Science of the Total Environment 784 (2021) 147159

Contents lists available at ScienceDirect

Science of the Total Environment

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

Copper promoting oyster larval growth and settlement: Molecular insights from RNA-seq

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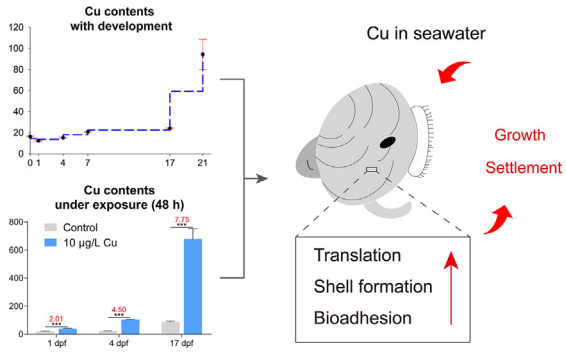
HIGHLIGHTS

- Cu demand increased sharply at the late pelagic stage of oyster larval development.
- 10 μg/L Cu exposure enhanced the process of translation, shell formation and larval growth.
- Cu participating in larval settlement was related to chitin binding proteins and proteinaceous matrix.
- A putative mechanism of Cu internalization and re-distribution in oyster larvae was proposed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 February 2021
Received in revised form 10 April 2021
Accepted 11 April 2021
Available online 17 April 2021

Editor: Daqiang Yin

Keywords:
Oysters
Copper
Growth
Settlement
RNA-seq

ABSTRACT

As a cofactor of key enzymes, Cu is required in living organisms, although Cu levels in the natural environment are typically low. In this study, the promotion of growth and settlement on the larvae of oyster *Crassostrea angulata* was observed at an environmentally relevant concentration (10 μg/L Cu). Interestingly, Cu accumulation in the soft tissue of oyster larvae increased during the larval development and exhibited a sharp increase at the late pelagic stage. With the help of RNA-seq, we constructed a high-quality transcriptional database of the oyster *C. angulata* larvae (24,257 genes with an average length of 1594 bp) via de novo assembly, which provided the basic molecular changes during the larval development. Network analysis of five early developmental stages and differential expression under Cu exposure were integrated to examine the roles of Cu in oyster larvae. Our molecular analysis demonstrated that both ion channels and organic transporters contributed to Cu internalization from the external environment, including zinc transporters and amino acid transporters. The followed distribution of Cu across cells was achieved by ATP7A, the circulatory system, and the Cu transporters (CTRs). Cu exposure enhanced the ribosome and the calcium binding proteins with a higher rate of translation and shell formation, giving rise to faster growth of oyster larvae. Furthermore, Cu facilitated the settling process by upregulating the chitin binding genes and then promoting the formation of the proteinaceous matrix between larvae and substrate. Our study presents the molecular basis for Cu promotion (i.e., hormesis) effects on oyster larval growth and settlement.

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https://doi.org/10.1016/j.scitotenv.2021.147159
0048-9697/

1. Introduction

Oysters are widely distributed and cultured across the world as important commercial species, with the production of about 6 million tons and up to 7 billion US dollars in 2018 (Duncan, 2003; FAO, 2018). There are increasing concerns about oysters' additional values, such as providing habitats for other species, controlling eutrophication, improving water quality, and buffering wave energy. Oysters are also regarded as an ideal biomonitor of metal pollution across coastal environments (Lu et al., 2019). The life history of oysters involves a short pelagic stage (2–3 weeks after fertilization), with dynamic physiology and molecules (Ginger et al., 2013; Lillis et al., 2013). Given the relatively fragile condition and metamorphosis, larvae are among the most vital stages during the whole life history of oysters and determine the population recruitment and formation of oyster reefs (Hunt and Scheibling, 1997). The development of oyster larvae encompasses many biological processes, including growth, cell proliferation, organ generation/degeneration, and rapid shell formation.

