Metabolomic and transcriptomic profiling reveals the alteration of energy metabolism in oyster larvae during initial shell formation and under experimental ocean acidification

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Marine bivalves secrete calcified shells to protect their soft bodies from predation and damages, which is of great importance for their survival, and for the safety of the coastal ecosystem. In recent years, larval shell formation of marine bivalves has been severely affected by ocean acidification (OA), and previous study indicated that OA might affect such process by disrupting endogenous energy metabolism. Developmental stages from trochophore to D-shape larvae are extremely important for initial shell formation in oyster since a calcified shell was formed to cover the chitin one. In the present study, metabolomic and transcriptomic approaches were employed to investigate the energy metabolism of oyster larvae during initial shell (prodissoconch I, PDI shell) formation and under experimental OA treatment. Totally 230 chemical compounds were identified from the present dataset, most of which were highly expressed in the "middle" stage (early D-shape larvae) which was critical for PDI shell formation since a calcified shell was formed to cover the chitin one. Several compounds such as glucose, glutaryl carnitine (C5), β-hydroxyisovalerylcarbnitine, 5-methylthioadenosine (MTA), myristoleate (14:1n5) and palmitoleate (16:1n7) were identified, which were involved in energy metabolic processes including amino acid oxidation, glycolysis, pentose phosphate pathway and fatty acid metabolism. In addition, mRNA expressions of genes related to protein metabolism, glycolysis, lipid degradation, calcium transport and organic matrix formation activities were significantly down-regulated upon experimental OA. These results collectively suggested that formation of the initial shell in oyster larvae required endogenous energy coming from amino acid oxidation, glycolysis, pentose phosphate pathway and fatty acid metabolism. These metabolic activities could be severely inhibited by experimental OA, which might alter the allocation of endogenous energy. Insufficient endogenous energy supply then suppressed the mobilization of calcium and resulted in a failure or delay in PDI shell formation.

The calcified shells are extremely important for marine bivalves living in the intertidal zone since the shells can protect them from tidal, predator and other harsh environmental factors1,2. Shell formation of marine bivalves happens as early as trochophore larvae, which relies on the energy from eggs3,4. According to previous study, total

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Oyster larvae at three developmental stages (15 (“early”), 17 (“middle”) and 21 (“late”) hours post fertilization (hpf)) were collected for metabolomic analysis since they were the key stages for the formation of calcified shells in oyster larvae. The present dataset comprised a total of 230 chemical compounds of known identity. 149, 162 and 33 compounds were identified from “early”, “middle” and “late” stages, respectively. A total of 149 chemical compounds were found to differentially expressed at the “early” stage, with 139 up-regulated ones and 10 down-regulated ones (Fig. 1A). The “middle” stage was critical for the formation of calcified shell in oyster larvae, and most of the differentially expressed compounds were identified from this stage including 142 up-regulated ones and 20 down-regulated ones (Fig. 1A,B). At the “late” stage, the calcified shell was formed and relatively fewer compounds were identified, which consisted of 11 up-regulated ones and 22 down-regulated ones (Fig. 1A). The identified chemicals were shown in Table S1.

Energy demand at key “middle” stage of larval development. Using Random forest and the Biochemical Importance Plot methods, several compounds related to energy metabolism were identified, most of which were highly expressed in the key “middle” stage at 17 hpf, during which the calcified shell was formed to cover the chitin one. Chemical compounds such as glucose, glutarylcarnitine (C5), 3-hydroxyisovaleroylcarnitine, 3-methylglutaryl carnitine (C6) and acetylcarnitine (C2). On the other hand, ribose, a metabolite associated with the anabolic pentose phosphate pathway, was also significantly elevated during the “middle” developmental stage (Fig. 2).

