Effects of hypoxia in the gills of the Manila clam Ruditapes philippinarum using NMR-based metabolomics

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ABSTRACT

Coastal hypoxia affects the survival, behavior, and reproduction of individual local marine organisms, and the abundance, biomass, and biodiversity of coastal ecosystems. In this study, we investigated the chronic effects of hypoxia on the metabolomics in the gills of Ruditapes (R.) philippinarum. The results indicated significant alterations in the metabolite profiles in the gills of the hypoxia-treated clams, in comparison with those maintained under normoxia. The levels of betaine, taurine, glycine, isoleucine, and alanine were significantly reduced, suggesting a disturbance of osmotic balance associated with hypoxia. Meanwhile, metabolites involved in energy metabolism, such as alanine and succinate, were also affected. Dramatic histopathological changes were observed in the gills and hepatopancreases of R. philippinarum grown in hypoxic waters, demonstrating tissue damages apparently caused by long-term exposure to hypoxia. Our findings suggest that hypoxia significantly affects the physiology of R. philippinarum, even at a sub-lethal level, and impedes health of the clams.

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1. Introduction

Global warming and eutrophication of the marine environment accelerate the expansion of the coastal hypoxic zone, to the detriment of marine ecosystems (Vaquer-Sunyer & Duarte, 2008; Pörtner et al., 2005). Hypoxification of the regional marine environment not only triggers a habitat loss for local marine organisms, reducing marine biological population, and altering the structure and function of marine ecosystem, but also interferes with the global biogeochemical cycling process (Deutsch et al., 2011). Impacts of marine hypoxia start from the sediment-water interface, causing a mass mortality of macrobenthos (Danise et al., 2013; Montagna & Ritter, 2006). Exposed to hypoxia, marine organisms may develop multiple physiological defects in respiration, metabolism, growth, and reproduction. The low levels of dissolved oxygen in seawater are associated with a decline in zooplankton (Elliott et al., 2012) and remarkable undesirable changes in the growth rates, mortality, reproduction, and predator-avoidance behavior of nektons (Gotanda et al., 2011; Richards, 2011; Rouhi et al., 2010). The adverse effects of marine hypoxia, in synergy with other factors such as abnormal temperature and overfishing, cause not only irreversible damage to the native ecosystem, but impose severe limitations on the productivity of local fisheries (Jackson, 2008). An example of this

is the elimination of the Norway lobster by marine hypoxia in the 1980s in the southeast Kattegat sea area (Rosenberg, 1985; Petersen & Pihl, 1995).

At cellular level, cells respond to hypoxia by altering gene expression and protein levels according to signals from oxygen sensors and relevant signal transduction pathways (Mendelsohn et al., 2008). These include a large number of proteins in mammalian cells, such as those involved in angiogenesis, vascular remodeling, glucose-transport, glycolysis, cell proliferation and apoptosis (Semenza, 2009; De et al., 2011), which are required to maintain cell metabolism, survival, and proliferation under bioenergistic stress (Majmundar et al., 2010; Semenza, 2007). Similarly in marine macrobenthos, metabolic pathways are re-structured to maintain energy balance under hypoxia (Zhang et al., 2010; Zhang et al., 2009).

Most studies on the effects of hypoxia on marine life have focused on metabolic processes associated with energy consumption (Ekau et al., 2010; Childress & Seibel, 1998; Seibel, 2011). A thorough understanding of the hypoxia-induced whole-body stress and the mechanisms underlying the adaption of the marine organisms to the hypoxic environment remains to be achieved. Accurately predicting the impacts of hypoxia on individual specifies and the ecosystem is difficult. Therefore, multidisciplinary research combining traditional physiology, molecular biology, metabolomics, and transcriptomics is required to examine acute and long-term effects of hypoxia on marine organisms and to delineate mechanisms that could confer sensitivity or tolerance.

