\gamma CONH_2$, COOH	4.00 (dd), 2.87(dd), 2.96(dd),	54.3, 37.6, 176.3, 177.3	G
14	Lysine	αCH, CH_2, γCH_2, δCH_2, εCH_2, COOH	3.76(t), 1.92(m), 1.48(d), 1.73(m), 3.03(t)	56.9, 32.6, 23.9, 29.1, 42.1, 178.3	G, H
15	Choline-*O*-sulfate	αCH_2, CH_2, N−CH_3	4.33, 3.69, 3.12(s)	69.5, 55.6	G
16	Phosphorylcholine	αCH_2, βCH_2, N−CH_3	4.26, 3.59, 3.21(s)	70.4, 56.7	G, H
17	Choline	αCH_2, βCH_2, N−CH_3	4.07, 3.53, 3.23(s)	68.9, 63.4, 56.8	G, H
18	Arginine	CH_2, γCH_2, δCH_2, εC, COOH	1.92(m), 1.66(m), 1.73(m), 3.25(t)	30.2, 26.4, 43.4, 159.9, 177.5	G, H
19	Betaine	CH_3, CH_2, COOH	3.27(s), 3.91(s)	56.2, 69.1, 171.9	G, H
20	U1		3.42(s)	57.9, 102.3	G, H
21	Taurine	CH_2SO_3, CH_2NH_2	3.27(t), 3.43(t)	50.4, 38.4	G, H
22	Glycine	αCH_2, COOH	3.57(s)	44.5, 175.3	G, H
23	β-Glucose	C_1H, C_2H, C_3H, C_4H, C_5H, C_6H	4.67(d), 3.25(t), 3.48(dd), 3.40(m), 3.46(m), 3.89(m), 3.73(m)	98.8, 77.1, 78.6, 72.8, 78.6, 63.8	G, H
24	α-Glucose	C_1H, C_2H, C_3H, C_4H, C_5H, C_6H	5.24(d), 3.54(dd), 3.71(m), 3.40(m), 3.82(m), 3.83(m)	95.0, 74.0, 75.0, 72.8, 74.5, 63.8	G, H
25	Uracil	C_2, C_4, C_5H, C_6H	5.81(d), 7.54(d)	156.1, 170.8, 103.4, 146.7	G
26	Uridine	C_2, C_4, C_5H, C_6H, $C_1'H$, $C_2'H$	5.91(d), 7.88(d), 5.92(d), 4.32	154.7, 169.4, 105.1, 144.6, 92.1, 76.2	G, H
27	Uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc)	C_3H, C_4H, C_6, $C_1'H$, $C_2'H$, $C_3'H$, $C_4'H$, $C_5'H$, G_1H, G_2H, G_3H, G_4H, G_5H, G_6H_2, CO, CH_3	5.97(d), 7.95(d), 5.99(d), 4.39(m), 4.36(m), 4.28(m), 4.23/4.17(m), 5.52(dd), 3.99(m), 3.81(m), 3.56(dd), 3.91(m), 3.84(dd), 2.07(s)	105.4, 144.3, 154.6, 91.1, 76.5, 72.3, 85.6, 67.7, 97.2, 56.2, 73.5, 72.1, 75.6, 62.8, 25.0, 177.6	G
28	Fumarate	COOH, CH	6.53(s)	177.5, 138.1	G, H
29	Tyrosine	αCH, βCH_2, C_1, Ring $C_{2,6}H$, Ring $C_{3,5}H$, Ring C_4, Ring COOH	3.95(dd), 3.2(dd), 3.06(dd), 7.2(d), 6.90 (d)	58.8, 38.4, 129.5, 133.6, 118.8, 157.5, 177.1	G, H
30	Histidine	C_2H, C_3, C_4H	7.93(d), 7.11(d)	138.8, 133.7, 120.0	G
31	Phenylalanine	αCH, βCH_2, C_1, Ring$C_{2,6}H$, Ring$C_{3,5}H$, RingC_4, Ring COOH	4.00(dd), 3.13(dd), 3.29(dd), 7.33(q), 7.43(t), 7.38(m)	59.1, 39.4, 137.9, 132.3, 132.1, 130.7, 177.1	G, H
32	Tryptophan	βCH_2, Ring C_2H, Ring C_3, Ring C_4H, Ring C_5H, Ring C_6H, Ring C_7H, Ring C_8, Ring C_9	3.23(dd), 3.47(dd), 7.33(s), 7.28(m), 7.19(m), 7.73(d), 7.54(d)	29.4, 128.1, 110.2, 125.0, 122.4, 121.3, 114.5, 139.2, 129.6	G, H
33	Inosine	C_2H, C_4, C_5, C_6, C_8H, $C_1'H$, $C_2'H$	8.23(s), 8.34(s), 6.10(d), 4.77, 4.44(dd)	149.3, 151.4, 127.0, 161.7, 143.1, 91.0, 76.7, 73.4	G, H
34	Maltose	C_4H, C_7H	3.64(m), 5.42(d), 3.59(m)	79.6, 102.6	G
35	Sugar and amino acids α-CH resonance	αCH resonances	3.25–4.0		G, H
36	Lipid	CH_3, $(CH_3)_n$	0.87(m), 1.28(m)		H
37	Sarcosine	N−CH_3	2.79(s)	36.0, 47.4	G, H
38	Dimethylglycine	N−CH_3	2.92(s)	45.9, 55.6	G, H

[a] Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dt, doublet of triples; m, multiplet.

