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Metabolic response of *Mercenaria mercenaria* under heat and hypoxia stress by widely targeted metabolomic approach

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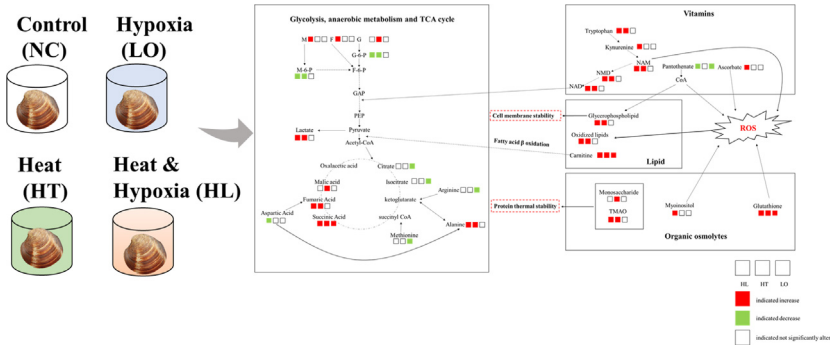
HIGHLIGHTS

- The metabolic response of hard clam under heat and hypoxia stress was explored.
- Glycolysis, anaerobic metabolism and TCA cycle were affected by stresses.
- Accumulation of carnitine may promote fatty acid β oxidation.
- Accumulation of glycerophospholipid may maintain cell membrane stability.
- Osmolytes played key roles in relieve ROS stress and maintain protein homeostasis.

GRAPHICAL ABSTRACT



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ABSTRACT

In the context of global climatic changes, marine organisms have been exposed to environmental stressors including heat and hypoxia. This calls for the design of multi-stressors to uncover the impact of oceanic factors on aquatic organisms. So far, little is known about the metabolic response of marine organisms, especially bivalves, to the combined effects of heat and hypoxia. In this study, we employed widely targeted metabolomic analysis to study the metabolic response of gills in hard clam, a heat- and hypoxia-tolerant bivalve. A total of 810 metabolites were identified. Results showed that the heat group (HT) and heat plus hypoxia group (HL) had a higher number of differential metabolites than the hypoxia group (LO). Glycolysis was affected by the heat and heat plus hypoxia stress. Moreover, anaerobic metabolic biomarkers were accumulated marking the onset of anaerobic metabolism. Environmental stresses may affect Tricarboxylic acid (TCA) cycle. Accumulation of carnitine and glycerophospholipid may promote fatty acid β oxidation and maintain cell membrane stability, respectively. The high content of oxidized lipids (i.e., Leukotriene) in HL and HT groups implied that the organisms were under ROS stress. The significantly differential metabolites of organic osmolytes and vitamins might relieve ROS stress. Moreover, accumulation of thermoprotective osmolytes (monosaccharide, Trimethylamine N-oxide (TMAO)) accumulation was helpful to maintain protein homeostasis. This investigation provided new insights into the adaptation mechanisms of hard clam to heat, hypoxia and combined stress at the metabolite level and highlighted the roles of molecules and protectants.

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1. Introduction

Marine ecosystem is affected by global climate change. The cultured marine organisms are facing different risks and even mass mortality from the impacts of global warming including high temperature and low dissolved oxygen (DO) (Huo et al., 2019; Soon and Zheng, 2019). In China, the marine heat wave frequency, duration, and mean intensity in Bohai Sea were twice more than the global average which have impacted fishery resources in the country (Yao et al., 2020). The content of dissolved oxygen decreased with the increased temperature (Götze et al., 2020). The dissolved oxygen (DO) concentration that was also a key factor of ocean change decreased with the increased temperature (Sampaio et al., 2021). Considering the potential combined effects, it's needed to design multi-stressors experiment to uncover the impacts of change in ocean (Sampaio et al., 2021).

A lot of energy was consumed for the basic maintenance of the aquatic animals under environmental stress and the energy balance in the organism was thus affected (Sokolova et al., 2012). Under moderate stress, the aerobic metabolism was restricted and the energy used for maintenance was increased (Sokolova et al., 2012). Under extreme environmental stress, marine organisms used all energy for their physiological maintenance and anaerobic metabolism became the main mode of energy supply (Sokolova et al., 2012). To cope with heat or hypoxia stress, marine organisms adopted different metabolic strategies. Accumulation of alternative anaerobic metabolite (succinate, propionate and malate) was one of hypoxia adaptative strategies in marine bivalve while the energy metabolism were species specific (Eymann et al., 2020). Other challenges that should be addressed under heat stress were the ROS damage (Rahman and Rahman, 2021), protein homeostasis and cell membrane stability (Chen et al., 2021). Overall, under heat or hypoxia stress organism could start anaerobic metabolism to supply energy (Eymann et al., 2020), accumulate antioxidant (Chen et al., 2021) or increase antioxidant enzyme activity to eliminate ROS (Rahman and Rahman, 2021), accumulate thermoprotective osmolytes (Chen et al., 2021) or up-regulate molecular chaperone expression (Zhang et al., 2020) to maintain protein homeostasis, and change phospholipid metabolism to protect cell membrane (Chen et al., 2021).

Hard clam *Mercenaria mercenaria* is a native marine bivalve in the United States of America. It is an important part of commercial shellfish farming (Garcia et al., 2014; Kraeuter and Castagna, 2001). After its introduction from the US, it becomes an important pond-cultured species in the coastal areas from Liaoning province to Guangdong province in China due to its adaptation to most pond culturing conditions. However, with global warming and increased hypoxia in shallow coastal waters (Li et al., 2019), heat and hypoxia became the main environmental stresses that threaten its physiological status in summer pond culture. The temperature tolerance range of adult hard clams is 1 to 34 °C (Bricelj et al., 2017). They can also survive two weeks under hypoxia condition (Knight, 2016). Therefore, hard clams can be used as an ideal model organism to study the metabolic adaptation mechanism for heat and hypoxia conditions.

Metabolites play key roles in the interaction of organisms with their environment. Metabolome can simultaneously identify and quantify several small molecular intermediate productions or end points (Nicholson et al., 2002). Environmental metabolome is a novel subdiscipline that explore the interactions of organisms with their habitat (Lankadurai et al., 2013). It could provide a deep insight of how to response to environmental changes in aquatic animals to natural and anthropogenic stressors (Viant, 2007). Different technological metabolomes have been performed to explore the physiological responses of bivalve to environmental stressors, including heavy metal (Liu et al., 2011; Zhang et al., 2011), ocean acidification (Wei et al., 2015; Götze et al., 2020), salinity challenge (Li et al., 2021), heat (Dunphy et al., 2018; Eymann et al., 2020; Götze et al., 2020; Jiang et al., 2020) and hypoxia (Zhang et al., 2017; Götze et al., 2020; Haider et al., 2020; Sun et al., 2021). However, little information is available on the metabolomic responses of hard clam to heat, hypoxia and combined stress.

Widely targeted metabolomics is the "new generation targeted metabolome". It combined species-specific targeted mass spectrometry (MS) databases with high throughput sequencing techniques so had the advantage of "universality" of nontargeted metabolomics and "accuracy" of targeted metabolomics (Chen et al., 2013). MS database was established by standard sample library construction, manual spectral analysis, literature and database matching. It could find more potential biological function metabolites by monitoring-enhanced product ions (MIN-EPI) method (Zhou et al., 2021). In this study, a widely targeted metabolomics was employed to explore the metabolic response to heat and hypoxia stress in hard clams. The identification of different metabolites in response to environmental stressors revealed the adaptive mechanism of organisms at the metabolic levels. Potential biomarkers were also identified in this study. The results of this study provided novel insights on the adaptive strategies for adverse environmental conditions at metabolic level, and assisted in the assessment of physiological status of the hard clam in aquaculture.