Manila clam Ruditapes (R.) philippinarum is one of the most commercialized aquaculture species worldwide, due to its high tolerance of a

wide range of salinities and temperatures (Wu et al., 2011). It is also frequently used as a bio-indicator in marine biology and toxicology research (Ji et al., 2006; Laing & Child, 1996; Matozzo et al., 2004; Hégaret et al., 2007; Moraga et al., 2002), particularly in the metabolomic characterization of marine organisms under environmental stress (Wu et al., 2011; Liu et al., 2013). In the present study, we determined the metabolomics of *R. philippinarum* exposed to hypoxia. We found that hypoxia interfered with the osmotic balance and energy metabolism of the clams, and led to significant tissue damage.

2. Materials and methods

2.1. Animals and hypoxia challenge

Healthy Zebra pedigree of *R. philippinarum* (shell length 33–35 mm) were purchased from a farm in Shouguang, China, and allowed to acclimate to artificial culture in filtered aerated seawater at 18 ± 2 °C for 2 weeks before using. The clams were fed with commercial diatoms (Leading Tec, Shanghai, China) and water replaced daily throughout the experiment. All chemicals used were purchased from Sangon Biotech (Shanghai, China) unless otherwise specified.

Hypoxia was achieved by bubbling nitrogen into the oxygen-saturated seawater to reduce the dissolved oxygen level to 2 mg L^{-1} O_2. The seawater surface in the tank was covered by a polyvinyl chloride plate to minimize oxygen uptake from the atmosphere. Eighty clams were maintained in hypoxic seawater for up to 20 days, with ten clams sampled on day 2, 5, 10 and 20 after the start of hypoxia challenge. The same number of clams were kept in normoxic seawater, with 8 mg L^{-1} O_2 and used as controls, and sampled in the same manner as the hypoxia-treated clams. At harvest, the gill tissues (~150 g each clam) were collected, quickly frozen in liquid nitrogen for 10 min, and then stored at −80 °C before metabolite extraction.

2.2. Histology of gill and hepatopancreas tissues

Gill and hepatopancreatic tissues were carefully removed from the hypoxia-treated and control groups of clams after 20 days, and fixed for 24 h in a Bonn's solution, which contained 71.4% chrysolepic acid, 23.8% formaldehyde, and 4.8% glacial acetic acid. Samples were then dehydrated through a series of ethanol solutions in water, with increasing percentages of ethanol, cleared in methyl benzoate, rinsed in benzene, and embedded in paraffin. Tissues were sectioned (6-μm thick) on a Leitz microtome (GMI, Ramsey, MN), mounted on albumin-coated slides, dried, and stored at room temperature until stained. The slides were stained with hematoxylin and eosin (H&E) as previously described (Martoja et al., 1970) and examined and imaged under a BX21 light microscope (Olympus, Tokyo, Japan) fitted with a Digital Sight DS-U3 camera (Nikon, Tokyo, Japan).

2.3. Metabolite extraction

Polar metabolites were extracted from the gills of clams as previously described (Ji et al., 2013). Briefly, the gill tissue was homogenized in 82.5% methanol in water at a solvent/tissue ratio of 4.85:1, using a Precellys 24 high throughput homogenizer (Berta, France). The homogenate (500 μL) was then mixed with 400 μL of chloroform and 400 μL of water and vortexed for 20 s. The mixture was incubated at 0 °C for about 10 min before centrifuging at 3000 ×g, 4 °C for 5 min. The methanol/water phase containing gill tissue metabolites was collected, freeze-dried overnight, and stored at −80 °C prior to use. Before analysis, the metabolite extracts were resuspended in 600 μL of phosphate buffer (150 mM Na_2HPO_4 and NaH_2PO_4, pH 7.0) prepared in D_2O, with 0.5 mM sodium 3-trimethylsilyl-2,2,3,3,-d$_4$-propionate (TSP) as a chemical shift standard. The solution was vortexed and centrifuged at 3000 ×g for 5 min at 4 °C, and the supernatant (550 μL) was transferred into a 5 mm nuclear magnetic resonance (NMR) tube before analysis.

