Journal of Oceanology and Limnology
Vol. 37 No. 1, P. 361-372, 2019
https://doi.org/10.1007/s00343-019-7268-0

Dynamic metabolite alterations of *Portunus trituberculatus* during larval development*

SHI Ce[1], ZENG Tinglan[1], LI Ronghua[1], WANG Chunlin[1], YE Yangfang[1, 2, **], MU Changkao[1, **]

[1] *Key Laboratory of Applied Marine Biotechnology*, *Ningbo University*, *Chinese Ministry of Education*, *Ningbo 315211*, *China*
[2] *Collaborative Innovation Center for Zhejiang Marine High-efficiency and Healthy Aquaculture*, *Ningbo 315211*, *China*

Received Oct. 8, 2017; accepted in principle Dec. 13, 2017; accepted for publication Jan. 4, 2018

Abstract A mass mortality often occurs from molting to the megalopa stage during the larval development of the swimming crab *Portunus trituberculatus*. Larvae with insufficient nutrient accumulation during the zoeal stages are probably unable to develop into juvenile swimming crabs. However, the nutritional information such as the primary metabolites is scarce for *P. trituberculatus* larvae. The aim of this work is to obtain an insight into the metabolite traits of *P. trituberculatus* at early developmental stages. ^{1}H nuclear magnetic resonance spectroscopy coupled with multivariate data analysis was used to determine how the metabolite profiles shift during larval development in *P. trituberculatus*. Our results show that the trend of total metabolites exhibited a rise from zoea 1 to zoea 3, followed by a drop from zoea 4 to megalopa and recovery during the first juvenile stage. A large-scale depletion of total metabolites in the zoea 4 and megalopa stages suggests a deep depression of metabolic activity, which may be linked to the mass mortality from molting to the megalopa stage. These findings provided essential metabolic information about the larval development of *P. trituberculatus* and important clues for understanding the nutritional requirements of *P. trituberculatus* in early developmental stages.

Keyword: *Portunus trituberculatus*; larval development; metabolite phenotype; nuclear magnetic resonance (NMR)

1 INTRODUCTION

The swimming crab *Portunus trituberculatus* is one of the most common edible marine crabs in China (Hao et al., 2015). To meet the gradual increase in market demand, the swimming crab has been artificially cultured along the coastal regions of East China since the 1990s (Xie et al., 2002). However, the survival rate of larvae is inconsistent and sometimes very low in artificial culture, which has been one of the obstacles for swimming crab hatcheries (Lim and Hirayama, 1991). The larval development of the swimming crab is complex and characterized by four zoeal instars and one megalopa stage before settling in the first-stage crab (Sun et al., 1984). A mass mortality often occurs from molting to the megalopa stage, which is termed as the "critical period" of swimming crab larval development (Lim and Hirayama, 1991). Nutrition has been regarded as one of the key factors for crustacean larvae undergoing these complex processes (Anger, 1998). Larvae with insufficient nutrient accumulation during the zoeal stages are probably unable to develop into juvenile swimming crabs (Dan et al., 2013). Therefore, the chemical composition of crustacean larval bodies has been extensively studied to understand the larval biology and nutrition (Anger, 2001; Ikeda et al., 2011). For example, the compositions of elements, amino acids, and lipids have been investigated in a variety of crustacean larvae, including red frog crab *Ranina ranina* (Minagawa et al., 1993), mud crab

* Supported by the National Natural Science Foundation of China (No. 41673076), the Major Agriculture Program of Ningbo (No. 2017C110007), China Agriculture Research System-CARS48, and the K. C. Wong Magna Fund in Ningbo University
** Corresponding authors: yeyangfang@nbu.edu.cn; muchangkao@nbu.edu.cn

Scylla serrata (Suprayudi et al., 2004), blue swimmer crab *Portunus pelagicus* (Wu et al., 2014), and tropical spiny rock lobster *Panulirus ornatus* (Ikeda et al., 2011; Conlan et al., 2014). To date, this information is scarce for *P. trituberculatus* larvae except regarding oxygen consumption (Morioka et al., 1988) as well as the carbon, nitrogen and phosphorus contents (Lim and Hirayama, 1991).

A body of evidence has indicated that the biochemical compounds give the important contributions to crustacean growth and development. For example, dietary cholesterol is required for better growth of green shore crab *Carcinus maenas* (Ponat and Adelung, 1983) and mud crab *S. serrata* (Sheen, 2000). The nutrients such as lipids and vitamins in diet may also play positive roles in the growth (Sheen and Wu, 1999) and reproduction (Millamena and Quinitio, 2000) of mud crab *S. serrata*. Furthermore, an appropriate ratio of decosahexaenoic acid and eicosapentaenoic acid in diets is important for the normal growth or survival of juvenile swimming crab *P. trituberculatus* (Hu et al., 2016). Besides the second metabolites, increasing evidence has shown that primary metabolites especially amino acids are also critical for crustacean physiology. For instance, arginine and its derivatives are major energy reserves of ATP in the muscles of crustaceans (Hird, 1986), whereas lysine is exclusively required for protein deposition (Boisen, 2003). Furthermore, proline, arginine, and alanine function in the osmoregulation of embryos and adults of the neotropical hololimnetic crab *Dilocarcinus pagei* (Augusto et al., 2007). Glutamic acid, glycine, alanine, and proline also play important roles as intracellular osmolytes when *Macrobrachium amazonicum* shrimp in the post-larvae stage are exposed to brackish water with salinity of 18 (Mazzarelli et al., 2015). In fact, the amino acid balance has been proven to be important to prevent molt death syndrome in juvenile American lobsters (Kean et al., 1985). Although limited information on the function of amino acids in *P. trituberculatus* larvae is available, these primary metabolites are as critical to the growth and successful metamorphosis of swimming crab larvae as lipids are in crustacean larvae (Suprayudi et al., 2004; Holme et al., 2009; Wu et al., 2014). However, most studies performed so far on crustacean larvae commonly overlook these primary metabolites.

In the present study, we used a nuclear magnetic resonance (NMR)-based metabolomic approach coupled with multivariate statistical analysis to analyze the metabolomic profiles of *P. trituberculatus* larvae at six specific stages. The main objectives of this work are to (1) unravel the changes in the metabolic phenotype over the course of the swimming crab ontogeny and (2) understand the metabolic traits of swimming crab at early developmental stages.

2 MATERIAL AND METHOD

2.1 Chemicals and reagents

Methanol, dipotassium hydrogen phosphate trihydrate and sodium dihydrogen phosphate dihydrate were purchased from China National Pharmaceutical Group (Beijing, China). Sodium 3-trimethylsilyl [2,2,3,3-$^2D_4$] propionate (TSP, internal standard for NMR analysis) and deuterated water (D_2O, 99.9% in D, field lock signal for NMR analysis) were purchased from Cambridge Isotope Laboratories (FL, Miami, USA). Phosphate buffer (K_2HPO_4/NaH_2PO_4, 0.15 mol/L, pH 7.54) was prepared in water containing 50% D_2O and 0.001% TSP (w/v) (Xiao et al., 2009).

2.2 Experimental system and animals

Several artificially cultured copulated female swimming crabs were reared in one indoor 30-m^3 cement pond sanded with approximately 10 cm thickness at the Jing-Ye Nursery Farm (Ningbo, China). The salinity and water temperature were maintained at 24±1 and 27±2°C, respectively. The crabs were fed daily on a diet with a combination of fresh mussels and frozen fishes, at a ratio of 5%–8% of the total body weight. An ovigerous crab with late-stage eggs (hatching within 2 days) was selected and transferred to a plastic basket equipped with aeration. This plastic basket was placed in the cement pond with free water circulation in and out of the basket. The ovigerous crab was not fed until the larvae hatched.