Identification of polyamines and nucleic acid metabolites. Many metabolites related to polyamines and nucleic acid metabolism were identified, including putrescine, 5-methylhioadenosine (MTA), adenosine, adenine, 2′-deoxyadenosine, cytidine and cytosine (Fig. 3). In particular, putrescine and MTA were involved in urea cycle and responsible for cellular proliferation process, while adenosine, adenine, 2′-deoxyadenosine, cytidine and cytosine were dramatically overexpressed in the “middle” stage and ranked the Top 30 chemicals identified in the present study.

Higher fatty acid oxidation in the “middle” and “late” stages. Free fatty acid metabolism was elevated in the “middle” and “late” stages comparing to the “early” stage (Fig. 4). The expressions of succinylcarnitine, acetylcarnitine and flavin mononucleotide (FMN) were significantly elevated in the “middle” stage, while propionylcarnitine, decanoylcarnitine, myristoylcarnitine, palmitoylcarnitine and stearoylcarnitine were highly expressed in the “late” stage. Besides, myristate (14:0), myristoleate (14:1n5), palmitoleate (16:1n7) and stearidate (18:4n3) were highly expressed in both “middle” and “late” stages.
The differentially expressed genes related to energy metabolism in the key “middle” stage.

For the significantly up-regulated genes identified from the “middle” stage, several GO terms related to energy metabolism and shell formation were identified, including lipid metabolic process (GO:0006629, FDR < 10.0E-3), cellular lipid metabolic process (GO:0044255, FDR < 19.0E-3), lipid biosynthetic process (GO:0008610, FDR < 96.0E-3), fatty acid metabolic process (GO:0006631, FDR < 69.0E-3), monocarboxylic acid metabolic process (GO:0032787, FDR < 120.0E-3), regulation of nucleoside metabolic process (GO:0009118, FDR < 140.0E-3) and regulation of purine nucleotide metabolic process (GO:1900542, FDR < 140.0E-3) (Fig. 5). Particularly, 10 and 7 genes characterized by overrepresented GO terms of lipid metabolic process and cellular lipid metabolic process were significantly overexpressed in the “middle” stage.

Key processes affected by OA during larval shell formation.

By analyzing the transcriptomic data of oyster larvae under experimental OA, expressions of genes related to key processes in larval shell formation were significantly inhibited, which included calcium transportation (CGI_10000261, CGI_10011110 and CGI_10008043), bicarbonate transport (CGI_10004992, CGI_10027742 and CGI_10020143), organic matrix (CGI_10011133, CGI_10003192 and CGI_10002674), glycolysis (CGI_10007559, CGI_10003670 and CGI_10025556), fatty acid metabolic process (CGI_10008805, CGI_10021437 and CGI_10001878), protein phosphorylation (CGI_10002840, CGI_10015358 and CGI_10015881), and ATP synthesis (CGI_10004070, CGI_10006577 and CGI_10005007) (Fig. 6, Table 1).

Discussion

Marine bivalves such as oysters and scallops secrete calcified shells as a supporting frame for their soft bodies and for protection from predators1,2, which is thought to be one of the key factors that trigger the expansion of bivalves at the dawn of the Cambrian times16. The early shell formation of bivalve larvae requires sufficient endogenous energy sources such as glucose, lipids and protein. In recent years, larval shell formation of marine bivalves has been severely affected by OA, resulting in vast mortality worldwide. Since previous study demonstrated that the level of reserves was only slightly higher than that required for shell formation in bivalve larvae10, it was hypothesized that OA might influence larval shell formation by disrupting the energy metabolic process. Although some progress has been made on revealing the metabolic bases of bivalve larvae under OA threat, much work is still needed to illustrate the precise metabolic pathways of lipids, carbohydrates and proteins during the formation of initial shell (PDI shell). In the present study, metabolomic and transcriptomic approaches were employed to study the metabolic variations in oyster larvae during formation of calcified shell and upon experimental OA, hoping to identify metabolites related to lipid/carbohydrate/protein metabolism during PDI shell formation and to reveal the negative effects of OA on oyster larvae from a point of view of energy metabolism.