2.4. Proton (^{1}H) NMR spectroscopy

NMR spectroscopic profiling of the gill metabolites from the clams was performed on an AV 500 NMR spectrometer (Bruker, Billerica, MA), operated at 500.18 MHz (at 298 K). One-dimensional (1D) spectra were acquired using an 11.9 μs pulse, 0.1 s mixing time, and 3.0 s relaxation delay with a standard 1 D NOESY pulse sequence. All spectra were recorded using a spectral width of 6009.6 Hz, and 128 transients collected with 16,384 data points. The resulted datasets were zero-filled to 32,768 points and Fourier-transformed with a weighted exponential line-broadening factor of 0.3 Hz. The spectra were manually phased, baseline corrected, and calibrated (TSP at 0.0 ppm) using TopSpin (version 2.1, Bruker).

2.5. Spectral data pre-processing and multivariate analysis

The NMR spectra were converted to a format for pattern recognition in a multivariate analysis using ProMetab, custom-programed in a Matlab software package (version 7.0, MathWorks, Natick, MA) (Purohit et al., 2004). A supervised partial least squares discriminant analysis (PLS-DA) was performed to maximize the separation between different groups of the gill tissue samples (Xu, 2004; Wold et al., 1984). The PLS-DA included a measured matrix X and a response matrix Y consisting of dummy variables. The quality of the PLS-DA model was assessed using a leave-one-out cross-validation method (Rubingh et al., 2006). The latent variables producing increases in Q^2 were considered as significant components, where:

$$Q^2 = 1 - \frac{\sum (Y_{predicted} - Y_{true})^2}{\sum Y^2_{true}}$$

The sensitivity and specificity of the PLS-DA model were calculated. Prior to PLS-DA, data were mean-centered using the PLS Toolbox (version 4.0, Eigenvector Research, Manson, WA). One-way analysis of variance (ANOVA) was implemented on the bin areas from NMR spectral peaks crucial for differentiating the sample groups to test the significances of alterations in the amount of corresponding metabolites (Liu et al., 2014).

Metabolites were assigned and quantified according to the tabulated chemical shifts and by using a Chenomx software (Chenomx, Edmonton, Canada). The concentrations were tested for normal distribution (Ryan-Joiner's test) and homogeneity of variances (Bartlett's test). The significance of differences was determined using one-way ANOVA and multiple comparisons. A P-value ≤ 0.05 was considered significant.

3. Results

3.1. Histopathological changes in the gills and hepatopancreas

H&E staining of the gills from the hypoxia-treated and untreated clams is illustrated in Fig. 1. The gills from the untreated clams exhibited a well-preserved structure, with ciliated columnar epithelial cells with ovoid nuclei lining the wall of the gill filament, and loose connective tissue filling the core (Fig. 1A). Under hypoxia; however, the gills of clams showed obvious destruction of structure (Fig. 1B); the epithelia of the gill filaments were incomplete and the interlayer structure disappeared, the gill filaments were intricately connected by cells of unknown identity.