Once swimming zoea 1 (Z1) larvae were released, the larvae freely scattered into the cement pond and the ovigerous crab was moved away from the cement pond. The larval culture was conducted according to the conventional protocol (Wu et al., 2014) with some modification. In detail, rotifers (*Branchionus plicatilis*) 30–40/mL and microalga (*Platymonas subcordiformis*) (5–10)×10^4/mL were provided as food every two hours for the crab larvae in the Z1 and zoea 2 (Z2) stages whereas *Artemia* nauplii 2–3 ind./mL were provided to the zoea 3 (Z3), zoea 4 (Z4), and megalopa (M) stages. The seawater in the rearing

pond was not renewed during the whole culture period.

To determine the metabolite composition of the various stages of *P*. *trituberculatus* larvae, the newly hatched Z1, Z2, Z3, Z4, M, and the newly settled first-stage crabs (C) were sampled. The samplings of the Z1, Z2, Z3, Z4, M, and C crabs were carried out when 70%–80% of the population had t molted to the desired developmental stage, which was visually determined according to Sun et al. (1984). All samples were immediately rinsed with filtered seawater, snap-frozen in liquid nitrogen and then stored at -80°C for metabolomic analysis. To investigate the crab metabolite alterations with the larval development, the same stage crabs were divided into ten portions, with each portion as one replicate. Each portion of the Z1, Z2, Z3, Z4, M, and C group contained approximately 2300, 430, 350, 530, 180, and 12 larvae, respectively.

2.3 Tissue extraction

Crab samples for NMR were prepared according to the previously defined protocol (Ye et al., 2014). Briefly, each crab sample was extracted with aqueous methanol (methanol/water=2:1) twice. After removal of methanol in vacuo, the crab extract was lyophilized. The resultant powder was dissolved in phosphate buffer.

2.4 NMR spectroscopy

All NMR spectra were recorded at 298 K on a Bruker Avance III 600 MHz NMR spectrometer equipped with an inverse detection cryogenic probe (Bruker BioSpin, Rheinstetten, Germany). A standard one-dimensional NMR experiment with water pre-saturation was performed for all crab extracts using a pulse sequence (recycle delay-90°-t_1-90°-t_m-90°-acquisition). The recycle delay was set to 2 s and the mixing time (t_m) to 100 ms. A t_1 was fixed to 6.5 μs. The 90° pulse length was adjusted to approximately 10 μs. For each crab sample, 64 scans were recorded in 32 k data points with a spectral width of 20×10^{-6}. In addition, standard acquisition parameters were employed in the acquisition of the two-dimensional NMR experiments on selected samples for metabolite identification, including ^{1}H-^{1}H correlation spectroscopy, ^{1}H-^{1}H total correlation spectroscopy, ^{1}H-^{13}C heteronuclear multiple bond correlation, and ^{1}H-^{13}C heteronuclear single quantum correlation spectra (Aue, 1976a, b; Braunschweiler and Ernst, 1983).

2.5 NMR data reduction and multivariate data analysis

Prior to Fourier transformation, an exponential line broadening function of 0.5 Hz was utilized to free induction decays. All ^{1}H NMR spectra were manually corrected for phase and baseline distortion, and calibrated to proton signal from TSP at δ 0.0 with the TOPSPIN software package (v2.0, Bruker Biospin, Germany). The spectral region δ 0.7–9.3 was selected for multivariate data analysis. The spectral regions of water (δ 4.70–5.20) and methanol (δ 3.35–3.37) were discarded to eliminate variations in water suppression and methanol removal. The remaining spectral region of δ 0.7–9.3 was segmented into regions of 0.004×10^{-6}. The obtained segments were further normalized to the wet weight of the corresponding crab sample to reduce variations in the sample weight inconsistencies.

For multivariate data analysis, the ^{1}H NMR spectral datasets were imported to SIMCA-P^{+} software (v12.0, Umetrics AB, Umeå, Sweden). To detect the general trend and sample outliers, an unsupervised principal components analysis (PCA) with mean-centered scaling was first performed. Each point represented one sample on the PCA scores plot. Subsequently, a supervised orthogonal projection to latent structure-discriminant analysis (OPLS-DA) was further applied to analyze the ^{1}H NMR spectral data using a unit variance method (Trygg and Wold, 2002; Van Den Berg et al., 2006). The validity of each OPLS-DA model was checked using a seven-fold cross validation method, and Q^2s was calculated to assess the quality of the models (Trygg et al., 2007). A cross validation-analysis of variance (CV-ANOVA) approach was also utilized to further check the model validity with $P<0.05$ as the significant level (Eriksson et al., 2008). To improve the interpretability of the OPLS-DA model, back-scaled transformations of the loadings of each variable contributing to the model were conducted (Cloarec et al., 2005). Subsequently, the coefficient plot of the OPLS-DA model was illustrated to highlight the significantly different metabolites between the two groups using a MATLAB script with some modifications. The color code of the correlation coefficient (R) of the metabolite indicated the weight of the discriminatory variable. A cut-off value of 0.602 was used in this study and the metabolites with an absolute R value above 0.602 were considered statistically significant ($P<0.05$).

To illustrate the metabolite variations with larval development, the relative changes of the typical metabolites of swimming crab at the Z2, Z3, Z4, M,