One of the well-known traits of oysters is their ability to hyperaccumulate Cu (Tan et al., 2015; Wang et al., 2011). Cu concentrations in adult oysters are further accelerated by Cu pollution in the water due to industrialization, leading to the coloration of oysters in the heavily polluted estuaries (e.g., up to 15,000 μg/g Cu in *C. hongkongensis*, Tan et al., 2015). As an essential element in living organisms, Cu is required at a relatively small amount (Wang et al., 2018) by serving as the cofactor of key enzymes and participating in development, energy production, reactive oxygen species (ROS) scavenging, and neural function (Hart et al., 1999; Hwang et al., 2014; Lee et al., 2001). Previous studies demonstrated that Cu contributed to amebocytes' antimicrobial activity in oyster *Crassostrea virginica* (Fisher, 2004) and the hemolymph in oyster *Crassostrea angulata* (Shi et al., 2019). In the larval stages, Cu was considered an indispensable element during metamorphosis (i.e., settlement). Prytherch (1934) first reported that the larval settlement of oyster *C. virginica* was proportional to the Cu level in the seawater within the range of 0.05 and 0.6 mg/L. Much later, Nell and Holliday (1986) reported that Cu (200 μg/L) exposure resulted in a higher larval settlement (9.2%) of Sydney rock (*Saccostrea commercialis*) compared to the control group (2%). Recently, Weng et al. (2019) reported the novel pattern of Cu accumulation with the life history of oyster *C. hongkongensis* and described the subcellular distribution of Cu in oyster larvae by NanoSIMS, speculating the significant role of Cu in the settlement via the cell apoptosis, gill generation, and energy metabolism.

Next-generation sequencing (NGS) is a revolutionary technique and provides massive information on nucleotide sequences (Basantani et al., 2017; van Dijk et al., 2014; Zhang et al., 2012). Among these techniques, RNA sequencing (RNA-seq) has been developed as a powerful tool to obtain the sequences and the corresponding expression with bioinformatics. It has been used to reveal the molecular effects of pollutants (Li et al., 2020; Meng et al., 2018), disease (Costa et al., 2013), and developmental process (Alyagor et al., 2018; Foulon et al., 2019). Several key genes have been identified from RNA-seq via the comparison among different developmental stages in the oysters, relating to settlement, metamorphosis, shell formation, and growth (De Wit et al., 2018; Eierman and Hare, 2015; Liu et al., 2020; Qin et al., 2012; Xu and Zhang, 2020). For example, chitin synthase and fibronectin-like were related to the shell formation in the Pacific oyster as referred from temporal RNA-seq with developmental stages (Zhang et al., 2012).

In the present study, the effects of different levels of Cu on the larvae of oyster *C. angulata* at the pelagic stage were first evaluated. The stimulation of both growth and settlement at 10 μg/L Cu was observed. Such promotion may have been related to the molecular functions within cells, including transporters responsible for the active Cu accumulation with larval development. Therefore, RNA-seq and a series of bioinformatic analyses were employed to investigate the underlying mechanisms. Our study provided important information for the molecular basis of Cu facilitation of oyster larval growth and settlement.

2. Materials and methods

2.1. Oyster larvae and Cu impacts

Oyster *Crassostrea angulata* was obtained from a breeding farm in Zhangzhou, Fujian Province, China. The fully develop and mature oysters (both male and female) were dissected to obtain the gametes. Only qualified spermatozoa (swimming actively) and eggs (pear shape) were mixed with a ratio of 3:1. Afterwards, the fertilized eggs were reared in 100 L pre-filtered natural seawater (pH 8.1, 25 psu) at 24–26 °C. The rearing density was adjusted with the stage of development, i.e., 15–20 larvae/mL for D-shape, 4 larvae/mL for umbo, 1–2 larvae/mL for pediveliger. They were fed with mixed algae (*Isochrysis galbana* and *Chlorella sp.*) twice daily. The seawater was renewed every 2 d. In the meantime, the shell length of oyster larvae and the number of oyster larvae were measured.

The 48 h toxicity of Cu to the oyster larvae was first established. The D-shape larvae (about 30 h post fertilization) were exposed to different levels of Cu (control, 2, 5, 10, 20, 50, 100, and 200 μg/L), each with three independent wells in a 6-well plate. Each well contained about 100 larvae, and these oyster larvae were not fed with algae. After 48 h, the surviving number of oyster larvae was counted under the dissecting microscope (Leica, Germany). Larvae without cilia beating were identified as dead.