Oyster larvae at three developmental stages (15 hpf ("early"), 17 hpf ("middle") and 21 hpf ("late")) were collected for metabolomic analysis since they were the key stages for the formation of calcified shells in oyster larvae. Totally 230 chemical compounds were identified from the present dataset, most of which were highly expressed in the "middle" stage. Overexpression of these metabolites indicated that a high level of energy metabolism was
demanded for the formation of calcified shell. Using Random Forest and the Biochemical Importance Plot methods, a vast array of differentially expressed compounds related to energy metabolism at the “middle” stage were identified, such as glucose, glutarylcarnitine (C5), β-hydroxyisovaleroylcarnitine, 3-methylglutarylcarnitine (C6) and acetylcarnitine (C2) and ribose were highly expressed in the “middle” stage, which was crucial for the formation of calcified shell.

Figure 2. Energy demand at the key “middle” stage. (A) Expression of chemicals related to energy metabolism in different developmental stages. (B) Change fold of key chemicals related to energy metabolism. (C) The pentose phosphate pathway. Chemical compounds such as glucose, glutarylcarnitine (C5), β-hydroxyisovaleroylcarnitine, 3-methylglutarylcarnitine (C6) and acetylcarnitine (C2) and ribose were highly expressed in the “middle” stage, which was crucial for the formation of calcified shell.

Figure 3. Metabolites of polyamines and nucleic acids metabolism. Many metabolites related to polyamines (A) and nucleic acid (B) metabolism were identified, including putrescine, 5-methylthioadenosine (MTA), adenosine, adenine, 2′-deoxyadenosine, cytidine and cytosine.
and acetylcarnitine (C2) and ribose (Fig. 2). β-hydroxyisovalerylcarnitine, 3-methylglutarylcarnitine, glutaryl-carnitine and acetylcarnitine are key intermediates of protein phosphorylation and amino acid oxidation17, while putrescine, 5-methylthioadenosine (MTA) and spermine were involved in the urea cycle18. Amino acids, derived largely from protein in the diet or from degradation of intracellular proteins, are the final class of biomolecules whose oxidation makes a significant contribution to the generation of metabolic energy 19. Meanwhile, amino acid catabolism results in waste ammonia, which needs a way to be excreted since they are toxic 20. As for most aquatic organisms, they excrete ammonia by diluting it by water outside the organism or converting it into a less toxic substance such as urea or uric21. In the present study, metabolites of both amino acid oxidation pathway and urea cycle pathway were overexpressed at the early D-shape larvae (“middle”) stage, which was key period for the PDI shell formation since a calcified shell was formed to cover the chitin one2. Besides, experimental OA was able to significantly suppress the mRNA expression of genes including DNA-directed RNA polymerase II submit spb 7, sparc-related modular calcium-binding protein 1-like, calcium-dependent protein kinase 31, ATP synthase lipid-binding mitochondrial-like, and ATP synthase subunit mitochondrial-like, which were related to protein metabolism and ATP synthesis22. Besides, research in marine bivalves proved that the interaction of seawater acidification and elevated temperature led to further expression of amino acid metabolism23. Therefore, results in the present study suggested that protein metabolism by amino acid oxidation could be a critical source of endogenous energy for the formation of initial shell in oyster larvae. OA inhibited larval shell formation by suppressing amino acid metabolism and resulted in a lack of ATP synthesis, which might then cause a failure of delay in PDI shell formation.