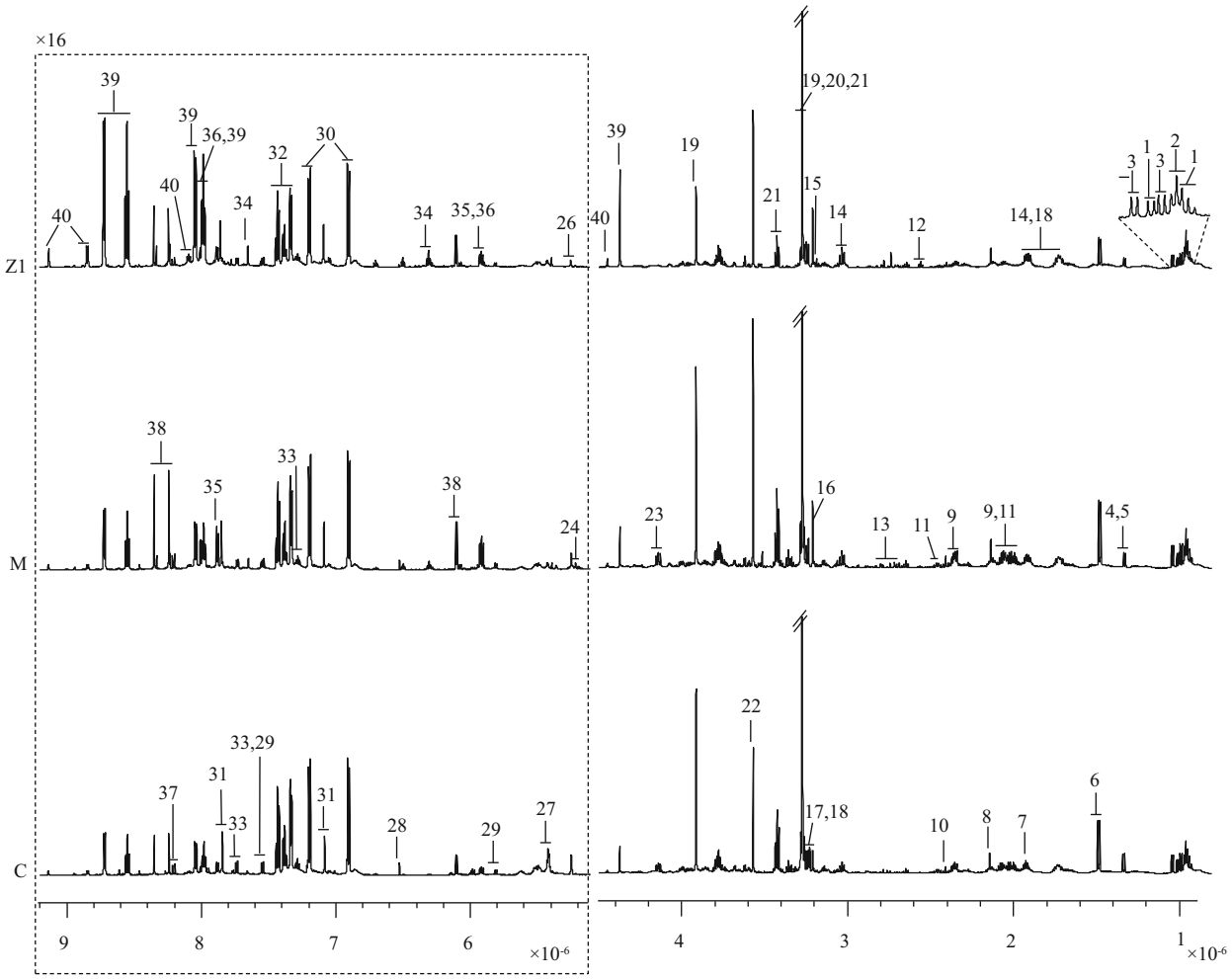

Fig.1 Three typical 600 MHz ^{1}H NMR spectra of *P. trituberculatus* extracts at zoea 1 (Z1), megalopa (M), and the first juvenile (C) stages

Compared to the chemical shift range at δ 0.8–4.5, the spectra in the region δ 5.1–9.2 are displayed at 16-fold magnification. Resonance assignments are provided in Supplementary Table S1. Keys: 1: isoleucine; 2: leucine; 3: valine; 4: lactate; 5: threonine; 6: alanine; 7: acetate; 8: methionine; 9: glutamate; 10: succinate; 11: glutamine; 12: β-alanine; 13: aspartate; 14: lysine; 15: choline-O-sulfate; 16: phosphorylcholine; 17: choline; 18: arginine; 19: betaine; 20: trimethylamine-N-oxide; 21: taurine; 22: glycine; 23: proline; 24: trehalose; 25: β-glucose; 26: α-glucose; 27: maltose; 28: fumarate; 29: uracil; 30: tyrosine; 31: histidine; 32: phenylalanine; 33: tryptophan; 34: thymidine; 35: uridine; 36: guanosine; 37: hypoxanthine; 38: adenosine; 39: 2-pyridinemethanol; 40: trigonelline.

and C stages were calculated against those at the Z1 stage in the form of $(C_i–C_{Z1})/C_{Z1}$, where C_i represented the metabolite level in the Z2, Z3, Z4, M, or C group and C_{Z1} represented that in the Z1 group.

3 RESULT

3.1 Metabolite identification from ^{1}H NMR spectra of crab extracts

Figure 1 shows representative ^{1}H NMR spectra of aqueous extracts obtained from swimming crab larvae. Both the ^{1}H and ^{13}C NMR resonances for detectable metabolites were assigned according to the reference to literature (Fan, 1996; Ye et al., 2014) and further confirmed by a series of two-dimensional NMR spectra. The detectable metabolites in the ^{1}H NMR spectra of swimming crab larvae comprised amino acids, nucleosides and nucleotides, organic acids, sugars, and organic amines. Some metabolite variations were clearly observed from the visual inspection of these NMR spectra. For example, an elevation in the betaine level was observed in both the M and C groups compared to those in the Z1 group, which was accompanied by a reduction in the 2-pyridinemethanol and trigonelline levels.

3.2 Metabolite changes in crab extract with larval development

The overview of the total metabolite changes in swimming crab throughout larval development was

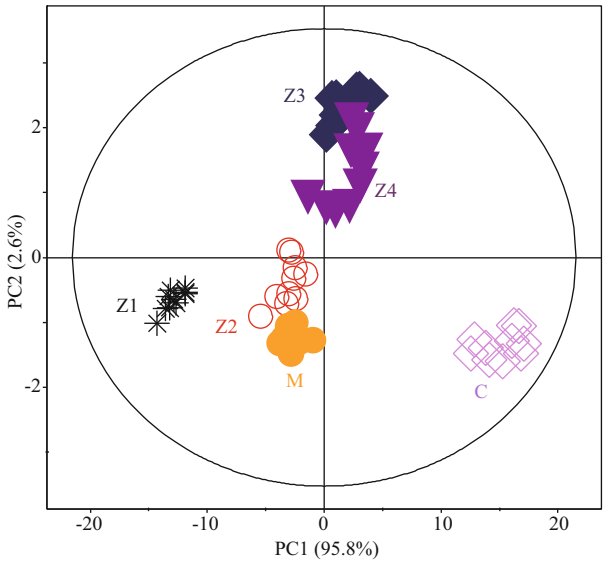

Fig.2 PCA scores plot constructed from ^{1}H NMR spectra of aqueous larval extracts of *P. trituberculatus* at zoea 1 (Z1, black stars), zoea 2 (Z2, red circles), zoea 3 (Z3, blue diamonds), zoea 4 (Z4, violet inverted triangles), megalopa (M, orange dots), and the first juvenile (C, pink open diamonds) stages

constructed using a PCA of all the normalized ^{1}H NMR data collected. The averaged PCA scores were calculated for the first two principal components (PC1 and PC2), which jointly explained 98.4% of the total variance (Fig.2). The PCA scores plot showed a clear separation amongst the six crab groups and displayed a larval development-dependent trend in the metabolic profiles of swimming crab. A substantial variation in the metabolic profiles of swimming crab existed from the Z1 to Z3 stages, owing to the discrimination amongst these three groups based on the PC1. Thereafter, one turn was observed from the Z4 to the megalopa stage. Finally, a considerable variation was observed from the megalopa to the first juvenile stage. Such changes in the metabolic profiling indicate different nutritional requirements of swimming crab in different larval development stages.