The second experiment examined the chronic toxicity of Cu on oyster larval growth over 20 d of exposure. The 1 dpf (days post-fertilization) oyster larvae were collected and exposed under six different Cu concentrations with three replicates. In each replicate, about 750 D-shape larvae were placed in a 100 mL beaker containing 75 mL adjusted seawater with Cu. The seawater with different Cu concentrations was renewed, and dead oyster larvae were removed every 2 d. At different periods of exposure, the shell length was measured until the oyster larvae were attached to the substrate. The growth rate of oyster larvae was calculated as length per day (μm/d).

The third experiment examined the influences of Cu exposure on the process of settlement. Specifically, 20 dpf oyster larvae with pseudopods were harvested from the rearing tank and then distributed to a 50 mL beaker. There were about 40 oyster larvae in each beaker containing 25 mL adjusted seawater with different levels of Cu (control, 2, 5, 10, 20, and 50 μg/L Cu). A small piece of oyster shell was placed for the settlement of oyster larvae. Three replicates were carried out simultaneously. The number of settled larvae was evaluated under a dissecting microscope after 24 h of exposure.

The alkali digestion method was employed to separate the soft tissue of larvae from the shell (Fig. 1), slightly modified from the previous study (Weng et al., 2019). The moisture in biological samples was removed via freeze-dryer (SCIENTZ, China) firstly. About 0.1 g dried larvae were digested by 2 mL tetramethylammonium hydroxide solution in water (TMAH, 0.2 M, Sigma), followed by centrifugation (800g, 10 min) to extract supernatant from the mixture. Two more operations were conducted as before, and the insoluble component (shell) was washed twice by Milli-Q water, achieving better extraction. All the supernatants were collected and mixed in a tube. Finally, both the mixed solution and in-soluble components (shell) were freeze-dried again. The weight of soft tissue was calculated as the difference between the dry weight of total larvae and the shell. The dried tube containing soft tissue samples was added by 65% HNO_3 under 80 °C for 12 h. Inductively coupled plasma-mass spectrometry (ICP-MS, NexION 300, PerkinElmer) was served to determine the metal concentration of samples, seawater, and algae. The Cu and Zn concentrations in the oyster larvae under 10 μg/L Cu exposure were simultaneously measured. The reliability of the above method in oyster larvae (both alkali and acid digestion) was checked by certified reference materials SRM 1566b, with three replicates. The recoveries were 101.7 ± 4.6% for Zn and 100.5 ± 3.4% for Cu, respectively. Meanwhile, six adult oysters (3 male and 3 female) were also processed for Cu determination by the above methods.