Normal hepatopancreas of clams was covered by epithelium attached to a thin layer of fibrous connective tissue (Fig. 1C). The epithelium consists mainly of digestive and secretory cells. The digestive cells are cube-shaped or long columnar, and are responsible for absorption and intracellular digestion. In the clams treated with hypoxia, deep purple round spots were observed in the digestive cells of the
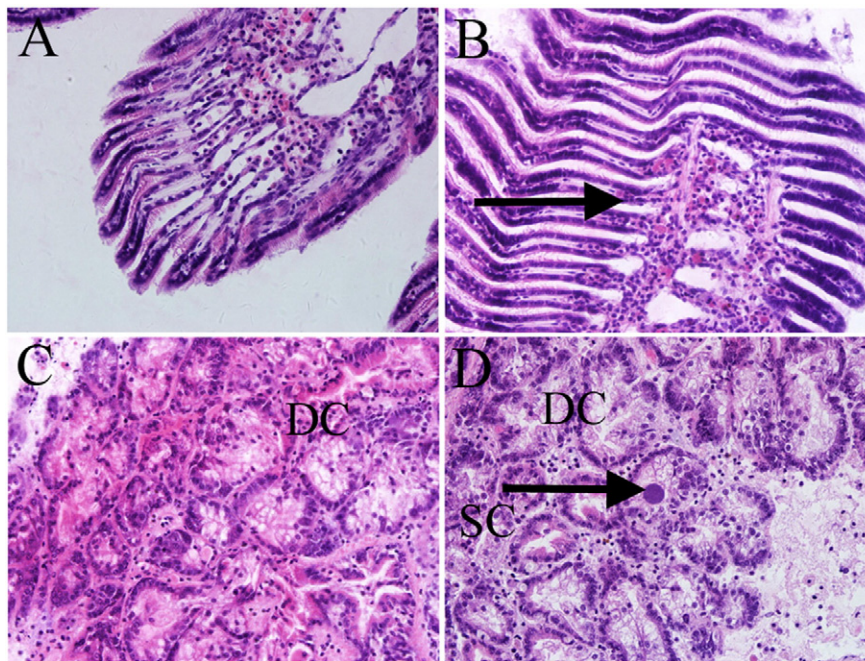
Fig. 1. Histopathological changes associated with hypoxia in *R. philippinarum*. Gills (A, B) and hepatopancreas (C, D) from the untreated (A, C) and hypoxia-treated (B, D) clams were collected at day 20 after the start of hypoxia challenge, fixed, and dissected (6 μm in thickness) prior to H&E staining. Magnification: 400×. Arrow, abnormal tissue. SC, secretory cells. DC, digestive cells.

hepatopancreas, which might be due to the abnormal lipid metabolism and lipid deposition (Fig. 1D).

3.2. Pattern recognition on the NMR spectra of gill tissue extracts

Supervised classification using PLS-DA demonstrated time-dependent metabolic effects of hypoxia on the gills of clams. The sample distribution plots showed significant differences in the metabolic profiles between the hypoxia-challenged and untreated control clams (Fig. 2A, C, E and G). The PLS-DA models proved to be robust, as evidenced by the >0.4 Q^2 values, and were thus used throughout the rest of the study.

The bin areas of potentially significant NMR spectral peaks allowed identification of metabolites that could differentiate the treated and control clam groups (Fig. 2B, D, F and H). The most obvious differences in the metabolite profiles of the gills of clams over the 20-day period were the losses in isoleucine, alanine, succinate, betaine, and taurine in the hypoxia group relative to the control group. Hypoxia was also associated with aberrant changes in glycine levels during the first 10 days; early accumulation, but gradual recession to the baseline of tyrosine and phenylalanine; an early decrease in arginine, and a strong increase in phenylalanine and aspartate during the later stages.

3.3. Effects of hypoxia on the metabolite profiles of clam gills

The levels of metabolites, including isoleucine, alanine, succinate, betaine, taurine, glycine, tyrosine, phenylalanine, arginine, and aspartate, in the gill tissues of clam under hypoxia was determined to characterize in detail the effect of hypoxia on the metabolite profiles of *R. philippinarum*. After the hypoxia treatment, the levels of isoleucine in the clam gills of the hypoxia group on days 2, 5, 10, and 20 were 0.59, 0.32, 0.51, and 0.68 μmol g^{-1} wet tissue on the 2nd, 5th, 10th and 20th day, respectively. These were significantly lower ($P < 0.01$) than that of the averages in the control group (about 0.90 μmol g^{-1} wet tissue at each time point; Fig. 3A). Hypoxia also induced a significant decrease of alanine, from an average of 15.84 μmol g^{-1} wet tissue in the controls to 6.66, 6.43, 6.68, and 7.41 μmol g^{-1} wet tissue at day 2, 5, 10 and 20, respectively, after hypoxia treatment (Fig. 3B). Correlatively,