Detailed information on the larval development-related metabolic changes was mined from the OPLS-DA comparisons of the ^{1}H NMR profiles of swimming crabs derived from two neighboring development stages. Five OPLS-DA models generated large Q^2 values with small P values obtained from the CV-ANOVA, suggesting a distinct metabolite difference between two neighboring stages (Figs.3, S1). Compared to Z1, the metabolic profiles of swimming crabs in Z2 were highlighted by higher levels of lactate, fumarate, choline, phosphorylcholine, betaine, taurine, trimethylamine-N-oxide (TMAO), glucose, maltose, uracil, uridine, hypoxanthine, inosine, and a range of amino acids, including isoleucine, leucine, valine, alanine, glutamate, glutamine, lysine, proline, phenylalanine, and tryptophan, whereas lower levels of 2-pyridinemethanol and trigonelline were observed (Figs.3d, 4). The development to Z3 also resulted in an increase in lactate, fumarate, betaine, taurine, TMAO, glucose, maltose, uridine, inosine and many amino acids, including isoleucine, leucine, valine, alanine, glutamate, lysine, proline, phenylalanine, and tryptophan, together with a decrease in trigonelline compared to Z2. Furthermore, more amino acids, including methionine, glycine, tyrosine, and histidine, were significantly accumulated in larvae at the Z3 stage (Figs.S1c, 4). However, the tendency of metabolite accumulation stopped at the Z4 stage. Conversely, a marked decrease was observed in Z4 relative to Z3 in terms of the levels of choline, maltose, thymidine, inosine, trigonelline and a range of amino acids including isoleucine, leucine, valine, methionine, glutamine, lysine, tyrosine, histidine, and phenylalanine. Simultaneously, the phosphorylcholine level was much higher in Z4 (Figs.3e, 4). More metabolites were depleted in the megalopa stage compared to Z4, including lactate, fumarate, phosphorylcholine, betaine, taurine, glucose, uracil, hypoxanthine, inosine, 2-pyridinemethanol, and trigonelline, as well as a range of amino acids, including isoleucine, leucine, valine, alanine, glutamate, lysine, tyrosine, histidine, phenylalanine, and tryptophan. Moreover, no significantly increased metabolites were observed in the Z4 and megalopa stages (Figs.S1d, 4). However, the second increase in the metabolite levels of swimming crabs was observed after metamorphosis to the first juvenile stage, which was highlighted by higher levels of lactate, fumarate, betaine, taurine, TMAO, glucose, maltose, hypoxanthine, 2-pyridinemethanol, and trigonelline, as well as a range of amino acids, including isoleucine, leucine, valine, alanine, methionine, lysine, tyrosine, histidine, phenylalanine, and tryptophan (Figs.3f, 4). The r values of significantly altered metabolites associated with larval development are shown in Fig.4 and listed in Table S2.

The temporal changes of some metabolites with larval development by the concentration ratios from swimming crabs in the later five development stages relative to those at the Z1 stage are illustrated in Fig.5. Overall, a significant elevation in the levels of glucose, fumarate, betaine, taurine, phosphorylcholine, uracil, uridine, hypoxanthine, inosine, and many amino

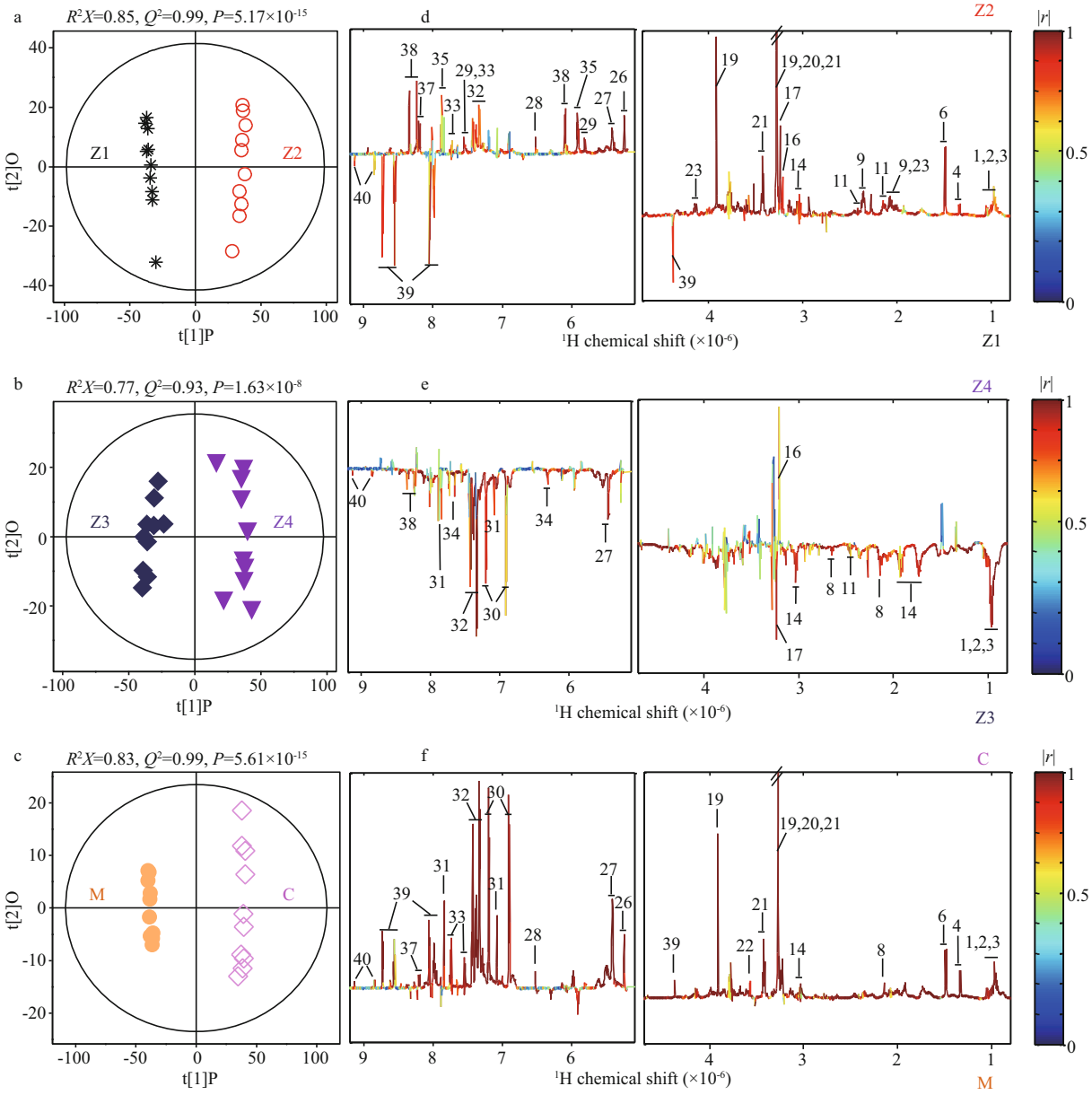

Fig.3 OPLS-DA scores plots (a, b, & c) and corresponding color-coded correlation coefficient loadings plots (d, e, & f) derived from NMR data for *P. trituberculatus* extracts associated with larval development

Z1: zoea 1, black stars; Z2: zoea 2, red circles; Z3: zoea 3, blue diamonds; Z4: zoea 4, violet inverted triangles; M: megalopa, orange dots; C: the first juvenile, pink open diamonds; A: Z2 vs Z1; B: Z4 vs Z3; C: L vs M. See Fig.1 for metabolite identification key.

acids, including isoleucine, valine, alanine, glutamate, glutamine, methionine, glycine, tyrosine, and phenylalanine, was observed in the later five stages compared to those at the Z1 stage. However, such an elevation shifted with larval development. The highest levels of most of these elevated metabolites were mainly observed in Z3. By contrast, the highest levels of alanine, fumarate, phosphorylcholine, uracil, and hypoxanthine were observed in Z4, whereas glycine, tyrosine, phenylalanine, betaine, and taurine were elevated in the first juvenile stage. Contrary to these increased metabolites, 2-pyridinemethanol and trigonelline presented a lasting depletion, as highlighted by 57.9% and 73.9% decreases in the megalopa stage, respectively. In addition, we also noted a significant fluctuation in the levels of leucine, lysine, tryptophan, histidine, and maltose during larval development.