Apart from amino acid oxidation, glycolysis and pentose phosphate pathway were also found to be significant energy sources for initial shell formation in oyster larvae11,24. Glucose and ribose were highly expressed in the “middle” stage. Glucose is the most important source of energy in all organisms. In the present study, metabolites related to glucose metabolism through glycolysis and pentose phosphate pathway were identified during initial shell formation, and transcriptomic analysis illustrated that the expression genes related to glycolysis, such as pyruvate kinase, hexokinase and aldolase, was obviously inhibited upon experimental OA. These results were inconsistent with previous reports that OA could up-regulate energy metabolism in both adult and larval marine bivalves. For example, it was said that when oyster C. gigas received an acute OA treatment, the alanine and ATP levels in mantle tissue decreased significantly whereas an increase in succinate levels was observed in gill tissue25. Thus, results from the present and previous studies suggested that glucose metabolism through glycolysis and pentose phosphate pathway should be another crucial source of energy supply for initial shell formation in oyster, which could be severely affected by OA and resulted in a failure or delay in larval shell formation.

Furthermore, fatty acids metabolism was found to be the third energy source for PDI shell formation in oyster larvae. On one hand, metabolites related to fatty acids metabolism including succinylcarnitine, acetylcarnitine,
Figure 5. Overrepresented GO terms related to energy metabolism during calcified shell formation. Several GO terms related to energy metabolism were identified, including lipid metabolic process, cellular lipid metabolic process, fatty acid metabolic process, monocarboxylic acid metabolic process, regulation of nucleoside metabolic process, and regulation of purine nucleotide metabolic process. The overrepresented GO terms were displayed with Cytoscape 3.6.1 (http://cytoscape.org/).

Figure 6. Key processes affected by experimental ocean acidification (OA) during larval shell formation. Experimental OA could suppress several key biological processes related to larval shell formation including calcium transportation, bicarbonate transport, organic matrix, glycolysis, fatty acid metabolic process, protein phosphorylation, and ATP synthesis.
RNA interference increased the intracellular Ca²⁺ metabolism, and cellular lipid metabolic process (Fig. 5). In addition, experimental OA could suppress related were also identified from the transcriptomic data, including phospholipid metabolic process, fatty acid (18:4n3) were highly expressed in the “middle” stage. On the other hand, several GO terms related to fatty acids mitochondrial, which were key molecules for fatty acid metabolism26. Fatty acids yield the most ATP on an energy per gram basis, when they are completely oxidized to CO₂ and water by beta oxidation and the citric acid cycle27. The metabolites identified in the present study were responsible for the activation of fatty acid degradation and could induce an increase of ATP synthesis. However, the metabolism of lipids could be negatively regulated by OA. In the pearl oyster P. fucata, genes associated with the “fatty acid biosynthesis” pathways were significantly enriched under acidification treatment13. And, unigenes involved in “fatty acid metabolism”, but not “glycerol metabolism”, are differentially expressed upon pH 7.8 treatment in P. fucata18. Like proteins and glucose, balanced lipid metabolism was also critical for PDI shell formation in oyster larvae24. And, results in the present study indicated that OA might influence initial shell formation of oyster larvae by inhibiting the processes of fatty acid metabolism. Furthermore, activities related to “fatty acid metabolic process” was slightly inhibited upon moderate acidification treatment (pH 7.7) and severely inhibited upon severe acidification treatment (pH 7.4) comparing with normal group, suggesting that moderate acidification treatment could barely influence fatty acid metabolism, while such process could be dramatically suppressed under severe acidification treatment.