2. Materials and methods

2.1. Experimental animals and stress challenge

The hard clams used in this study were collected from the culture ponds in Binzhou (Shandong Prince, China). They had a shell length of 44.46 ± 4.83 mm while their weights were 28.99 ± 8.85 g. Before the start of experiment, hard clams were acclimation for more than two weeks in the laboratory aquarium containing sand-filtered and aerated seawater (temperature: 18 ± 2 °C, salinity: 30 ± 0.5‰). Spirulina powder was added in the laboratory aquarium once a day as source of food for the hard clams. There was no mortality of the hard clams during the laboratory acclimation. The following experimental groups were established: negative control, NC (20 °C, 6 mg/L); hypoxia group, LO (20 °C, 0.2 mg/L); heat group, HT (35 °C, 6 mg/L) and heat plus hypoxia group, HL (35 °C, 0.2 mg/L). During the stress period, hard clams were not fed to minimize bacterial growth. The stress challenge experiments were carried out using a novel hypoxia simulation device (Li et al., 2019). In brief, there were four same environmental simulation systems in this device. A supply tank where to adjust of the DO concentration and water temperature was under four replicate experimental tanks. It contained a heater and sensors for control DO and temperature. Temperature adjustment was achieved by heater heating and DO adjustment was achieved by bubbling in nitrogen under the control of solenoid valves, respectively (Li et al., 2019). Each treatment contained four replicates, and each replicate contained 20 hard clams. The hard clams were abruptly exposed to the experimental conditions of temperature and dissolved oxygen and remained in these experimental conditions for 2 days.

2.2. Survival rate

Hard clams were considered dead if the shells opened and the mantles did not react after stimulation. The numbers of dead hard clam in each group were counted and recorded during the stress time. To prevent water deterioration caused by decay, the clams were checked for survival at an interval of 2 h.

2.3. Tissue sampling

After 2 days of exposure to stress (the mortality of hard clams in the HL group was more than 50%, Fig. 1). Eighteen alive hard clams (six biological replicates × three hard clams per biological replicates) from each group were dissected promptly after the 2 days test period. Their gills were removed, preserved in liquid nitrogen, and stored in refrigerator at −80 °C before use. In this study, gills were selected as the target

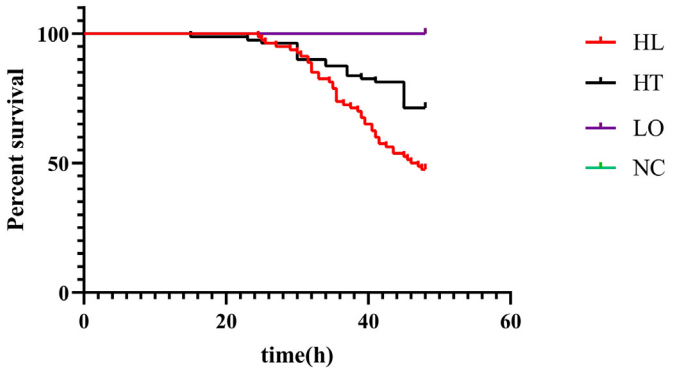

Fig. 1. Survival curve of hard clams during experiment. HL indicating heat and hypoxia group, HT indicating heat group, LO indicating hypoxia group and NC indicating negative control group. Survival curve was tested by log rank test.

tissue because they were the major respiratory organ in marine bivalves. Furthermore, gills were directly exposed to the fluctuations in the ambient oxygen levels hence sensitive to environmental stress.

2.4. Metabolomic profiling

Extraction of metabolites was carried out based on standardized flow in Wuhan MetWare Biotechnology Co., Ltd. (www.metware.cn). The gill samples were first thawed on ice. An approximate of 50 ± 2 mg of gill was taken from each sample. The samples were homogenized by precooled steel balls for 3 min (30 Hz). 1 mL 70% methanol (internal standard extract) was added into the homogenized sample. The mixture was whirled for 5 min, and then was centrifuged for 10 min (4 °C, 12,000 rpm). 400 μL of supernatant was taken and stored at −20 °C overnight. The supernatant was then centrifuged (4 °C, 12,000 rpm) for 3 min. Lastly, 200 μL of the supernatant was taken into the injection bottle for analysis. Metabolite detection, identification, and quantification were carried out in Wuhan MetWare Biotechnology Co., Ltd. (www.metware.cn). A LC-ESI-MS/MS system was used for the extract sample analysis. The UPLC, ESI and MS experimental conditions was similar with Zhou's study (Zhou et al., 2021).

2.5. Bioinformatic analysis

The mass spectrum data was processed using Analyst 1.6.3 software. All data analysis was based on the self-built MWDB database (Metware Biotechnology Co., Ltd. Wuhan, China). The relationships of the identified metabolites among the tested samples were determined using Principal component analysis (PCA). To obtain a higher level of group separation and to improve understanding of the variables responsible for classification, supervised orthogonal projections for latent structures-discriminant analysis (OPLS-DA) was generated using R package MetaboAnalystR (v1.0.1). The OPLS-DA was employed to identify the differential metabolites between the samples after the data was log transformed (log2) and mean centered. Significantly regulated metabolites between groups were determined by variable important in projection (VIP) ≥ 1 and absolute Log2FC (fold change) ≥ 1. VIP values were extracted from OPLS-DA result.

The differential metabolites were annotated by KEGG compound database (http://www.kegg.jp/kegg/compound/) and then mapped to the KEGG pathway database (http://www.kegg.jp/kegg/pathway.html). Pathways were fed into metabolite sets enrichment analysis (MSEA), their significance was determined by hypergeometric test's *p*-values, where $p < 0.05$ was considered to be significant.

The Receiver Operating Characteristic curve (ROC) analysis was performed in https://www.metaboanalyst.ca/ using 10 increase and 10 decrease metabolites with the largest absolute $\log_2$ (Fold change) value in the experimental groups (HL, HT and LO) and the negative control group (NC). The Area Under the Curve (AUC) were calculated. The metabolites were considered as biomarkers if their AUC values were more than 0.9.

3. Results

3.1. Survival rate

The survival rate of hard clam was found to vary with different environmental stress ($p < 0.01$, Fig. 1). All the hard clams in the negative control (NC) group and low oxygen (LO) group were found alive, showing 0% mortality after 2 days of the test duration. In the heat plus hypoxia (HL) group, the first death of hard clam was recorded after 25 h of the test period and the mortality rose up to 52.5% after 48 h of test duration. In the heat (HT) group, the first death of hard clam was recorded after 15 h and the mortality was 3.75 and 28.75% after 25 and 48 h of the test period.

3.2. Widely targeted metabolomic profile

In this study, we employed widely targeted metabolomics analysis to explore the potential adaptation mechanism from the metabolomics levels. A total of 810 metabolites were identified and their PCA analysis conducted to determine the metabolites variation in all groups (HL, HT, LO and NC). The analysis confirmed the association of the different metabolites with the environmental stresses (Fig. 2). It was found that the metabolites in the four groups had good aggregation. The first principal axis (PC 1) showed 26.07% variability. Further, the metabolites of hard clams in the high temperature groups (HL and HT) were different from the metabolites of hard clams in the normal temperature groups (LO and NC). Therefore, in consistence with their survival rates, the hard clams might have a better tolerance in hypoxia than in heat stress. In other word, heat related stress had a much more effect in metabolic response of hard clams than the effects of stress from hypoxia.

OPLS-DA analysis for the metabolites showed that experimental groups (HL, HT and LO) and negative control group (NC) were readily separated and did not indicate any specific outliers in the samples (Fig. S1). These results suggested that the heat plus hypoxia, heat and hypoxia stresses could have affected the gill metabolome of hard clams.

3.3. Differential metabolite selection

In this study, VIP ≥ 1 and absolute Log2FC ≥ 1 were used as the threshold to screen the differential metabolites. We focused on the differential metabolites identified between the experimental groups and the negative control group. From the HL vs NC comparison analysis, it was found that a total of 109 metabolites were significantly increased and 21 metabolites were significantly decreased. From the HT vs NC comparison analysis, it was found that a total of 143 metabolites were significantly increased while 21 metabolites were significantly decreased. Lastly, it was found that 20 metabolites were significantly increased while 26 metabolites were significantly decreased during LO vs NC comparison analysis.