4 DISCUSSION

This study showed the changes in the metabolic phenotype of *P. trituberculatus* during larval development. We found that the metabolic phenotype of *P. trituberculatus* larvae significantly changed

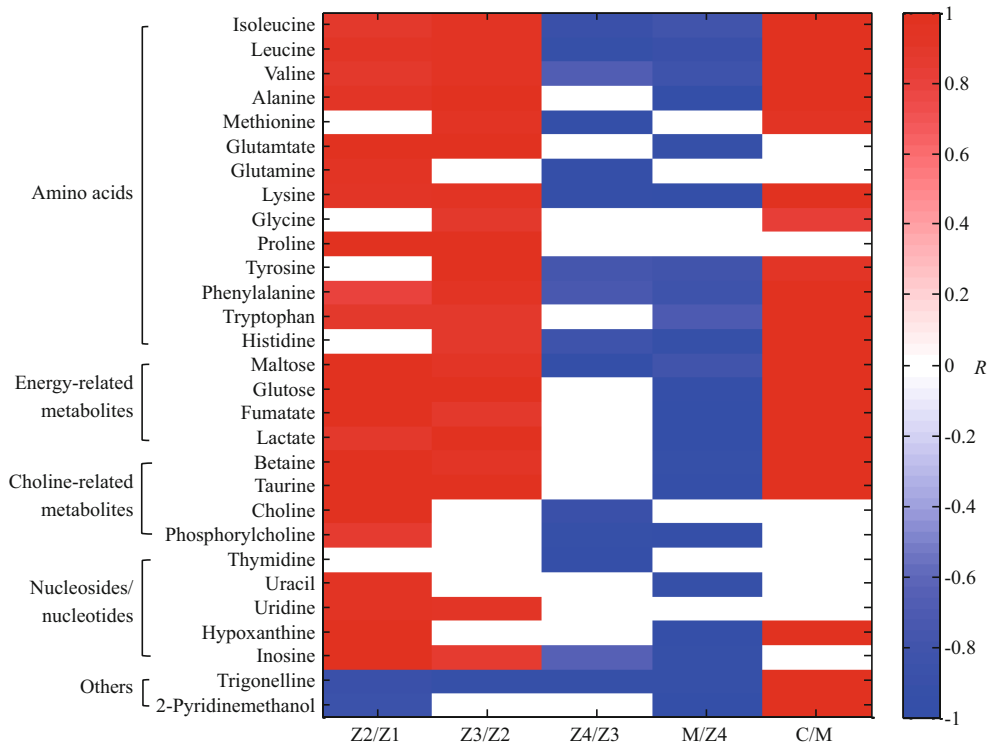

Fig.4 Dynamic alterations of the values of the correlation coefficients derived from significantly altered metabolites of *P. trituberculatus* associated with larval development

The color indicates a correction coefficient as scaled on the right-hand side. Red denotes an increase in the metabolite levels of swimming crab at this time point against the level at its proximate former one, whereas blue denotes a decrease.

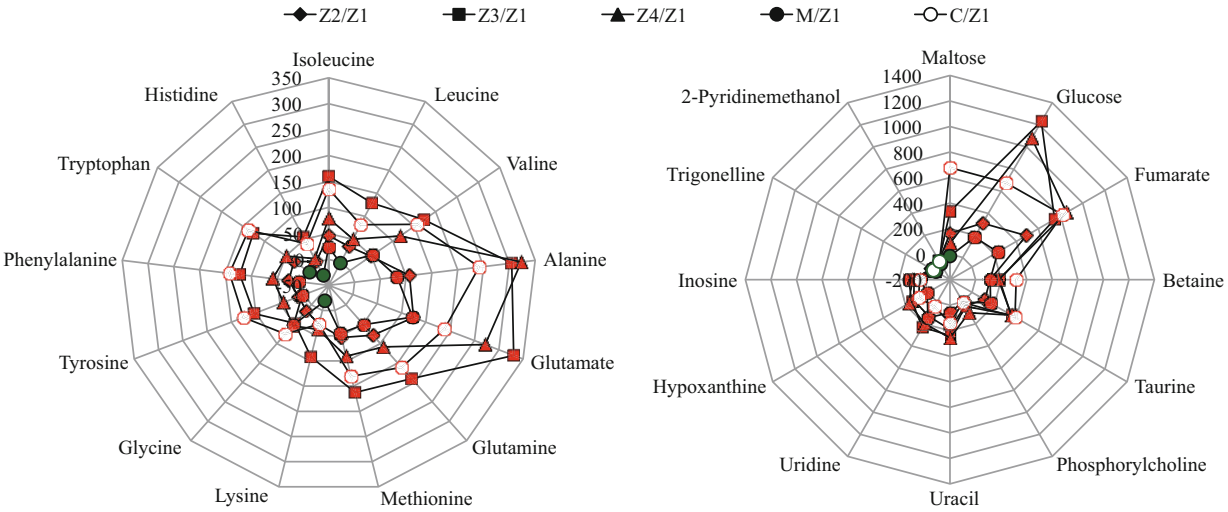

Fig.5 Ratios of changes for the metabolites of *P. trituberculatus* at the later development stage against those at zoea 1 stage

Z1: zoea 1; Z2: zoea 2; Z3: zoea 3; Z4: zoea 4; M: megalopa; C: first juvenile. Red denotes an increase in the metabolite levels of swimming crab at the Z2, Z3, Z4, M, or C stage relative to those at the Z1 stage, whereas green denotes a decrease.

during larval development, as highlighted by a significant reduction in the metabolite levels from the Z4 to the M stage. These metabolic changes are involved in many pathways, including energy metabolism, amino acid metabolism, nucleic acid metabolism, osmoregulation, and antioxidation (Fig.6).

A significant fluctuation in the amino acid levels was observed during larval development, with a dramatic rise from Z1 to Z3, sharp drop from Z4 to megalopa as well as a clear recovery in the first-stage crab. However, there was only a minor difference in