Table 2
Significantly altered metabolites in crayfish gill and hepatopancreas extracts with WSSV infection.

Metabolites	δ (ppm)	Correlation coefficient (r)[a], $\|r\| > 0.602$, $p < 0.05$		
		Gill		Hepatopancreas
		1 h	6 h	6 h
isoleucine	1.01(d)	–	–	0.78
leucine	0.96(d)	–	–	0.81
valine	1.04(d)	–	–	0.78
lactate	1.33(s)	–	–	0.76
alanine	1.49(d)	0.76	0.77	0.74
methionine	2.14(s)	0.65	0.68	0.76
glutamate	2.36(dt)	0.82	–	0.78
phosphorylcholine	3.21(s)	–	0.78	–
betaine	3.27(s)	–	0.83	–
glucose	4.67(d)	0.78	0.96	–
uracil	5.81(d)	0.73	0.94	–
fumarate	6.53(s)	–	0.78	–
tyrosine	6.90(d)	–	0.68	0.75
histidine	7.11(d)	–	0.69	–
phenylalanine	7.43(t)	–	–	–
tryptophan	7.73(d)	–	0.77	–
inosine	8.34(s)	0.92	–	–
threonine	4.26(m)	–	–	0.74
glutamine	2.46(m)	–	–	0.78
phenylalanine	7.43(t)	–	–	0.79
lipid	0.87(m)	–[b]	–[b]	0.72

[a] The correlation coefficients shown are based on OPLS-DA analysis of a two-class model, r values indicate an increase of metabolite in the W group relative to control strain. "–" means the correlation coefficient |r| is less than cutoff value.
[b] The metabolite is not detected.

infection led to significantly increased levels of inosine, glucose and uracil, as well as three amino acids (glutamate, alanine and methionine) in the first 1 h post infection (Table 2; Fig. S3). In comparison with 1 hpi, 6 hpi of active WSSV infection caused more extensive alterations of metabolites. The levels of glucose, uracil, betaine, phosphorylcholine and fumarate were markedly increased, along with five amino acids including alanine, tryptophan, histidine, tyrosine and methionine (Table 2; Fig. S3).

The NMR data manifested that some metabolites concerning important host pathways, including glycolysis (glucose), nucleotide metabolism (uracil and inosine), amino acid biosynthesis (alanine, valine, leucine and isoleucine) and the TCA cycle (fumarate) were up-regulated. Compared with control group, glucose were elevated in gill post infection for 1 h and 6 h (Table 2; Fig. S3), indicating the promotion of glycolysis. Su et al. also detected a significant increase of glucose in WSSV-injected shrimp hemocytes at 12 hpi, what is more, other metabolites related to glycolysis were at a high level as well (Su et al., 2014). Likewise, glucose was elevated in shrimp plasma infected by IHHNV for 7 d, might because it was used as an energy source by shrimp to immediately impulse the activation of immune response to control the virus' replication (Alvarez et al., 2012). Another prominent finding was a rise in the fumarate level after infection for 6 h (Table 2; Fig. S3). The increase of fumarate, which is an intermediate metabolite of TCA cycle means TCA is enhanced. Taken together, we could conclude the energy metabolism was enhanced post infection, which on the one hand showed host immune was activated to eliminate virus, on the other hand the enhanced energy metabolism in return contributed to WSSV infection.

Meanwhile, the results mirrored an enhanced production of amino acid and nucleotide biosynthesis metabolism (uracil and inosine) after WSSV infection (Table 2; Fig. S3). Inosine is a naturally occurring purine formed from the breakdown of adenosine. These boosts occurred presumably because it favored viral genome replication and protein synthesis. Su et al. reported that WSSV infection induced an increase in several of the detected amino acids, including alanine, aspartate, glutamate, histidine, proline and tryptophan in shrimp hemocytes at the early stage (12 hpi). They surmised that the strongly increased amino acid metabolites may be useful for protein synthesis and benefit viral genome replication (Su et al., 2014).