These metabolites could be divided into thirteen classes including fatty acyl (FA), heterocyclic compounds, organic acid & its derivatives, carboxylic acids & derivatives, aldehyde & ketones & esters, hormones & related compounds, glycerides class (GL), glycerophospholipids (GP), coenzyme & vitamins, alcohol & amines, nucleotide & its metabolomics, benzene & substituted derivatives and amino acid & its metabolomics. The differential metabolites in HL vs NC, HT vs NC and LO vs NC were as shown in the supplemental file 1, supplemental file 2 and supplemental file 3 respectively.

Fig. 3A represented the composition percentages of thirteen classes of the metabolites in three pairs of pairwise comparisons. It was evident that fatty acyl (FA), showed a large percentage (21.54% in HL vs NC, 16.46% in HT vs NC and 32.61% in LO vs NC). Most acylcarnitine were

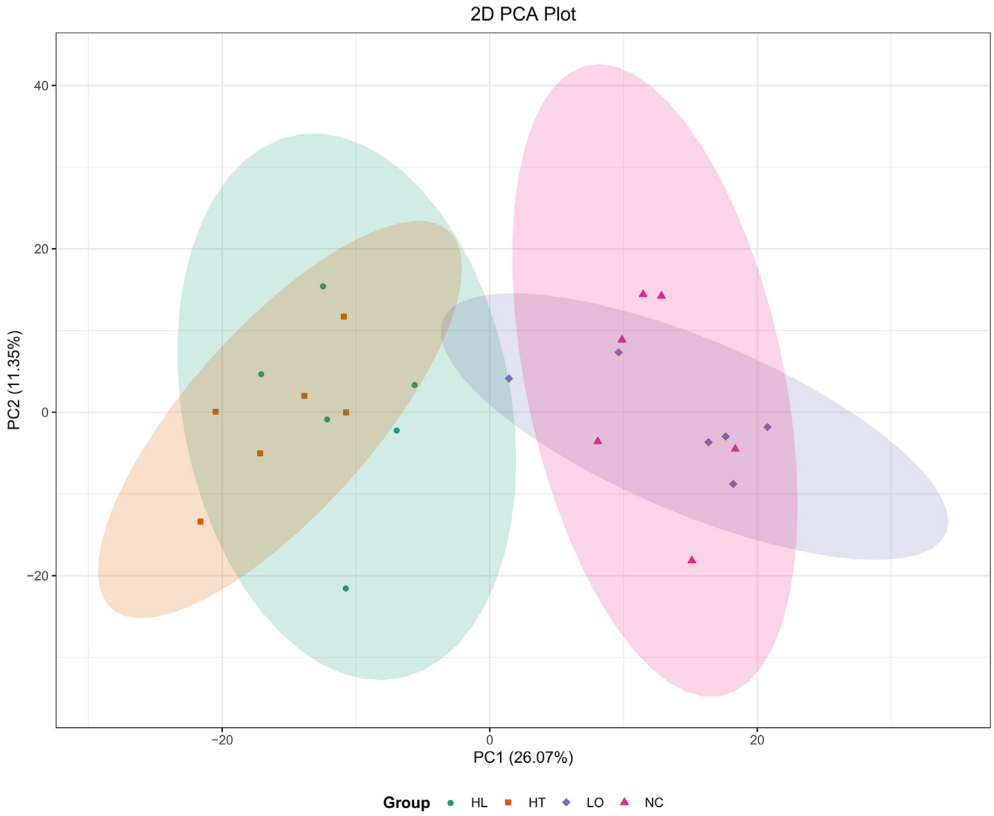

Fig. 2. Principal component analysis (PCA) of the metabolites in gills.

increased in experimental groups. Interestingly, oxidized lipids were not found in FA class in LO and NC pair of pairwise comparisons. However, oxidized lipids such as leukotriene C4, 14, 15-leukotriene C4 had accumulated significantly in HL vs NC and HT vs NC pairs of pairwise comparisons.

Glycerophospholipids (GP) was another difference between heat comparisons pairs (HL vs NC and HT vs NC) and normal temperature group (LO vs NC). Glycerophospholipids was found to be 10% and 20.12% in HL vs NC and HT vs NC, respectively while none differential GP metabolites were found in LO vs NC. The percent of amino acid and its metabolomics was 20.77 and 23.17 in HL vs NC and HT vs NC comparisons pairs respectively while 8.70% in LO vs NC comparison. The overlapping differential metabolites between three pairs of pairwise comparisons was represented using venn diagram shown in the Fig. 3B. A total of 217 non-repetitive differential metabolites were obtained. There were 16 overlapping differential metabolites in the three pairs of pairwise comparisons. All these metabolites showed similar trends in experiential group than negative control group, including 15 increase metabolites (including 1 free fatty acid, 9 kinds of acylcarnitine, 4 kinds of organic acid & its derivatives and 1 small peptide) and 1 decrease metabolite (thyroxine).

3.4. KEGG pathway enrichment analysis of differential metabolites

For better understanding of the function of different metabolites, KEGG enrichment analysis of metabolites which were significantly different in three pairs of pairwise comparisons were carried out. Fig. 4A showed the propanoate metabolism, nicotinate and nicotinamide metabolism, pyruvate metabolism, inositol phosphate metabolism, vitamin digestion and absorption, African trypanosomiasis, longevity regulating pathway-worm and oxidative phosphorylation were significantly enrichment in HL vs NC ($P < 0.05$). Fig. 4B showed the propanoate metabolism, thyroid hormone synthesis, pyruvate metabolism, nicotinate and nicotinamide metabolism, longevity regulating pathway-worm, oxidative phosphorylation, insulin resistance and fructose and mannose metabolism were significantly enrichment in the comparative analysis of HT and NC ($P < 0.05$). Fig. 4C showed that the citrate cycle (TCA cycle), central carbon metabolism in cancer, glyoxylate and dicarboxylate metabolism, glucagon signaling pathway, caffeine metabolism and propanoate metabolism were significantly enriched in the comparative analysis of LO and NC ($P < 0.05$).

3.5. Biomarkers selection by ROC analysis

The ROC analysis was carried out using 20 metabolites (10 increase and 10 decrease) with the largest absolute log2 (Fold change) value between specific experimental group (HL, HT and LO) and the negative control group (NC). All the AUC values of the selected metabolites in the HL and NC group comparison were more than or equal 0.9. This indicated that the metabolites could be the potential biomarkers for heat plus hypoxia stress (Fig. 5A). In HT and NC group comparative analysis, all the selected metabolites could be the potential biomarkers for heat stress (Fig. 5B) except uredoisobutyric acid, 2-((3-Oxo-3-phenylpropyl) amino) acetic acid) and L-thyroxine. Further, the selected metabolites in the LO and NC group comparison could be the potential biomarkers for hypoxia stress except caffeine, carnitine C23:5, 1, 5-diaminopentane, 2-(dimethylamino) guanosine, 5′-deoxy-5′-fluoroadenosine, eritadenine and shikimic acid (Fig. 5C).