concentration of succinate decreased by about 2 folds at days 2 and 5, and remained significantly lower than the controls until the end of the treatment (Fig. 3C). Clustered with this profile, the betaine contents decreased to 38.80, 40.63, 36.43 and 36.25 μmol g-1 wet tissue on the 2nd, 5th, 10th, and 20th day after hypoxia treatment, respectively (Fig. 3D).

Alterations in some metabolites (e.g., arginine, glycine, tyrosine, phenylalanine, and aspartate) in the clam gills that were associated with hypoxia were limited to certain periods only. Reduction of arginine in the clam gills occurred during the early stage of hypoxia treatment, at day 5, from 1.86 to 1.26 μmol g^{-1} wet tissue ($P < 0.01$) while no apparent aberrations were found at other time points (Fig. 3E). The levels of glycine in the gills of clams diminished to 6.72, 7.68 and 7.05 μmol g-1 wet tissue ($P < 0.01$) on the 2nd, 5th and 10th day post hypoxia challenge, respectively, but resumed to normal at the 20th day of hypoxia treatment (Fig. 3F). Inversely, a temporal elevation of tyrosine (0.37 μmol g^{-1} wet tissue) was detected 2 days after hypoxia treatment compared with the controls (0.27 μmol g^{-1} wet tissue), but its concentration returned to the baseline level shortly afterward (Fig. 3G). Phenylalanine levels in the gills of clams treated by hypoxia were remarkably higher than the untreated (0.23 μmol g^{-1} wet tissue) on days 2, 10, and 20 (Fig. 3H). In addition, a late accumulation of aspartate in the clam gills (6.33 μmol g^{-1} wet tissue) was observed on the 20th day of hypoxia exposure, with a 40% increase over the control group (Fig. 3I). These data together suggested aberrant changes occurred in the metabolomics profiles of the Manila clam under the stress of hypoxia.