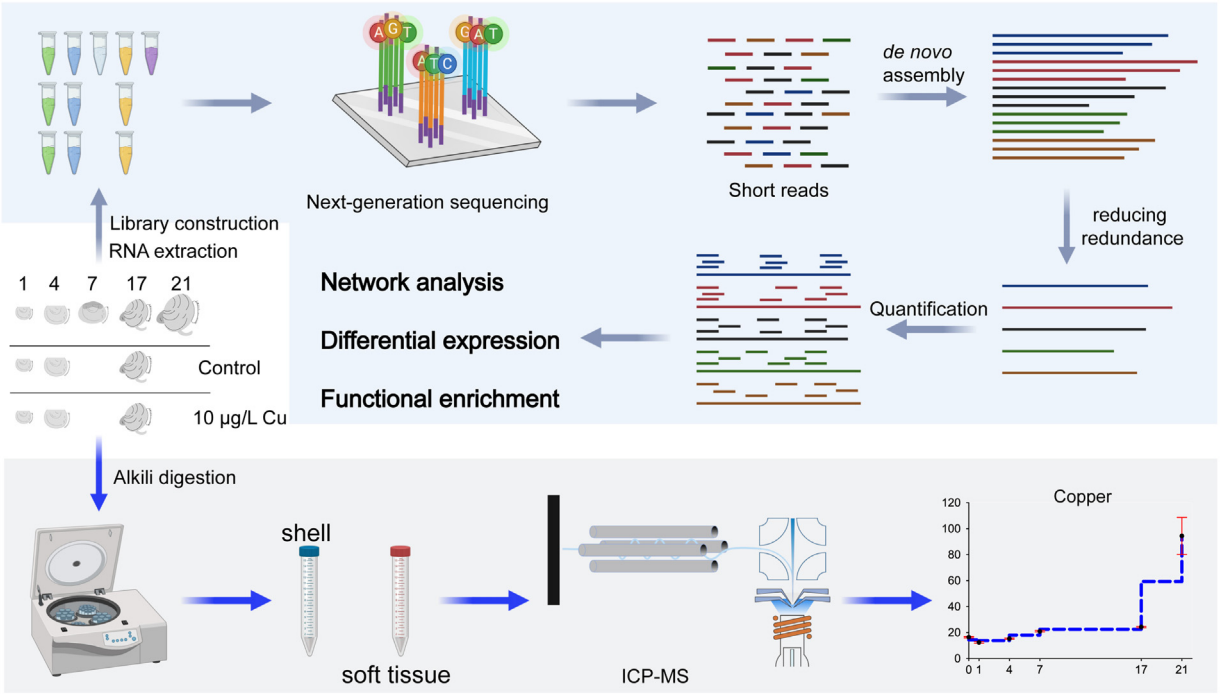

Fig. 1. The general workflow on oyster larvae, including bioinformatics and metal determination. Created with BioRender.com.

In this study, the metal concentrations in oysters were calculated by the dry weight (dw).

2.2. Cu exposure for molecular studies

Our results (shown below) suggested that Cu indeed promoted the growth and settlement of oyster larvae at 10 μg/L Cu. The following molecular study, therefore, specifically focused on Cu exposure at this concentration. Three stages of larvae (1 dpf, 4 dpf, and 17 dpf) were exposed to 10 μg/L Cu for 48 h, which was designed to study the molecular changes under environmental Cu exposure. In addition, five developmental stages of oyster larvae (1 dpf, 4 dpf, 7 dpf, 17 dpf, and 21 dpf) were harvested for RNA sequencing and metal determination, which aimed to reveal the temporal pattern of Cu content and gene expression with development. Samples for RNA sequencing were fixed by RNAlater™ Stabilization Solution (Invitrogen, USA), and samples for metal determination were frozen under −20 °C. The general workflow in this study is shown in Fig. 1.

2.3. RNA isolation and sequencing

Total RNA of oyster larvae was isolated by TRIzol (Invitrogen, USA) based on manufacturing guidelines. The quality and yield of RNA were measured by RNA Nano 6000 Assay Kit with Agilent 2100 bioanalyzer (Agilent Technologies, USA) and Qubit® RNA Assay Kit with Qubit® 2.0 Fluorometer (Life Technologies, USA), respectively. One μg high-quality RNA (not degraded) per sample was processed by NEBNext® Ultra™ RNA Library Prep Kit (NEB, USA) to construct the library. Afterwards, Hiseq 4000 (Illumina, USA) platform was employed to obtain 150-bp Paired-End (PE) reads. All raw reads were deposited in the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI), with accession number PRJNA668688 and PRJNA668690.

2.4. Bioinformatic analysis

The workflow of bioinformatic analysis was modified from Li et al. (2020). Clean reads were obtained by trimmomatic software (Bolger et al., 2014), removing adapters and low-quality reads (N content > 5% and quality value of base < 20). Trinity software was employed in de novo assembly with the default parameters except for min_ker_cov: 3 (Grabherr et al., 2011). As the sister species of *C. angulata*, Pacific oyster *Crassostrea gigas* was annotated fully with genome draft, which reduced the redundant isoforms obtained from de novo assembly (Ono et al., 2015; Wang et al., 2010; Zhang et al., 2012). In more details, blastx was applied to seek the matched sequences against the protein database of *C. gigas* firstly, and then the subject with the highest score was selected by Python 3.6 (Camacho et al., 2009). The newly constructed mRNA sequences were deposited in the National Center for Biotechnology Information (NCBI), with accession number PRJNA695413. The completeness of the obtained transcriptional profile was evaluated by the BUSCO against the Mollusca Odb10 dataset (Seppey et al., 2019). Also, the Gene Ontology annotations and KEGG orthologs were obtained based on the genome information of *C. gigas*.