3.6. Systemic analysis of metabolome response to environmental stress (HL, HT and LO)

Potential metabolomic response and adaption mechanism of hard clam in the HL, HT and LO groups was as shown in Fig. 6. Glycolysis metabolites including fructose, mannose were more significantly increased in HL group than in NC group. Glucose were significantly increased in the heat (HT) group than in the negative control (NC) group. Glucose-6-phosphate and mannose-6-phosphate were significantly decreased

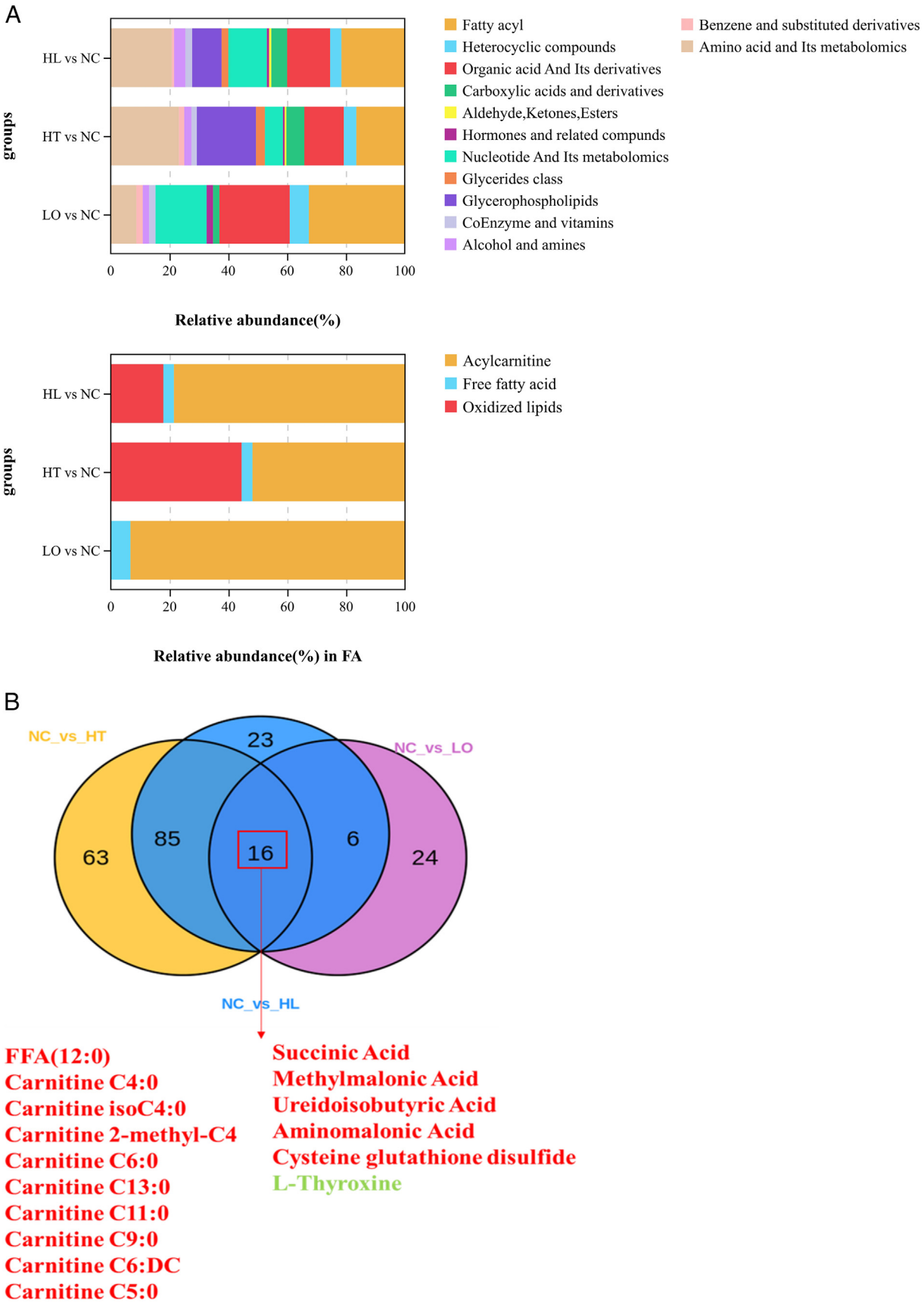

Fig. 3. Differential metabolite analysis based on pairwise comparison.
A. Composition and percentage of differential metabolites under environmental stresses.
B. Venn diagram of differential metabolites pairwise comparison. Red indicated increase; green indicated decrease.

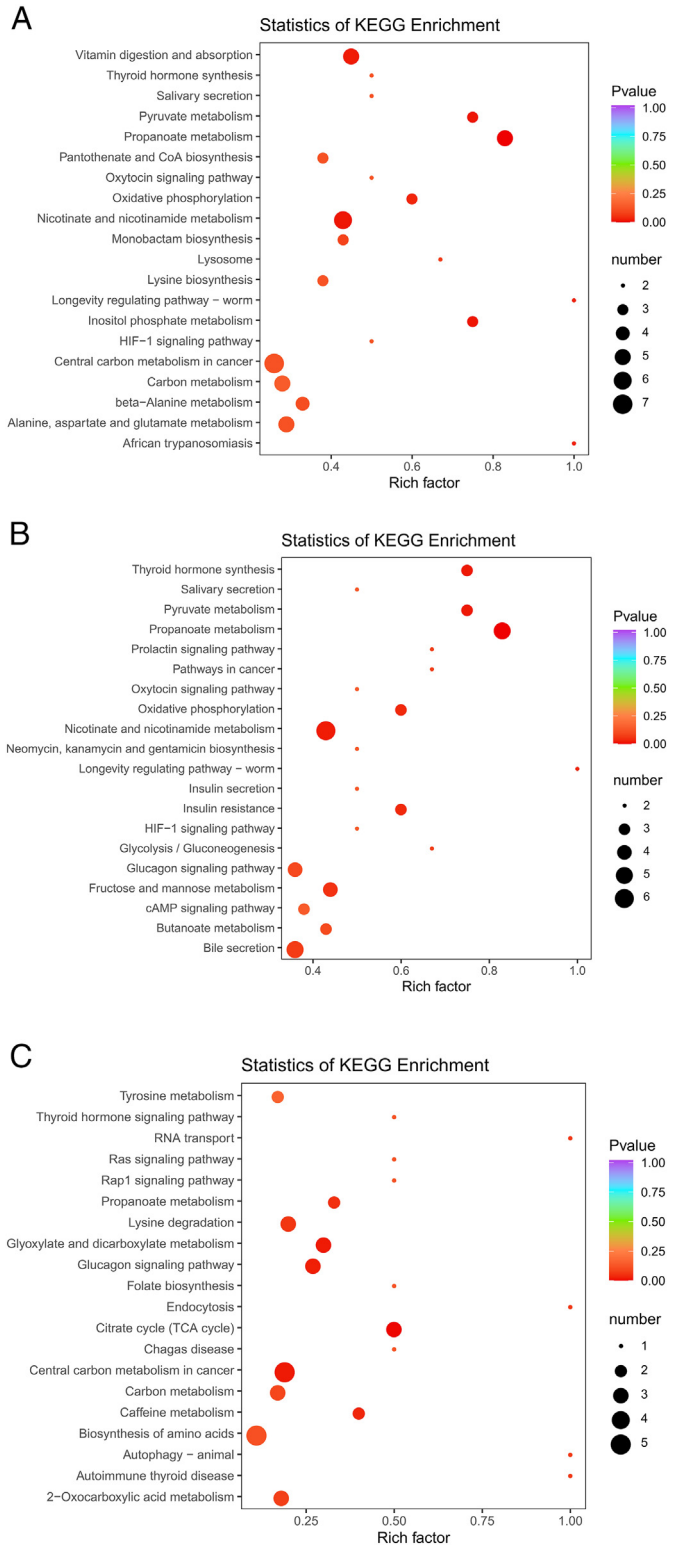

Fig. 4. Enriched KEGG pathways for the differential metabolites. (A) HL vs NC; (B) HT vs NC; (C) LO vs NC.

in the HL and HT group than in NC group. Glycolysis related coenzyme nicotinamide adenine dinucleotide (NAD) and its precursors (nicotinamide (NAM) and nicotinamide mononucleotide (NMD)) were significantly increased in HL and HT groups. These indicated that glycolysis was affected under heat plus hypoxia and heat stress. Anaerobic metabolic biomarkers (alanine, succinic acid, fumaric acid and malic acid) were accumulation and anaerobic substrate (aspartate) were decreased in HL group, marking the onset of anaerobic metabolism. Lactic acid was increased in HL and HT group than NC group. Citrate, isocitrate and arginine (could turn to ketoglutarate), methionine (could turn to succinyl CoA) were down-regulation in LO group and TCA cycle might be significantly affected. The oxidized lipids (leukotriene) increased in HL and HT implied that organism was under ROS stress. The accumulation of carnitine might promote fatty acid β oxidation for energy supply. The increased content of glycerophospholipid might play an important role in cell membrane stability. Organic osmolytes played roles in protein thermal stability (monosaccharide and Trimethylamine N-oxide (TMAO) increased) and ROS elimination (myoinositol and glutathione increased). Vitamin including ascorbate, pantothenate and nicotinamide played important antioxidation roles. Moreover, pantothenate probably helped maintain the cell integrity through CoA promoting phospholipid synthesis.