4. Discussion

In response to environmental changes, organisms adjust their metabolic physiology to adapt to the new material and energy requirements. Studying the metabolic alterations helps us understand the subtle physiological changes that different organisms undergo during various natural and manmade stresses, ahead of the appearance of biological and ecological consequences. Metabolomics allows a systematic view of the metabolic shift triggered by environmental changes, by measuring levels of endogenous low-molecular-weight metabolites (Wang et al., 2003; Plumb et al., 2003). It facilitates assessment of the metabolic
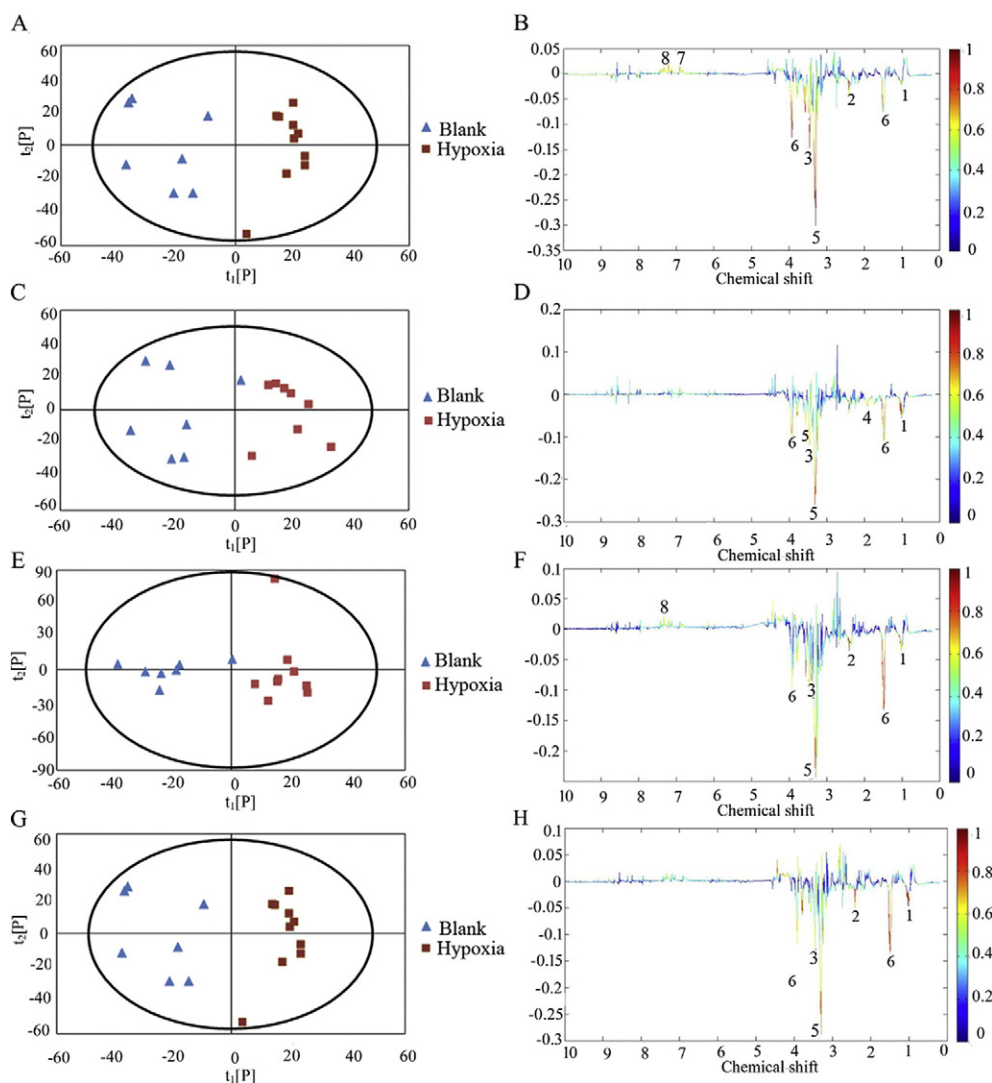
Fig. 2. PLS-DA showing differentiable metabolite profiles of *R. philippinarum* exposed to hypoxia (red squares) for 2(A), 5(C), 10(E), and 20 days (G). The representative 1D ^{1}H NMR spectra of gill tissue extracts at different timepoints are shown in B, D, F, and H. Metabolites of interest: 1, isoleucine; 2, alanine; 3, succinate; 4, betaine; 5, arginine; 6, glycine; 7, tyrosine; 8, phenylalanine; and 9, aspartate.

status of cells, tissues, organs, and whole organisms (Lin et al., 2006; Stentiford et al., 2005). In the present study, NMR spectroscopy was performed to profile the metabolic alterations in the gills of *R. philippinarum* that occur in response to hypoxia. Our data indicated a wide range of changes in the metabolite profiles, and significant histopathological damage to the gills, caused by lack of oxygen. These results hint at the potential dramatic effects of coastal hypoxia on the local marine ecosystem.

Consistent with previous studies (Hanana et al., 2014; Zhang et al., 2011), the NMR spectra of the gills from the clam *R. philippinarum* was dominated by organic osmolytes (Fig. 3). These small organic molecules regulate the osmotic balance in marine organisms (Liu et al., 2011). The levels of several organic osmolytes, such as betaine (Fig. 3D), glycine (Fig. 3F), isoleucine (Fig. 3A), and alanine (Fig. 3B) were significantly lower in the clams treated with hypoxia, compared with the control group. These were major parameters that differentiated the hypoxic and untreated metabolite profiles in the gills of clams in the PLS-DA analysis (Fig. 2). These differences suggested disturbances in osmotic regulation associated with hypoxia, which have also been demonstrated when *R. philippinarum* is exposed to other stresses, such as salinity irregularity and environmental pollution (Wu et al., 2011; Liu et al., 2014; Zhang et al., 2011; Liu et al., 2011). The alterations in the amount of these osmolytes reflect an innate response of the clams to balance

their intracellular osmolarity with the local environment, as previously demonstrated for other marine organisms (Viant et al., 2003).