Interestingly, a marked increase in betaine level was observed at 6 hpi in gill (Table 2; Fig. S3). Betaine, one of the most abundant osmolytes in marine invertebrates participates in regulating osmotic balance between intracellular and external saline environments (Burg and Ferraris, 2008; Perrino and Pierce, 2000). It is generally known that gill has a role in transport and osmoregulatory functions (Ali et al., 2015), as the change of some metabolites triggered by the virus infection destroyed the stability of osmotic pressure, the betaine was increased to balance the osmotic pressure. WSSV infection of gill for 6 h also clearly caused a significant up-regulation of phosphorylcholine, which was related to choline metabolism and participated in membrane biosynthesis. As progeny WSSV virions were detected in the nucleus of infected crayfish Hpt cells as early as 12 hpi (Wu et al., 2015), furthermore, the transcript of one of the WSSV envelope protein VP28 started to be synthesized from 3 hpi in red claw crayfish Hpt cells (Liu et al., unpublished data). Other studies also reported that the transcripts of WSSV envelope proteins like wsv238 in crayfish *Cambarus clarkii* gill and VP12 in muscle of crayfish *Cambarus clarkii* could be detected from 2 hpi post WSSV infection (Lan et al., 2006; Li et al., 2013a). So we speculated that the improved choline metabolism might benefit virus envelope synthesis and therefore be in preparation for the progeny virus assembly.

3.3. *WSSV infection induced hepatopancreas metabolomics changes*

^{1}H NMR spectral data sets from hepatopancreas were also analyzed by PCA and OPLS-DA as those of gill extracts. Only one of the models passed the strict test of CV-ANOVA. The model and its corresponding OPLS-DA coefficient-coded loading plots are illustrated in Fig. S4. We did not detect metabolic changes at 1 hpi and 12 hpi, suggesting that WSSV had no significant effect on the hepatopancreas metabolome change after infection for 1 h and

12 h. Compared with the corresponding hepatopancreas collected from CFS-injected group, the WSSV-infected group showed a metabolic response at 6 hpi.

Hepatopancreas presented dramatically elevated metabolites including lactate and lipid, together with ten amino acids (alanine, valine, leucine, isoleucine, glutamate, glutamine, phenylalanine, tyrosine, threonine and methionine) (Table 2; Fig. S4). While Liu et al. found that glucose was elevated in company with acetate, lactate, *N*-acetyl glycoprotein signals, lysine, tyrosine, and lipid was significantly decreased in hepatopancreas of *L. vannamei* post WSSV infection at 48 hpi, which was defined as late infection stage (Liu et al., 2015). Due to the different infection stages, the metabolites were found to be various.

In our present study, the increased lactate which represented the improvement of glycolysis, accompanied with the elevated amino acids indicated energy metabolism was enhanced in hepatopancreas challenged by WSSV, as well as in gill. These variations "satisfied" the virus' requirement for energy during its genome replication and the energetic cost of activated crayfish immune responses (Wang et al., 2012a, 2012b). Combine this and the results of gill, we could hypothesize that glycolysis accounts for rapidly energy supply in gill and hepatopancreas during virus invasion.

Also, WSSV infection in hepatopancreas for 6 h caused a significant up-regulation of lipid (Table 2; Fig. S4), which acted as a source of energy and macromolecular precursors for envelope synthesis. Previous research indicated that hepatitis C virus replication was linked to host cholesterol synthesis (Kapadia and Chisari, 2005). Similarly, a marked increase in fatty acid biosynthesis occurred in MRC-5 fibroblasts after HCMV infection and inhibition of fatty acid biosynthesis suppressed the replication of enveloped viruses: HCMV and influenza A virus (Munger et al., 2008). Research also showed that most of the long chain fatty acids (LCFAs) were at extremely high level in the stomach cells of WSSV-infected shrimp at 24 hpi, what is more, C75 (fatty acid synthase inhibitor) damaged the new virion formation to a certain extent, therefore they speculated that WSSV appeared to induce lipid biosynthesis in order to support viral morphogenesis (Hsieh et al., 2015). Taken together, we could infer that WSSV may trigger the hepatopancreas cell lipid metabolism to fulfill its successful infection.

In summary, we observed the metabolic responses in crayfish gill and hepatopancreas infected by WSSV. The NMR data manifested that some metabolites concerning important host pathways, including glycolysis (e.g. glucose), nucleotide metabolism (e.g. uracil), amino acid biosynthesis (e.g. valine, leucine, isoleucine) and the TCA cycle (e.g. fumarate) were up-regulated. Besides, betaine and lipid also were found at drastically elevated levels. These findings provided a comprehensive insight into the metabolic responses of crayfish gill and hepatopancreas post WSSV infection, and our study also highlights the value of metabolomics in elucidating the host metabolic mechanisms towards pathogen infection.

Acknowledgments

This work was financially supported by National Basic Research Program of China (973 Program, No. 2012CB114403), NSFC (31222056, 41176114, 41476117), SFFPC (2013J06010), Collaborative Innovation Center for Zhejiang Marine High-Efficiency and Healthy Aquaculture, and the K. C. Wong Magna Fund at Ningbo University.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.04.006.

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