4. Discussion

Water temperature and DO are important environmental factors in aquatic environment that affect animal physiology. Increasing seawater temperatures and decreasing DO have been identified as two of the main causes of mortality in bivalve (Soon and Zheng, 2019). Metabonomic approaches provide high-throughput tools to characterize changes of metabolite and reveal the physiological alterations in aquatic animals in stressful environment (Huo et al., 2019). In this study, widely targeted metabolomic analysis was used to evaluate the physiological response of gills in hard clam, a heat- and hypoxia-tolerant bivalve. To adapt to the adverse environmental conditions (heat, hypoxia and heat plus hypoxia), *M. mercenaria* employed a variety of metabolites alterations. In order to clearly identify possible physiological response and metabolomic adaption mechanisms of adverse environment in hard clam, as shown in Fig. 6. Adverse environment potential biomarkers were also identified through ROC analysis.

4.1. Glycolysis, anaerobic metabolism and TCA cycle

The primary challenge for the organism under harsh environmental conditions was to optimize its energy requirement to maintain the basic physiological processes (Kyeong et al., 2020). Organism under harsh environmental conditions should overcome the increased energy demands due to the effects of temperature on metabolic rate (Somero, 2002). Glycolysis-related genes, including *hexokinase*, *phosphofructokinase*, *glyceraldehyde-3-phosphate dehydrogenase* have been found to increase significantly under air exposure stress in oyster, *Crassostrea gigas* (Meng et al., 2018). The glycolysis metabolites content including glucose-1-phosphate, glucose-6-phosphate and the glycolysis limited enzymatic activities of hexokinase and pyruvate kinase enzymes in periwinkle snail, *E. malaccana* increased at extremely high temperature (Chen et al., 2021). This indicated that glycolysis was activated under extreme thermal stress (Chen et al., 2021). The results showed that glycolysis played a key role in physiological adaptation to environmental stress in shellfish. In the most animals, glucose is the major monosaccharide that plays an essential role in energy synthesis (Wasserman, 2009). Other monosaccharide such as fructose and mannose can enter the glycolytic pathway by being converted into intermediates. Fructose was directly converted into fructose-6-phosphate while mannose formed mannose-6-phosphate before converting into fructose-6-phosphate. In our study, the content levels of fructose and mannose were significantly higher in the heat plus hypoxia group than in the negative control group. Therefore, it was plausible that the accumulated mannose and fructose can serve as immediate sources of energy under heat plus hypoxia stress conditions (Yancey, 2005). The nicotinamide adenine dinucleotide (NAD) that acted as hydride-accepting and hydride-donating coenzyme in glycolysis also accumulated in HL and HT group. Nicotinamide adenine dinucleotide (NAD) had a role in redox reactions of energy transduction and acted as a substrate in

regulatory reactions that lead to its degradation (Dölle et al., 2013). In summary, glycolysis was affected in hard clam under heat plus hypoxia and heat stress environmental conditions.

Regulation of anaerobic metabolism played a key role in hypoxia tolerance in marine mollusks (Larade and Storey, 2009). During warming, the transition to partial anaerobiosis occured well before the lethal temperature was reached (Eymann et al., 2020). Alanine and aspartate took part in anaerobic metabolism, alanine was the early anaerobiosis biomarker (Eymann et al., 2020) and aspartate was one of anaerobic substrate for producing alanine. Alanine was increased in HL and HT group. Aspartate depleted in HL group which was similar with the previous study of mussel and oyster (Haider et al., 2020). In our study, the content levels of alanine was higher while that of aspartate was lower. This showed that hard clam had started the anaerobic metabolism. The level of three biomarkers of anaerobic metabolism: succinate, fumarate and malate (Eymann et al., 2020), increased in HL and/or HT groups. However, the content level of succinate was higher in hypoxia (LO) group than in the negative control (NC) group. An increase in anaerobic metabolism could be a physiologically adaptive response to a short-term heat, hypoxia or combination of heat and hypoxia conditions, to ensure adequate energy.

The TCA cycle is a common pathway for the oxidative decomposition of sugars, amino acids and lipids. A review published in Nature communications highlighted on the role of metabolites of TCA cycle in cell fate and function and change in their abundance during regulation physiology and disease (Martínez-Reyes and Chandel, 2020). In the current study, besides that succinate content level was higher in hypoxia (LO) group, the citrate and isocitrate were significantly decreased. The content level of arginine and methionine also decreased in LO group which could have turned to ketoglutarate and succinyl CoA, respectively. Furthermore, in the comparative study between LO and NC groups, the TCA cycle pathway was significantly enriched (Fig. 4). Therefore, these results implied that hypoxia condition can alter the TCA cycle.

4.2. Lipid metabolism and glycerophospholipid metabolism

Lipids which are important sources of energy (Toprak, 2020). Lipid metabolism had an important role in hypoxia stress (Sun et al., 2020a; Sun et al., 2020b; Zhao et al., 2020) and *Vibrio*-resistance (Su et al., 2021). Metabolites related to lipid metabolism comprised made up a large percent of different metabolites under HL, HT and LO groups (Fig. 3A). This indicated that lipid related metabolites had a strong response under environmental stress.

Acylcarnitines were intermediate oxidative metabolites that comprise a fatty acid esterified to a carnitine molecule (Reuter and Evans, 2012). It played a functional role in transportation of the long-chain fatty acids (LCFAs) across the mitochondrial membrane for β-oxidation (Rinaldo et al., 2002). In a study of metabolomic effects of noise exposure on rats, the content level of Carnitine isoC4:0 was significantly increased in serum of rats in the noise stress group (Ji et al., 2020). Our results were consistent with this. What's more, of the 16