Bowtie2 and RSEM were employed in quantification to map the reads into the constructed mRNA sequences and determine the expression of genes, respectively (Langmead and Salzberg, 2012; Li and Dewey, 2011). The differential expressed genes (DEGs) were evaluated in DESeq2 (Love et al., 2014), with the criteria: $|\log_2\text{fold-change}| > 1$ and false discovery rate (FDR) < 0.05.

Weighted gene co-expression network analysis (WGCNA) was employed to construct a transcriptional network (Langfelder and Horvath, 2008). In more details, the matrix consisted of the transcripts per million (TPM) value of 16,030 genes from 15 samples in five stages (1 dpf, 4 dpf, 7 dpf, 17 dpf, and 21 dpf), with the non-zero TPM value. Genes with similar expression patterns were clustered as the same module, with the suggested parameter in the operation manual. The Cu content and the developmental stage during the larval development were selected to study the relationship against 29 identified modules, which was designed to investigate the dynamic change at mRNA level with the Cu accumulation and the developmental stages of oyster larvae. The psych (R package) finished the principal component analysis based on the TPM values of genes in the samples (Revelle, 2020).

Genes in the module of interest during development and the genes with differential expression under Cu exposure were subjected to GOseq package for functional analysis, including GO and

KEGG (Young et al., 2010). TBtools software and ggplot2 (R package) were employed to generate graphs in this work (Chen et al., 2020; Wickham, 2016).

2.5. RT-qPCR verification

Real-time quantitative polymerase chain reaction (RT-qPCR) is a suitable tool to compare the expressional difference among samples, which is also a method to check the reliability of next-generation RNA-seq. Primers were synthesized by TSINGKE Biological Technology (China), and the sequences are shown in Table S1. Elongation factor 1-beta (EF-1β) was selected as the internal reference among samples reported by the previous study (Yan et al., 2017). The relative expression of genes was calculated based on the $2^{-\Delta\Delta Ct}$ method. In this study, the expression level of 9 genes in different samples was quantified by RT-qPCR. Details of the workflow are described in a previous study (Li and Wang, 2021). As shown in Fig. S1, a linear relationship was observed between RNA-seq and RT-qPCR ($R^2 = 0.9157$), indicating that RNA-seq was a reliable tool to investigate the expression pattern in oyster *C. angulata*.

2.6. Statistical analysis

Most statistical analyses were carried out by the integrated algorithm in the software or package (i.e., hypergeometric test in GOseq package for functional enrichment, differential expression in DESeq2 package, Pearson correlation in WGCNA for the relationship among module-module and traits-modules). The independent student's *t*-test in SPSS 22.0 was applied to determine whether there was a significant difference between the means of data in two groups, including the Cu content in oyster larvae and the settling rate within 24 h. The correlation between the two groups was evaluated by the linear regression in SPSS 22.0, including the comparison between RT-qPCR and RNA-seq.

3. Results

3.1. Dynamic Cu contents with larval development

Oyster larvae exhibited dramatic changes in morphology within 22 days post fertilization, including the size, shape, and appearance (Fig. 2). Besides, the ontogeny pattern of Cu contents in the soft tissue of oyster larvae was novel. Cu concentration on 1d was 12.3 μg/g, which was the lowest among different stages and comparable to the Cu level in previous study (Moreira et al., 2018). The decreased Cu content between fertilized eggs (16.0 μg/g) and 1 dpf might result from the growth dilution. Within the first 24 h after fertilization, cell division and differentiation contributed to the formation of organs and calcified shells but with less Cu uptake. From 1 d to 17 d after fertilization, Cu content increased gradually, 12.3 μg/g in 1 dpf, 15.2 μg/g in 4 dpf, 20.8 μg/g in 7 dpf, and 24.2 μg/g in 17 dpf. Interestingly, there was a sharp rise of Cu concentration in 21 dpf larvae when they tended to attach to the substrate and begin the sessile life (i.e., up to 94.4 μg/g), which was 3.9–7.7 times as high as the pre-mentioned larvae. Furthermore, there were similar Cu levels in the soft tissue of adult oysters (110.3 μg/g in the female and 101.6 μg/g in the male) against the 21 dpf larvae.