Throughout the hypoxia treatment, the levels of alanine in the gills of the clams were merely 40.6% to 46.8% that of the clams under normoxia (Fig. 3B). This amino acid is one of the major end-products of the anaerobic breakdown of glucose (Carlsson & Gäde, 1986; Stokes & Awapara, 1968), and was logically expected to increase when the clams were maintained under hypoxia. However, the decrease of alanine observed in this study was consistent with the simultaneous reduction of succinate, which is another product of the anaerobic consumption of glucose (Carlsson & Gäde, 1986; Stokes & Awapara, 1968) (Fig. 3C). Of note, another anaerobic metabolic pathway that converts aspartate to succinate (Graham & Ellington, 1985) was also affected, as suggested by the accumulation of aspartate towards the end of the hypoxia treatment, which correlated with the reduction of succinate (Fig. 3C). Taken together, our data suggest a general inhibition of energy metabolism that could be associated with hypoxia in *R. philippinarum*.

The gills and hepatopancreas of bivalve species are responsive and sensitive to environmental stressors (Marigómez et al., 2002; Au, 2004; Abdel-Nabi et al., 2007). In the present study, we observed that the gills of *R. philippinarum* exposed to hypoxia lost their structures (Fig. 1). The irregularity of epithelium and loss of interlamellar tissue of the gill filaments may have significant effects on respiration and
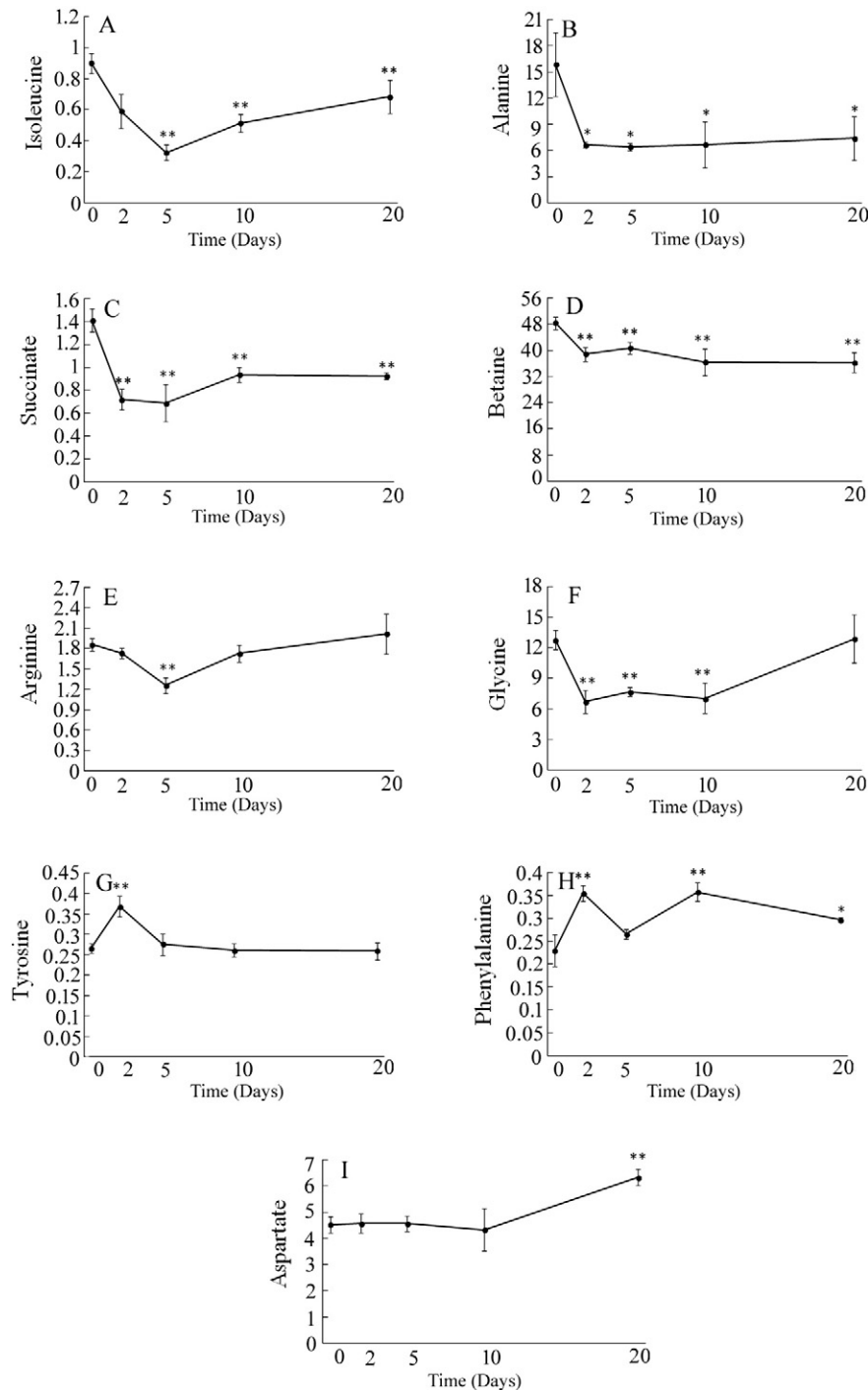
Fig. 3. Effect of hypoxia on the concentrations of isoleucine (A), alanine (B), arginine (C), succinate (D), glycine (E), betaine (F), tyrosine (G), phenylalanine (H), and aspartate (I) in the gills of *R. philippinarum* after 2, 5, 10, and 20 days of exposure to hypoxia. The levels of all metabolites are presented in μmol g^{-1} wet tissue.