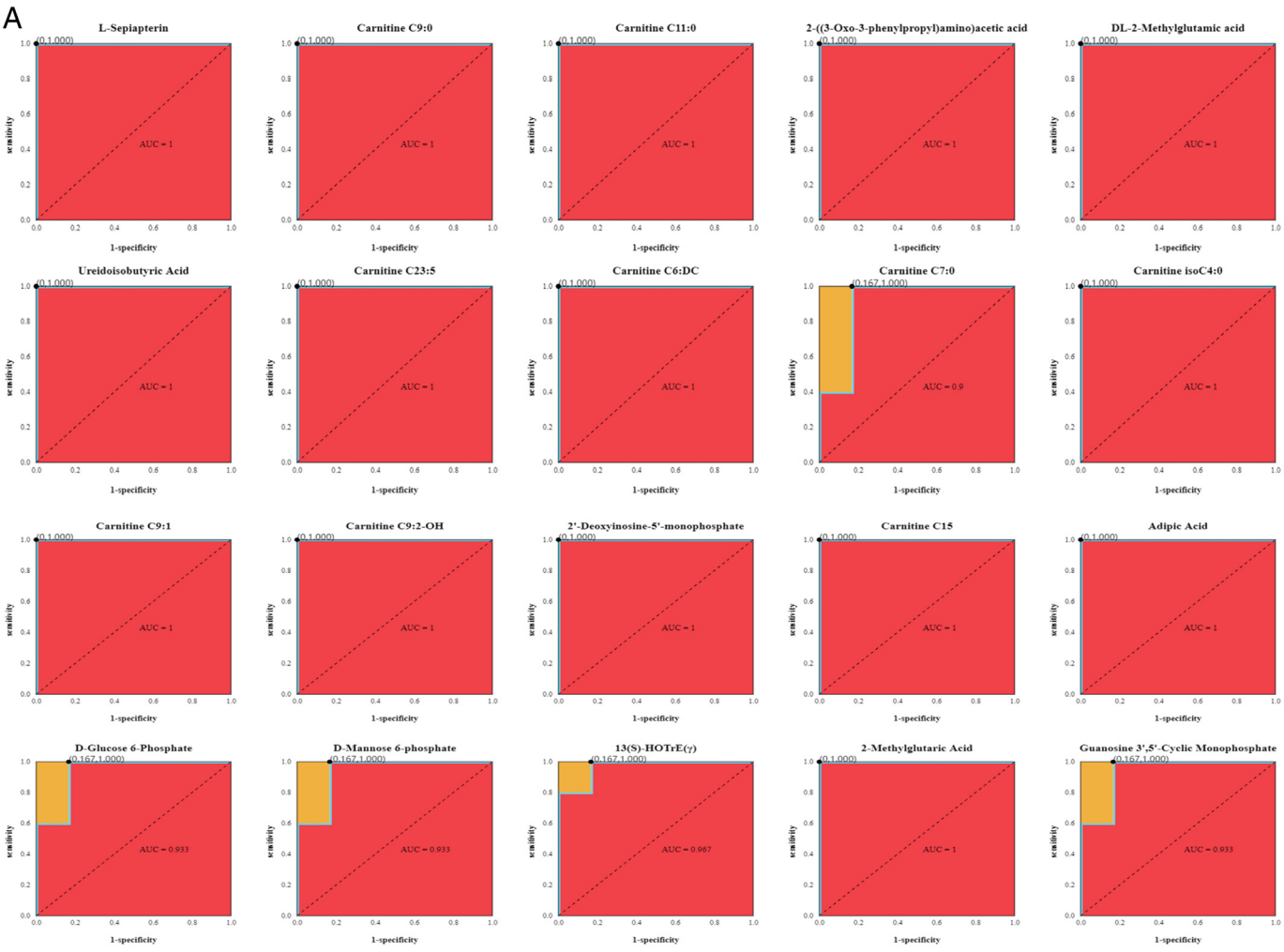

Fig. 5. Identification of potential biomarkers under HL (A), HT (B) and LO (C) by ROC analysis.

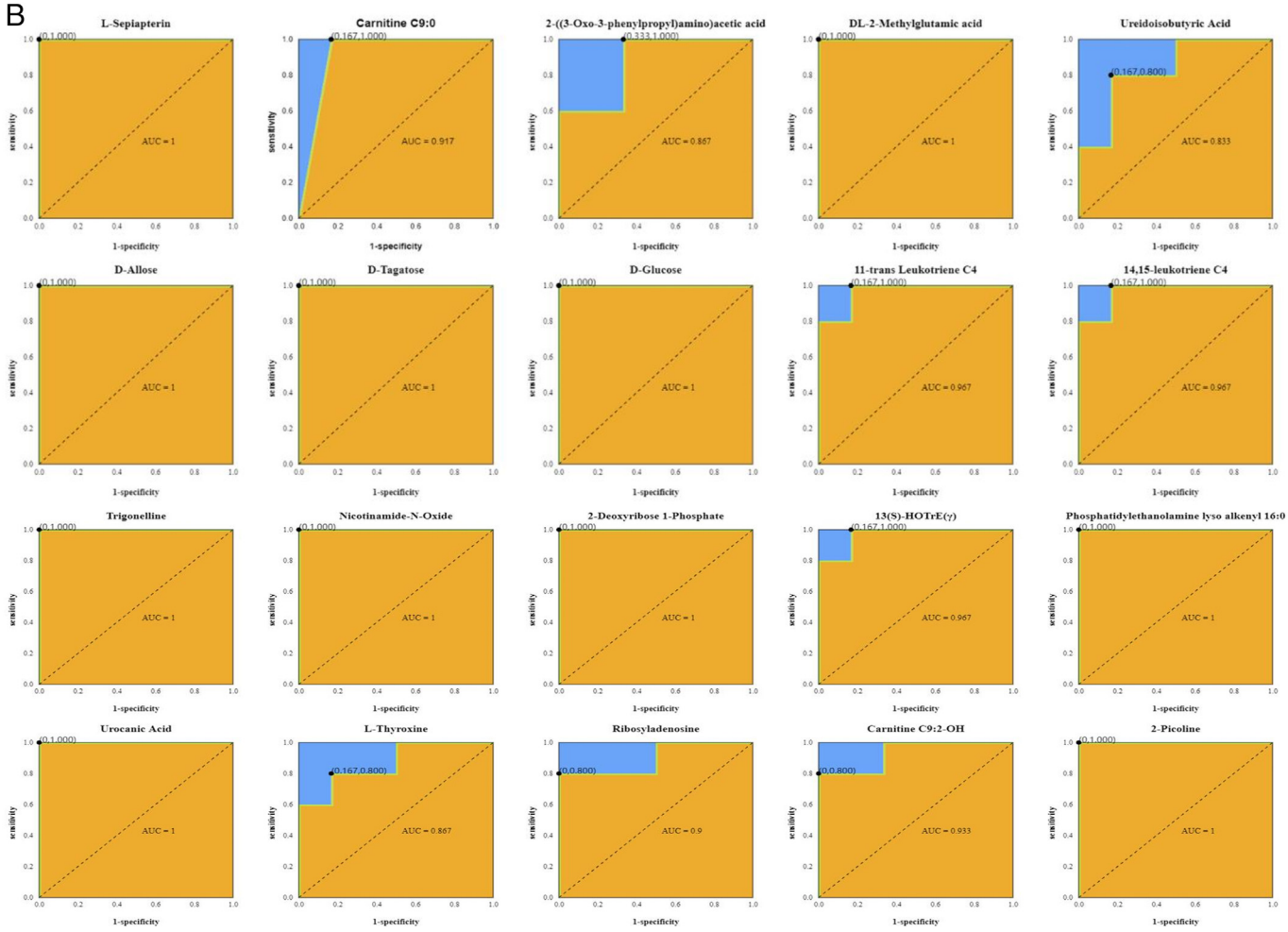

Fig. 5 (*continued*).

common differential metabolites, 9 were acylcarnitines (Fig. 3B). We speculated that acylcarnitine was involved in fatty acid β oxidation to produce energy during the stress period. The expression of the transcription factor of fatty acid oxidation systems, *Carnitine O-palmitoyltransferase 1* (Su et al., 2021) was upregulated in heat plus moderate hypoxia (35 °C, 2 mg/L) and heat plus severe hypoxia (35 °C, 0.2 mg/L) stress conditions. This result further supported hypothesis of our study.

Oxidized lipid was another class of Fatty acyl that was found in HL and HT groups but was not found in LO group (Fig. 3A). Among oxidized lipids, leukotriene C4 was a major indicator of ROS involved in endoplasmic reticulum stress (Dvash et al., 2015). In the present study, leukotriene C4, 14, 15-leukotriene C4 and 11-trans leukotriene C4 were oxidized lipids found accumulated in HL and HT groups. These showed that hard clams were subjected to oxidative stress when under high temperature conditions. Of note, the AUC value of 14, 15-leukotriene C4 and 11-trans leukotriene C4 were both more than 0.9 (Fig. 5B), hence the two could be used as biomarkers of heat stress in hard clam.