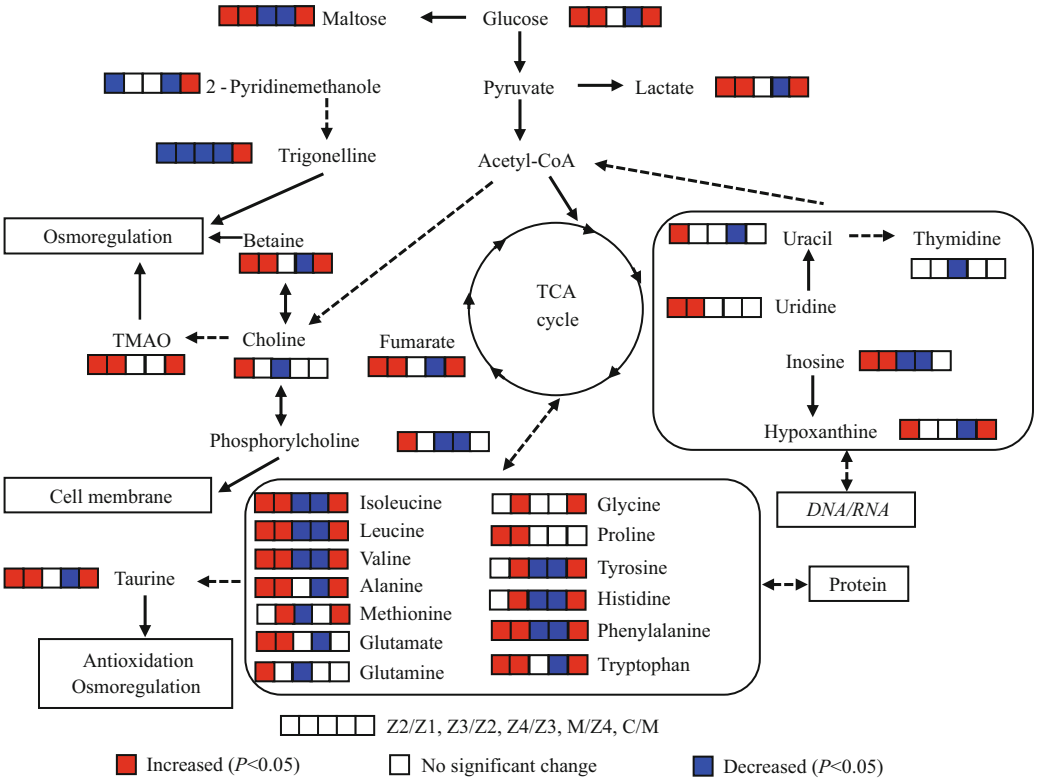

Fig.6 Summary of dynamic metabolic changes of *P. trituberculatus* during larval development

Metabolites with red or blue boxes represent significant increases or decreases in levels, respectively, at the significance level of $P<0.05$ at this time point against the level at its proximate former stage. Metabolites with empty boxes represent no significant increases or decreases in levels.

the number of changed amino acids among the different ontogeny stages. This observation is in agreement with the results reported by Andrés et al. (2010), who found a dramatic shift in the amino acid levels in the spider crab *Maja brachydactyla* during larval development. The Andrés et al. (2010) study also found that the levels of most of the amino acids also increased from the Z1 to the Z2 stage and decreased from the Z2 to the megalopa stage. However, some differences were found between the two studies, with a significant accumulation of 10 amino acids and no decrease in amino acids in the first juvenile stage in this study, whereas Andrés et al. (2010) found a decrease in 14 amino acids and an increase in arginine, aspartate, and serine. In fact, threonine, arginine, and aspartate exhibited no significant changes, and serine and cystine were not detected in this study, whereas these amino acids shifted significantly in the study by Andrés et al. (2010). In the present study, a differing dietary regime was designed for swimming crab larvae, in which rotifers and microalga were used for the Z1 and Z2 stages, whereas *Artemia* nauplii were used for the Z3, Z4, and megalopa stages. Nutrition has been found to affect the amino acid profile of some planktonic crustacean species (Brucet et al., 2005). Such variations in the amino acid profile seem to be related to the larval diet although the change in amino acids appears to occur on approximately a three-day-delay (Lim and Hirayama, 1991). However, other evidence indicates that a close relationship between the diet and amino acid composition does not exist in crustacean larvae, including mud crab *S. serrata* (Peñaflorida et al., 2004) and spider crab *M. brachydactyla* (Andrés et al., 2010). Thus, the variation in the amino acid profile in *P. trituberculatus* larvae probably results from the ontogeny advancement. In fact, under similar dietary condition, the major fatty acid content of *P. pelagicus* larvae was found to increase from the newly hatched zoea 1 stage to the newly molted zoea 4 stage, decrease from the newly molted zoea 4 stage to the newly molted megalopa stage, and increase again from the newly molted megalopa stage to the newly molted first crabs (Wu et al., 2014). In Wu et al. (2014) study, the highest fatty acid contents seemed to occur at zoea 4 in *P. pelagicus*. However, the highest levels probably occurred at zoea 2 or zoea 3 because the fatty acid contents of *P. pelagicus* at these two stages were not detected. Nevertheless, in this study, the highest levels

of almost all of the detected metabolites occurred at *P*. *trituberculatus* Z3 stage.

From the view of metabolism, a significant rise in the amino acid levels from Z1 to Z3 may indicate a promoted synthesis of amino acids. The amino acids may be generated by proteolysis of endogenous proteins, as swimming crab eggs in the heartbeat stage contains approximately 6.42 μg of proteins, which can be used to meet the amino acid requirements of larval crab at the Z1 and Z2 stages (Chen et al., 2007). In this study, the accumulation of most amino acids lasted to Z3, which may be attributed to the endogenous proteins. Of course, the exogenous proteins from the diet should contribute to amino acid pool of swimming crab larvae. Subsequently, well-defined drops were observed in the isoleucine, leucine, valine, methionine, glutamine, tyrosine, histidine, and phenylalanine levels from the Z4 stage and the alanine, glutamate, tryptophan levels from the megalopa stage (Fig.4). Obviously, the megalopa larvae had the lowest levels of amino acids (Fig.5). The depletion of so many amino acids may indicate the increased utilization of amino acids. After metamorphosis (the first juvenile), the depletion of amino acids was alleviated with the levels recovering to the Z3 levels (Fig.5). It seems that the first juvenile crab has a higher available amino acid pool after settling. The recovery of the amino acid levels is extremely significant as an amino acid balance is necessary for the survival and growth of crustaceans, especially for newly hatched larvae (Kean et al., 1985; Brucet et al., 2005). Whether the mass mortality from molting to the megalopa stage is related to the sharply depleted amino acid pool deserves further research. However, it is well known that amino acids are crucial for maintaining the structure and metabolism of the cells (Brucet et al., 2005). First of all, amino acids are the structural units that make up proteins. Secondly, glucogenic amino acids can be converted into glucose through gluconeogenesis. In addition, taurine, which has many fundamental biological roles, is synthesized from cysteine via the cysteine sulfinic acid pathway. Free amino acids have other important physiological functions, such as intracellular isosmotic regulation and signal transduction (Haond et al., 1999). Therefore, dietary amino acid supplementation has been considered in recent crustacean nutritional studies. For example, dietary supplementation of single amino acids, such as lysine (Jin et al., 2015) and arginine (Jin et al., 2016), improved the growth performance and feed utilization of juvenile swimming crabs. Furthermore, nutrition is regarded as one of the key factors for crustacean larvae undergoing the “critical period” of larval development (Anger, 1998). Therefore, dietary supplementation of amino acids may be helpful for the Z4 and megalopa larvae to successfully metamorphose into juvenile crabs. However, our metabolomic results also suggest that a range of amino acids may play a joint role during larval development. Therefore, dietary supplementation of multiple amino acids may be much better than supplementation with a single amino acid to maintain an amino acid balance in swimming crab larvae.

In addition, while arginine was detected in this study, no significant change was observed in the arginine level throughout larval development. Although arginine is thought to be an essential amino acid for all crustacean species (Committee on the Nutrient Requirements of Fish and Shrimp et al., 2011) and required by juvenile swimming crab (Jin et al., 2016), our observation suggests that the extra amount of this amino acid does not seem to be needed by swimming crab larvae under this dietary condition.