The above results evidenced that formation of the initial shell in oyster larvae required endogenous energy supplied by amino acid oxidation, glycolysis, pentose phosphate pathway and fatty acid metabolism. These metabolic activities were severely affected by experimental OA, resulting in a failure or delay in PDI shell formation. In order to reveal the link between energy metabolism and shell formation, the expression patterns of shell formation-related genes were further investigated. By analyzing the transcriptomic data of oyster larvae under experimental OA, expressions of genes related to key processes in larvae shell formation were significantly inhibited, which included calcium transportation, bicarbonate transport, and organic matrix. These results suggested that the mobilization of calcium in oyster larvae was significantly disrupted upon experimental OA treatment. According to our previous study, oyster carbonic anhydrases (CA) was able to modulate intracellular pH (pHi) of oyster haemocytes under CO₂ exposure28. Meanwhile, it was found that elevated CO₂ caused the decrease of intracellular Ca²⁺ in haemocytes. The inhibition of CA by acetazolamide and suppression of CgCa gene via RNA interference increased the intracellular Ca²⁺ in haemocytes29. The above results suggested that initial shell formation in oyster larvae required endogenous energy supplied by amino acid oxidation, glycolysis, pentose phosphate pathway and fatty acid metabolism. Experimental OA affected such process by disrupting the balanced energy metabolism, which might alter the allocation of metabolic energy and further suppress the mobilization of calcium in oyster larvae. Moreover, activities related to “organic matrix” and “glycocalyx” was activated upon moderate acidification treatment (pH 7.7) and inhibited upon severe acidification treatment (pH 7.4), which indicated that moderate acidification treatment could activate the stress response in oyster to sustain homeostasis upon acidifying environment, while the severe acidification treatment would significantly suppress normal physiological activities.

| Treatments | GO ID         | GO terms                                      | Nr. genes |
|------------|---------------|-----------------------------------------------|-----------|
| pH 7.8     | GO:0006426    | iron ion transport                            | 3         |
|            | GO:0010466    | negative regulation of peptidase activity     | 7         |
|            | GO:0030414    | peptidase inhibitor activity                  | 12        |
|            | GO:0030529    | ribonucleoprotein complex                      | 16        |
|            | GO:0042254    | ribosome biogenesis                            | 6         |
|            | GO:0043232    | intracellular non-membrane-bounded organelle   | 26        |
|            | GO:0044281    | small molecule metabolic process               | 36        |
|            | GO:1901564    | organonitrogen compound metabolic process      | 36        |
| pH 7.4     | GO:0006412    | translation                                    | 107       |
|            | GO:0008135    | translation factor activity, nucleic acid binding | 20       |
|            | GO:0008137    | NADH dehydrogenase (ubiquinone) activity       | 5         |
|            | GO:0009059    | macromolecule biosynthetic process             | 147       |
|            | GO:0010467    | gene expression                                | 149       |
|            | GO:0015986    | ATP synthesis coupled proton transport          | 9         |
|            | GO:0016651    | oxidoreductase activity, acting on NAD(P)H    | 8         |
|            | GO:0019538    | protein metabolic process                      | 162       |
|            | GO:0043170    | macromolecule metabolic process                | 228       |
|            | GO:0044249    | cellular biosynthetic process                  | 175       |
|            | GO:0044260    | cellular macromolecule metabolic process       | 206       |
|            | GO:0044267    | cellular protein metabolic process             | 147       |
|            | GO:1901576    | organic substance biosynthetic process         | 176       |

Table 1. Key significantly down-regulated genes under experimental acidification treatment.
In conclusion, metabolomic and transcriptomic approaches were employed to investigate the energy metabolism of oyster larvae during PDI shell formation and under experimental OA treatment (Fig. 7). Results in the present study suggested that formation of the initial shell required endogenous energy coming from amino acid oxidation, glycolysis, pentose phosphate pathway and fatty acid metabolism. These metabolic activities could be severely inhibited by experimental OA, which might alter the allocation of metabolic energy. Insufficient endogenous energy supply then suppressed the mobilization of calcium and resulted in a failure or delay in PDI shell formation.

Methods

Oyster larvae sample collection. Sexually mature oyster C. gigas (about 2-year old, averaging 150 mm in shell length) were collected from a local farm in Dalian, Liaoning Province, China, and maintained in the aerated seawater at 20°C for 14 days before processing. The eggs and sperms were scraped from different adult oysters and mixed well to minimize individual variability. After fertilization, the developing embryos were cultured in filtered and aerated sea water at 20 °C. The breeding protocol was performed according to previous description31.