Glycerophospholipid is an important component of cell membrane that is destroyed by high temperature (Calzada et al., 2016). Under thermal stress, *E. malaccana* increased the level of glycerophospholipid metabolism to maintain cellular membrane structure (Chen et al., 2021). However, in our sturdy, lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) were increased in HL group. The LPC, lysobisphosphatidic acids (LPA) and most of LPE (8 kinds of LPE increased and 2 kinds of decreased) were significantly decreased in HL group. Phosphatidylethanolamine (PE) (Calzada et al., 2016), the second most abundant phospholipid in the cell whose biosynthesis precursors was LPE (Vance, 2015). Although LPC was previously considered as a biomarker of disease, this is now controversial (Law et al., 2019). LPA was one of the smallest and simplest phospholipids discovered so far and it was the main precursor in the early stage of phospholipid biosynthesis in eukaryotic cells (Pyne et al., 2004). The accumulated LPA, LPC and LPE in hard clams under heat stress could have enhanced the stability of the cell membrane structure. However, their precise roles need to be explored further.

One of the most significant difference between the differential metabolites in hard clams of LO vs NC comparison from the rest (HL vs NC, HT vs NC) was the presence of oxidized lipids and glycerophospholipid (Fig. 3A). Therefore, it could be explained that hard clam had a strong hypoxia tolerance or hypoxia caused less damage to the hard clams than heat.

4.3. Organic osmolytes

Different environmental stress can lead to the overproduction of reactive oxygen species (ROS). Increased levels of oxidative stress leads to DNA, protein and lipid damage and even cell death (Boveris and Chance, 1973). The accumulation of oxidized lipids (leukotriene) in hard clams was an indication that they were under ROS stress. Organic solutes including polyols and sugars, amino acids and their derivatives, and majority of these organic solutes have cytoprotective properties, such as antioxidation (Yancey, 2005). The presence of myoinositol which is an active cytoprotectant acted as an antioxidant and scavenged free radicals generated during stresses (Yancey, 2005). It was observed that the concentration of myoinositol was significantly higher in the heat

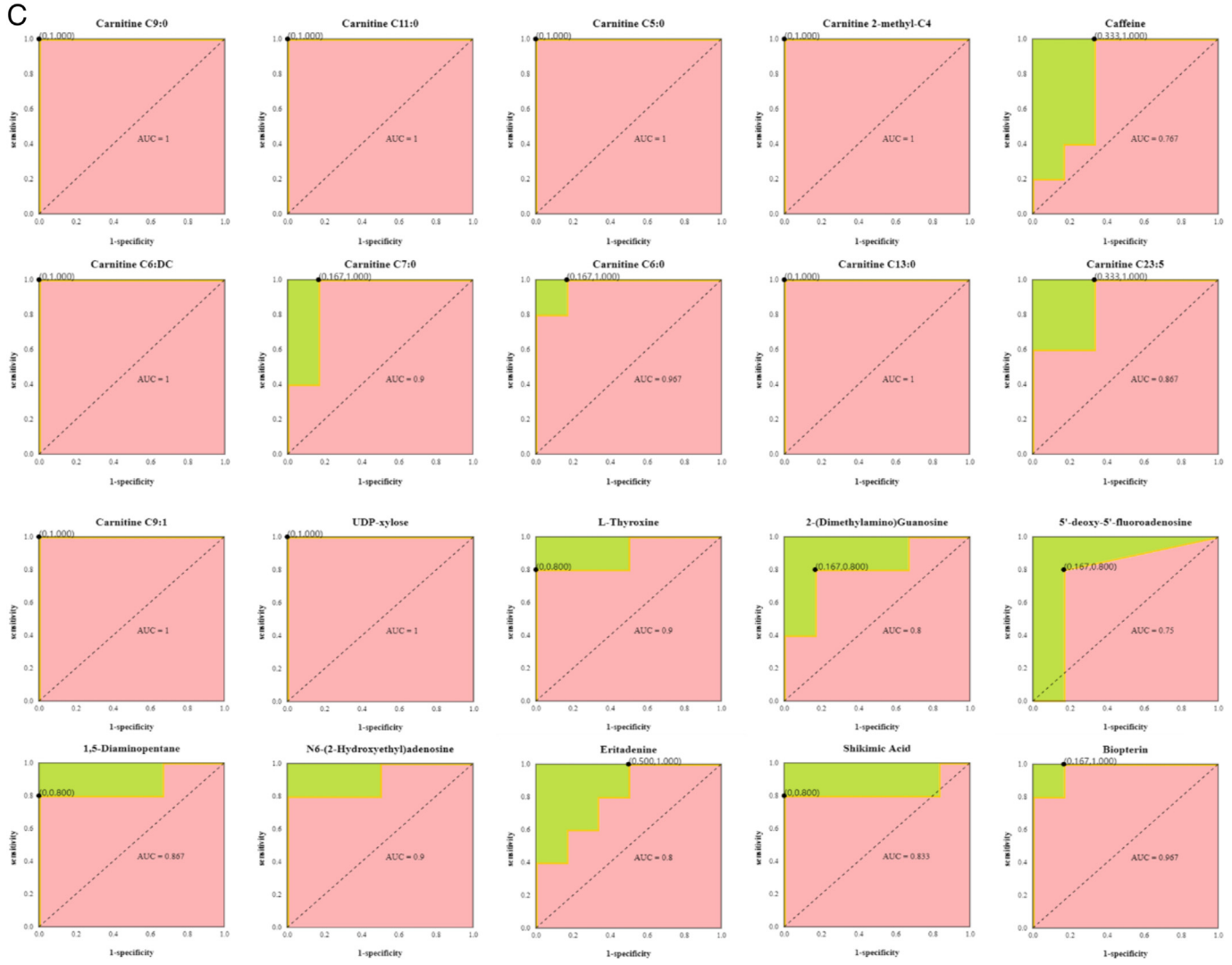

Fig. 5 (*continued*).

plus hypoxia (HL) group than in the negative control (NC) group. The concentration of cysteine glutathione disulfide was higher in all stress groups (HL, HT, LO) than in the negative control group (Fig. 3B). The reduced-form of glutathione only increased in heat (HT) group. These results indicated that antioxidative osmolytes can scavenge free radicals and ROS to maintain cellular homeostasis and protect organisms from oxidative tissue damage under stress.

Heat stress increased the risk of aberrant folding by negatively affect protein structure. Another cytoprotective property of organic solutes was stabilization of proteins. The concentrations of the thermoprotective osmolytes (glycine betaine, choline and carnitine) increased in *E. malaccana* under increased temperature (Chen et al., 2021). The *E. malaccana* is one of the most heat-tolerant eukaryotes and the increased thermoprotective osmolytes could have protected their protein structures of from denaturation by high temperatures (Chen et al., 2021). Almost all natural osmolytes solutes increased the protein thermal stability in vitro and some carbohydrate solutes may be used in living organisms to counteract disruption of proteins by high temperatures (Yancey, 2005). In this study, the concentration of various monosaccharide such as glucose, tagatose and allose were significantly increased in HT group than in the negative control (NC) group. Trimethylamine N-oxide (TMAO) can also enhance protein folding and ligand binding (Yancey, 2005). In this study, the concentration of TMAO was higher in HL and HT groups than in the negative control (NC) group. A study by Bennion et al. (2004) reported that TMAO can enhance water structure, causing greater organization through stronger hydrogen bonding among water molecules. Therefore, the results of the current study showed that the accumulation of thermoprotective osmolytes (monosaccharide, TMAO) promoted maintenance of the protein homeostasis in hard clams.