Another prominent finding was a trend similar to amino acids in the levels of a range of other metabolites as during larval development (Figs.3, 4). These metabolites include lactate, fumarate, glucose, maltose, choline, phosphorylcholine, uracil, hypoxanthine, and inosine, which are mainly utilized for energy metabolism (lactate, fumarate, glucose, and maltose), membrane-related metabolism (choline and phosphorylcholine), and nucleic acid metabolism (uracil, hypoxanthine, and inosine). For instance, some energy-related metabolites, including glucose, fumarate, and lactate, were highly increased at the Z2 and Z3 stages, decreased at the megalopa stage, and increased again at the first-crab stage. These observations may indicate significantly changes in glycolysis, tricarboxylic acid cycle activity, and lactate fermentation during larval development. A similar phenomenon also occurred in the levels of choline, phosphorylcholine, uracil, uridine, hypoxanthine, and inosine, which indicated a significant shift in the membrane-related metabolism and nucleic acid metabolism of swimming crab larvae. Due to the lack of data available concerning the variations of these primary metabolites and the limited information concerning their nutrition in crustaceans, the role that these metabolites play during larval development needs to be elucidated in the future. However, a significant drop in the levels of

these metabolites at the Z4 and megalopa stages may indicate a broad range of metabolic depression at these two stages of swimming crab larvae. Likely, there is an urgent requirement of swimming crab at the Z4 and/or megalopa stages for these metabolites. If so, dietary supplementation of these metabolites may be considered along with amino acids.

In addition, we noted a distinct trend in the trigonelline and 2-pyridinemethanol levels from the other metabolites detected in this study. The trigonelline level was reduced from the Z2 to the megalopa stage but recovered in the first juvenile stage. Trigonelline is a type of alkaloid, which is formed by the methylation of nicotinic acid (vitamin B_3) (Upmeier et al., 1988). Trigonelline can function as a compatible osmolyte in the green shore crab *C. maenas* under the highest seawater CO_2 stress (Hammer et al., 2012) and in adult swimming crab in response to low salinity (Ye et al., 2014). In this study, a decrease in the trigonelline level likely indicates a decreased role of this nicotinic acid betaine in the intracellular osmotic regulation of swimming crab larvae. However, other compatible osmolytes, including TMAO (Yancey et al., 2002), betaine (Preston, 1993), taurine (Avella et al., 2009), alanine, and proline (Brucet et al., 2005), also significantly changed during larval development. These observations possibly suggest a joint contribution of a range of functionally interchangeable osmolytes (Yancey, 2005) to the balance of the osmotic equilibrium in swimming crab larvae. In addition, we also noted a decrease in the 2-pyridinemethanol level from the Z2 to the megalopa stage followed by a recovery in juvenile crabs. A significant accumulation of 2-pyridinemethanol has been found in the muscle of swimming crabs as a response to low salinity (Ye et al., 2014). It seems that this picolinic acid-related compound plays an important role in swimming crab larvae although little information exists on its physiological function in marine crustaceans.

5 CONCLUSION

In summary, our study shows that the metabolic phenotype of *P. trituberculatus* were significantly different among various stages of larval development. The overall trend of metabolites exhibited a rise from Z1 to Z3, drop from Z4 to megalopa and recovery in the first juvenile stage. Several metabolites, such as a range of amino acids, glucose, betaine, and inosine, were good indicators of the metabolic activity of swimming crab larvae. Specifically, a large-scale depletion of metabolites in the Z4 and megalopa stages suggests a deep depression of metabolic activity, which may be linked to the mass mortality from molting to the megalopa stage. Finally, our metabolomic results provide important clues for understanding the nutritional requirements of swimming crab in early developmental stages and for optimizing the dietary regimes for *P. trituberculatus* larvae.

References

Andrés M, Estévez A, Hontoria F, Rotllant G. 2010. Differential utilization of biochemical components during larval development of the spider crab *Maja brachydactyla* (Decapoda: Majidae). *Marine Biology*, **157**(10): 2 329-2 340.

Anger K. 1998. Patterns of growth and chemical composition in decapod crustacean larvae. *Invertebrate Reproduction & Development*, **33**(2-3): 159-176.

Anger K. 2001. *The Biology of Decapod Crustacean Larvae*. A.A. Balkem, Lisse.

Aue W P, Bartholdi E, Ernst R R. 1976a. Two-dimensional spectroscopy. Application to nuclear magnetic resonance. *The Journal of Chemical Physics*, **64**(5): 2 229-2 246.

Aue W P, Karhan J, Ernst R R. 1976b. Homonuclear broad band decoupling and two-dimensional *J*-resolved NMR-spectroscopy. *The Journal of Chemical Physics*, **64**(10): 4 226-4 227.

Augusto A, Greene L J, Laure H J, McNamara J C. 2007. Adaptive shifts in osmoregulatory strategy and the invasion of freshwater by brachyuran crabs: evidence from *Dilocarcinus pagei* (trichodactylidae). *Journal of Experimental Zoology*, **307**A(12): 688-698.

Avella M, Ducoudret O, Pisani D F, Poujeol P. 2009. Swelling-activated transport of taurine in cultured gill cells of sea bass: physiological adaptation and pavement cell plasticity. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology*, **296**(4): R1 149-R1 160.

Boisen S. 2003. Ideal dietary amino acid profiles for pigs. *In*: D'Mello J P F ed. Amino Acids in Animal Nutrition. CABI, Edinburgh, United Kingdom. p.103-123.

Braunschweiler L, Ernst R R. 1983. Coherence transfer by isotropic mixing: application to proton correlation spectroscopy. *Journal of Magnetic Resonance* (1969), **53**(3): 521-528.

Brucet S, Boix D, López-Flores R, Badosa A, Quintana X D. 2005. Ontogenic changes of amino acid composition in planktonic crustacean species. *Marine Biology*, **148**(1): 131-139.

Chen S L, Wu X G, Cheng Y X, Wang C L, Zhu D F, Zhou B, Wang J F, Gong L J. 2007. Changes of proximate biochemical composition and energy source during embryonic development of swimming crab, *Portunus trituberculatus*. *Journal of Fishery Sciences of China*, **14**(2): 229-235. (in Chinese with English abstract)

Cloarec O, Dumas M E, Trygg J, Craig A, Barton R H, Lindon J C, Nicholson J K, Holmes E. 2005. Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in ^{1}H NMR spectroscopic metabonomic studies. *Analytical Chemistry*, **77**(2): 517-526.

Committee on the Nutrient Requirements of Fish and Shrimp, Board on Agriculture and Natural Resources, National Research Council. 2011. Nutrient Requirements of Fish and Shrimp. National Academies Press, Washington, DC. p.57-92.

Conlan J A, Jones P L, Turchini G M, Hall M R, Francis D S. 2014. Changes in the nutritional composition of captive early-mid stage *Panulirus ornatus* phyllosoma over ecdysis and larval development. *Aquaculture*, **434**: 159-170.

Dan S, Kaneko T, Takeshima S, Ashidate M, Hamasaki K. 2013. Variations in larval morphology and their relationships to survival during mass seed production by the swimming crab, *Portunus trituberculatus* (Brachyura, Portunidae). *Aquaculture*, **414**-**415**: 109-118.

Eriksson L, Trygg J, Wold S. 2008. CV-ANOVA for significance testing of PLS and OPLS® models. *Journal Chemometrics*, **22**(11-12): 594-600.

Fan T W M. 1996. Metabolite profiling by one-and two-dimensional NMR analysis of complex mixtures. *Progress in Nuclear Magnetic Resonance Spectroscopy*, **28**(2): 161-219.

Hammer K M, Pedersen S A, Størseth T R. 2012. Elevated seawater levels of CO_2 change the metabolic fingerprint of tissues and hemolymph from the green shore crab *Carcinus maenas*. *Comparative Biochemistry and Physiology Part D*: *Genomics and Proteomics*, **7**(3): 292-302.