food uptake, as previously reported (Marigómez et al., 2002; Au, 2004; Abdel-Nabi et al., 2007), although food clearance rates were not measured in the current study. In support of this, a previous study showed that repeated exposure to hypoxia reduces the clearance rate (Kozuki et al., 2013).

Hypoxia in eutrophic coastal water is a severe stressor and can detrimentally affect aquatic systems (Rosenberg, 1985; Long et al., 2014). To prevent the biological and ecological consequences of hypoxification, it is essential to monitor the coastal environment for the subtle physiological, behavioral, and reproductive aberrations in marine organisms which are relatively more susceptible to the impact of declining dissolvable oxygen. *R. philippinarum* is a commonly used bioindicator (Ji et al.,

2006; Liu et al., 2013; Kozuki et al., 2013) and its sensitivity to hypoxia has previously been demonstrated in the lab and in the field (Kozuki et al., 2013). Our data showed significant alterations in the metabolite profiles of the gills of *R. philippinarum* under hypoxia, adding a new dimension of impacts that the coastal water hypoxification has on this important aquaculture species.

5. Conclusions

In conclusion, this study used NMR spectroscopy to detect significant alterations in the metabolomics of the gill tissues of *R. philippinarum* that had been chronically exposed to hypoxia. The results

indicated that hypoxia has a dramatic effect on the osmotic balance of *R. philippinarum*, as well as energy metabolism. Associated with these effects, apparent deformity of the gills was observed. These findings suggest that *R. philippinarum* can be a sensitive bioindicator in coastal ecotoxicology.

Acknowledgement

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