4.4. Vitamins

Vitamin C (ascorbic acid) is an essential micronutrient for the normal growth and physiological function of most aquatic animals and is, therefore, used as a diet supplement to enhance tolerance to stress (Dawood and Koshio, 2018). Ascorbic acid was a robust antioxidant which can scavenge ROS (Bae et al., 2012) and regulated tolerance to environmental stressors (Merchie et al., 1995). As mentioned above, heat plus hypoxia stress induced ROS, and thus the increase in levels of ascorbic acid in HL group might an important response to cope with the ROS stress. The main physiological functions of pantothenate include to participate in the synthesis and degradation of fatty acids, as well as the synthesis of membrane phospholipids (Chen, 2005). Pantothenate was the precursor of coenzyme A (CoA), and CoA was the bioactive form of pantothenate that effects its functions. It has been shown that pantothenate can protect the membrane system against lipid peroxidation through different mechanisms (Chen, 2005). Hence, it

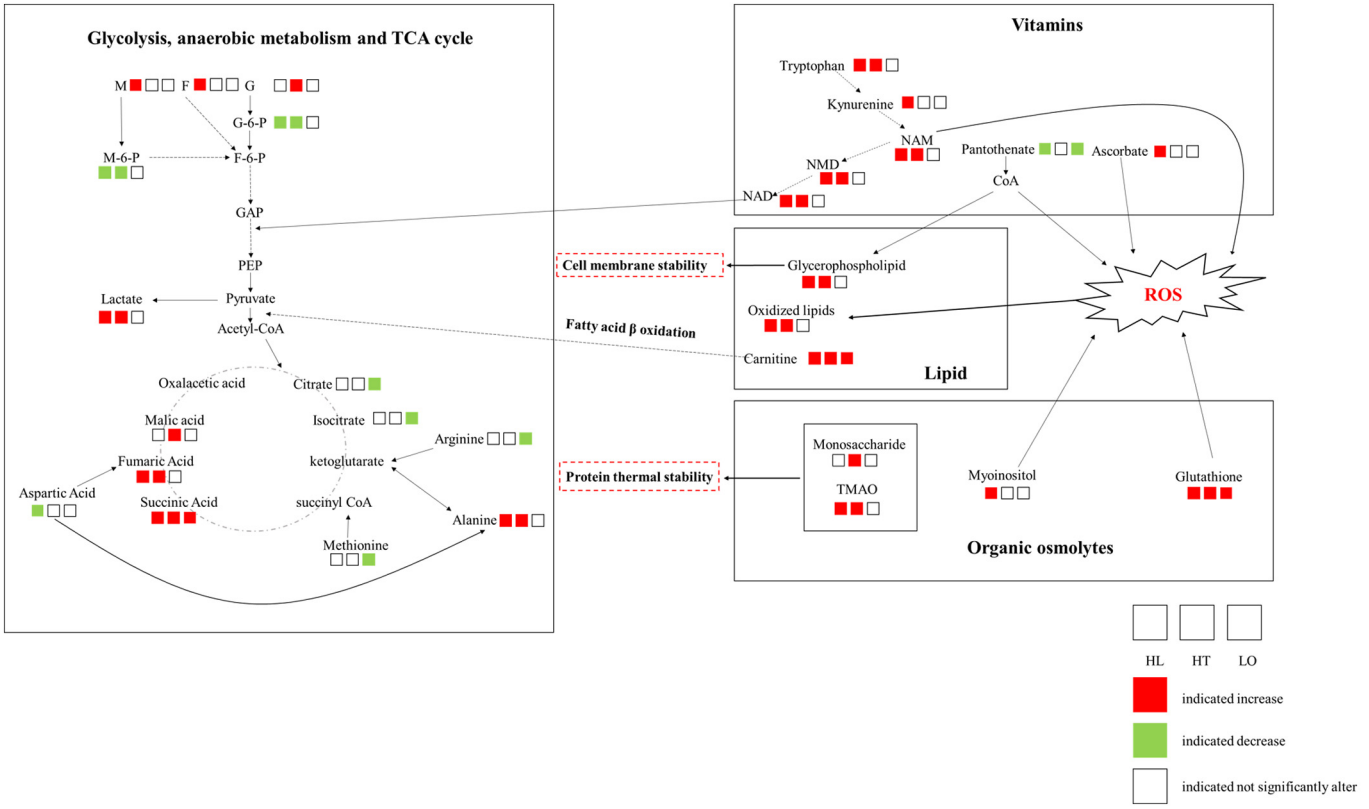

Fig. 6. The hypothetical metabolic response map under environmental stresses. Red indicated increase; green indicated decrease.

maintained integrity of the cell structure and function (Chen, 2005). Pantothenate and its derivatives also protected cytoplasmic membranes against free radical damage by increasing the amount of CoA, which in turn facilitated the repair of membranes by promoting phospholipid synthesis (Slyshenkov et al., 1995). In this study, the concentration of pantothenate was reduced under hypoxia and heat plus hypoxia stress, which may be a response to maintain cell membrane integrity.

Nicotinamide (NAM) is a form of Vitamin B3 which can suppress oxidative stress (Wei et al., 2020). Consumption of NAM-containing diet can alleviate oxidative stress by reducing ROS content in serum, increasing glutathione content and promoting glutathione metabolism in cow (Wei et al., 2018). NAM was also reported to maintain DNA integrity and membrane phosphatidylserine asymmetry hence improve integrity of cells structures and prevent cellular removal (Maiese and Chong, 2003). It has also been implicated in the control a number of pathways (e.g., PARP, Akt) to prevent DNA degradation (Maiese and Chong, 2003). Of note, NAM can be converted into nicotinamide mononucleotide (NMD) and then to nicotinamide adenine dinucleotide (NAD) (Wei et al., 2020). The generated NAD had vital roles in glycolysis and TCA cycle as hydride-accepting and hydride-donating coenzyme (Belenky et al., 2007). In this study, the content of NAM, NMD and NAD was higher in HL and HT groups compare to NC group. Interestingly, tryptophan (the biosynthesis initiator of NAM) and kynurenine (the vital intermediate product of NAM biosynthesis pathway) (Belenky et al., 2007) were also higher in HL than in NC group whereas tryptophan was higher and kynurenine was not significantly changed in HT group. These results indicated that the NAD metabolism pathway played a key role in the defense mechanisms against heat and heat plus hypoxia stress in hard clam.

4.5. Other physiological response

When exposed to environmental stress (salinity, hypoxia and temperature), aquatic animals underwent endocrine changes as the primary adaption response (Eissa and Wang, 2016). Compared to vertebrates, invertebrates had simpler endocrine systems, but robust stress hormones response (Adamo, 2012). Under environmental stress, hormones such as dopamine, epinephrine, norepinephrine, octopamine and serotonin were rapidly released (Adamo, 2012). Thyroxines may influence all major metabolic pathways and they are well-known to increase basal energy expenditure (Pucci et al., 2000). Thyroxine can stimulate ATP consumption, supply energy, and increase heat production. It did not regulate of metabolism immediately, but rather modulated the activities of metabolic pathways on a medium-or long-term basis, either by regulating hormones such as insulin, glucagon and catecholamines directly or indirectly (Pucci et al., 2000). In this study, the concentration of L-thyroxine, which was the sole differential hormone, was all decreased in HL, HT and LO stress groups than in NC group as shown in Fig. 3B. Thyroxine regulated responses to salinity by modulating lipid metabolism (Sheridan, 1989). The content of thyroxine (T4) was decreased within 3 h after exposure to heat stress in spotted sea bass *Lateolabrax japonicus* (meeting communications). This indicated that thyroxine levels were reduced under adverse environments to avoid unnecessary energy expenditure. The precise functions of thyroxine in the defense mechanism of hard clam against environmental stress require further investigation.

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Ethics standards

The experiment with invertebrate in this study do not need approval in ethics committee.

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CRediT authorship contribution statement

Zhi Hu: Investigation, Data curation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Jie Feng:** Data curation, Formal analysis, Writing – review & editing. **Hao Song:** Formal analysis, Writing – review & editing. **Cong Zhou:** Project administration, Resources. **Mei-Jie Yang:** Project administration. **Pu Shi:** Project administration. **Zheng-Lin Yu:** Project administration, Resources. **Yong-Jun Guo:** Resources. **Yong-Ren Li:** Resources. **Tao Zhang:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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