Hao G J, Lin F, Mu C K, Li R H, Yao J Y, Yuan X M, Pan X Y, Shen J Y, Wang C L. 2015. SNP E4-205 C/T in C-type lectin of *Portunus trituberculatus* is association with susceptibility/resistance to *Vibrio alginolyticus* challenge. *Aquaculture*, **442**: 125-131.

Haond C, Bonnal L, Sandeaux R, Charmantier G, Trilles J P. 1999. Ontogeny of intracellular isosmotic regulation in the European lobster *Homarus gammarus* (L.). *Physiological and Biochemical Zoology*, **72**(5): 534-544.

Hird F J R. 1986. The importance of arginine in evolution. *Comparative Biochemistry and Physiology Part B*: *Comparative Biochemistry*, **85**(2): 285-288.

Holme M H, Zeng C S, Southgate P C. 2009. A review of recent progress toward development of a formulated microbound diet for mud crab, *Scylla serrata*, larvae and their nutritional requirements. *Aquaculture*, **286**(3-4): 164-175.

Hu S, Wang J, Han T, Li X, Jiang Y, Wang C. 2016. Effects of dietary DHA/EPA ratios on growth performance, survival and fatty acid composition of juvenile swimming crab (*Portunus trituberculatus*). *Aquaculture Research*, **48**(3): 1 291-1 301.

Ikeda T, Smith G, McKinnon A D, Hall M. 2011. Metabolism and chemical composition of phyllosoma larvae, with special reference to the tropical rock lobster *Panulirus ornatus* (Decapoda; Palinuridae). *Journal of Experimental Marine Biology and Ecology*, **405**(1-2): 80-86.

Jin M, Wang M Q, Huo Y W, Huang W W, Mai K S, Zhou Q C. 2015. Dietary lysine requirement of juvenile swimming crab, *Portunus trituberculatus*. *Aquaculture*, **448**: 1-7.

Jin M, Zhou Q C, Wang M Q, Huo Y W, Huang W W, Mai K S. 2016. Dietary arginine requirement of juvenile swimming crab, *Portunus trituberculatus*. *Aquaculture Nutrition*, **22**(6): 1 174-1 184.

Kean J C, Castell J D, Boghen A G, D'Abramo L R, Conklin D E. 1985. A re-evaluation of the lecitihin and cholesterol requirements of juvenile lobster (*Homarus americanus*) using crab protein-based diets. *Aquaculture*, **47**(2-3): 143-149.

Lim B K, Hirayama K. 1991. Growth and elemental composition (C,N,P) during larval developmental stages of mass-cultured swimming crab *Portunus trituberculatus*. *Marine Ecology Progress Series*, **78**: 131-137.

Mazzarelli C C M, Santos M R, Amorim R V, Augusto A. 2015. Effect of salinity on the metabolism and osmoregulation of selected ontogenetic stages of an amazon population of *Macrobrachium amazonicum* shrimp (Decapoda, Palaemonidae). *Brazilian Journal of Biology*, **75**(2): 372-379.

Millamena O M, Quinitio E. 2000. The effects of diets on reproductive performance of eyestalk ablated and intact mud crab *Scylla serrata*. *Aquaculture*, **181**(1-2): 81-90.

Minagawa M, Chiu J R, Murano M. 1993. Developmental changes in body weight and elemental composition of laboratory-reared larvae of the red frog crab, *Ranina ranina* (Decapoda: Brachyura). *Marine Biology*, **116**(3): 399-406.

Morioka Y, Kitajima C, Hayashida G. 1988. Oxygen consumption, growth and calculated food requirement of the swimming crab *Protunus trituberculatus* in its early developmental stage. *Nippon Suisan Gakkaishi*, **54**(7): 1 137-1 141.

Peñaflorida V D. 2004. Amino acid profiles in the midgut, ovary, developing eggs and zoea of the mud crab, *Scylla serrata*. *Israeli Journal of Aquaculture-Bamidgeh*, **56**(2): 111-123.

Ponat A, Adelung D. 1983. Studies to establish an optimal diet for *Carcinus maenas*: 3. Vitamin and quantitative lipid requirements. *Marine Biology*, **74**(3): 275-279.

Preston R L. 1993. Transport of amino acids by marine invertebrates. *Journal of Experimental Zoology*, **265**(4): 410-421.

Sheen S S, Wu S W. 1999. The effects of dietary lipid levels on the growth response of juvenile mud crab *Scylla serrata*. *Aquaculture*, **175**(1-2): 143-153.

Sheen S S. 2000. Dietary cholesterol requirement of juvenile mud crab *Scylla serrata*. *Aquaculture*, **189**(3-4): 277-285.

Sun Y M, Yan Y, Sun J J. 1984. The larval development of *Portunus trituberculatus*. *Journal of Fisheries of China*, **8**(3): 219-226. (in Chinese with English abstract)

Suprayudi M A, Takeuchi T, Hamasaki K. 2004. Essential fatty acids for larval mud crab *Scylla serrata*: implications of lack of the ability to bioconvert C18 unsaturated fatty acids to highly unsaturated fatty acids. *Aquaculture*, **231**(1-4): 403-416.

Trygg J, Holmes E, Lundstedt T. 2007. Chemometrics in metabonomics. *Journal of Proteome Research*, **6**(2): 469-479.

Trygg J, Wold S. 2002. Orthogonal projections to latent structures (O-PLS). *Journal of Chemometrics*, **16**(3): 119-128.

Upmeier B, Gross W, Köster S, Barz W. 1988. Purification and properties of *S*-adenosyl-$_L$-methionine:Nicotinic acid-*N*-methyltransferase from cell suspension cultures of *Glycine max* L. *Archives of Biochemistry and Biophysics*, **262**(2): 445-454.

Van Den Berg R A, Hoefsloot H C J, Westerhuis J A, Smilde A K, Van Der Werf M J. 2006. Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics*, **7**: 142.

Wu X G, Zeng C S, Southgate P C. 2014. Ontogenetic patterns of growth and lipid composition changes of blue swimmer crab larvae: insights into larval biology and lipid nutrition. *Marine and Freshwater Research*, **65**(3): 228-243.

Xiao C N, Hao F H, Qin X R, Wang Y L, Tang H R. 2009. An optimized buffer system for NMR-based urinary metabonomics with effective pH control, chemical shift consistency and dilution minimization. *Analyst*, **134**(5): 916-925.

Xie Z M, Liu H J, Feng L. 2002. Breeding of swimming crab, *Portunus trituberculatus*. In: Xie Z M ed. Hatchery Technology of Marine Economic Crabs. China Agriculture Press, Beijing, China. p.1-304. (in Chinese)

Yancey P H, Blake W R, Conley J. 2002. Unusual organic osmolytes in deep-sea animals: adaptations to hydrostatic pressure and other perturbants. *Comparative Biochemistry and Physiology Part A*: *Molecular & Integrative Physiology*, **133**(3): 667-676.

Yancey P H. 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology*, **208**(15): 2 819-2 830.

Ye Y F, An Y P, Li R H, Mu C K, Wang C L. 2014. Strategy of metabolic phenotype modulation in *Portunus trituberculatus* exposed to low salinity. *Journal of Agricultural and Food Chemistry*, **62**(15): 3 496-3 503.

Electronic supplementary material

Supplementary material (Supplementary Tables S1–S2 and Fig.S1) is available in the online version of this article at https://doi.org/10.1007/s00343-019-7268-0.