In the acidification treatment experiment, trochophores collected at 15 hours post fertilization (hpf) were equally divided into two groups, taking three replicates for each group. Larvae cultured in normal sea water (pH = 8.10 ± 0.05) was designated as the control group (Normal group), while those in the CO₂ exposure groups (pH7.7 and pH7.4 groups) were bathed in acidified seawater (pH = 7.40 ± 0.05)32,33. The pH value of the CO₂

Figure 7. Schema illustrating how OA affected initial shell formation in oyster larvae. The formation of the initial shell in oyster larvae required endogenous energy coming from amino acid oxidation, glycolysis, pentose phosphate pathway and fatty acid metabolism. These metabolic activities could be severely inhibited by experimental OA, which might alter the allocation of metabolic energy. Insufficient endogenous energy supply then suppressed the mobilization of calcium and resulted in a failure or delay in PDI shell formation.
exposure group was controlled using an acidimeter (AiKB, Qingdao, China)34. Total alkalinity was determined by end-point titration of 25 mmol L−1 HCl on 50 mL samples. Carbonate parameters were calculated from the pH and total alkalinity.

The collection of larvae was performed according to Liu et al.31. Trophophore, early D-shape larvae and D-shape larvae were sampled at 15 hpf, 17 hpf, and 21 hpf, respectively. And, early D-shape larvae under experimental ocean acidification treatments were also collected. One microliter of Trizol (Invitrogen) was added to each tube containing larvae for RNA isolation, while larvae for metabolomic analysis were frozen directly in liquid nitrogen. Three replicates were conducted for RNA sequencing, while ten replicates were employed for each group in metabolomic analysis.

**Metabolite profiling.** Trophophore, early D-shape larvae and D-shape larvae sampled at 15 hpf, 17 hpf, and 21 hpf respectively were sent to metabolomic sequencing to explore the metabolomic patterns in oyster larvae during shell formation. The significant compounds were identified with liquid/gas chromatography coupled to mass spectrometry (LC/GC–MS) method. The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysis. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition. Raw data was extracted, peak-identified and QC processed using Metabolon’s hardware and software. The informatics system used in the present study was described by Luo et al.35. Random Forest method was used to analyze the metabolomic data36,37.

**Bioinformatical analysis of the transcriptomic data.** Trophophore, early D-shape larvae and D-shape larvae sampled at 15 hpf, 17 hpf, and 21 hpf, as well as D-shape larvae under moderate (pH 7.7) and severe (pH 7.4) acidification treatment were sent to RNA sequencing. The transcriptomic data was analyzed to explore the metabolic patterns in oyster larvae during shell formation. The analyzing protocol was similar to previous description36,39. Rstudio and Cytoscape ClueGO software were adopted to perform Gene Ontology (GO) overrepresentation analysis of the identified significantly up- and down-expressed genes. The hypergeometric test with FDR value of 0.01 was used to do the GO enrichment, and the differentially expressed genes were selected as test set, while all identified genes were taken as the reference set. The significantly overrepresented GO terms were calculates from test set, and displayed as a network using BiNGO plug-in to Cytoscape (http://cytoscape.org/)38.

**Statistical analysis.** Statistical analysis was performed and all data were given as Means ± S.D. Statistical significance was determined by two-tailed Student’s t-test, or by one-way analysis of variance (ANOVA) followed by S-N-K post hoc test for multiple comparisons. Statistically significant difference was designated at p < 0.05, indicating by asterisks.

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Author contributions

Zhaoqun Liu, Zhi Zhou designed the experiments. Zhaoqun Liu, Yukun Zhang, Yan Zheng, Yanan Zong, and Zhi Zhou performed the experiments. Zhaoqun Liu, Zhi Zhou, Chang Liu, Ning Kong, and Lingling Wang analyzed the data. Qiang Gao, Lingling Wang, and Linseng Song contributed reagents/materials/analysis tools. Zhaoqun Liu, Zhi Zhou, Lingling Wang, and Linseng Song wrote the manuscript. All the authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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