3.2. Cu toxicity on oyster larvae

In the present study, the main source of Cu in oyster larvae was waterborne. As shown in Table S2, the actual Cu concentrations in the experiment fluctuated around the nominal ones. Cu concentrations in natural seawater and the control treatment were typically low, ranging

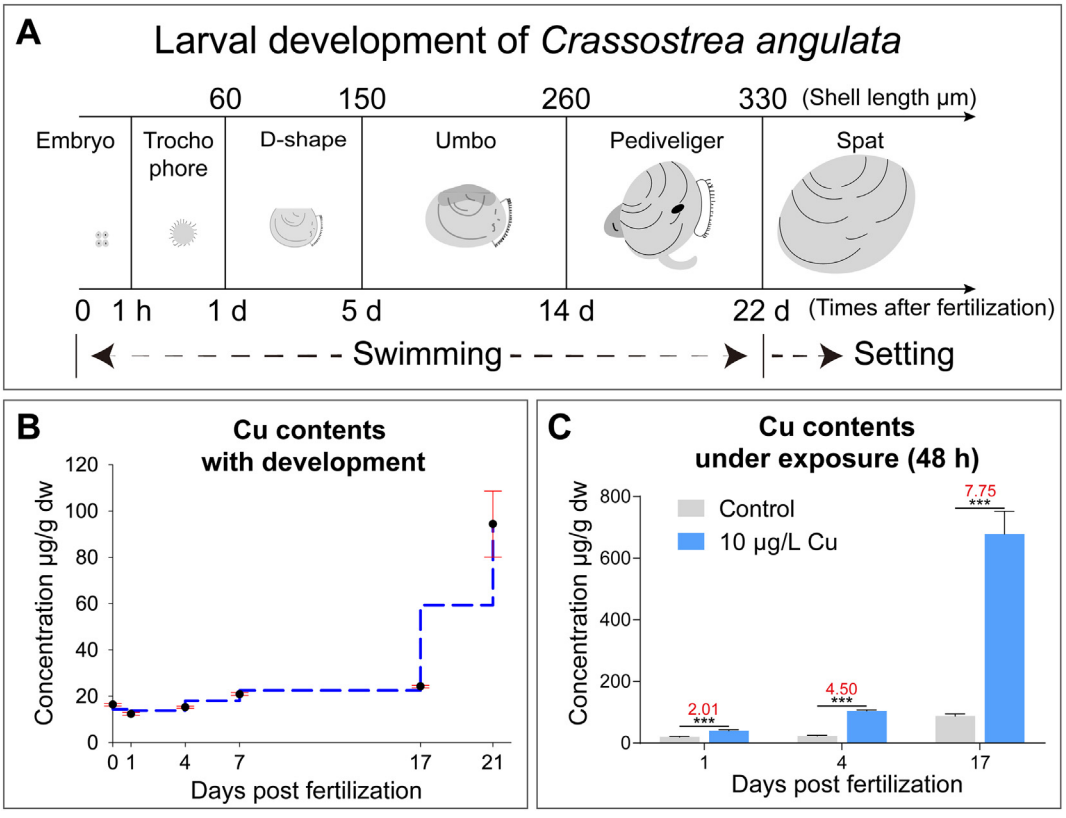

Fig. 2. The dynamic changes during the developmental stages of oyster larvae. (A) Generally, the swimming habit of oyster larvae would last about 22 d before they attach to substrates. (B) Cu contents in six developmental stages of oyster larvae. 0 means the fertilized embryo. (C) Cu contents in three stages of oysters under Cu exposure for 48 h. *** means a significant difference in independent student's *t*-test analysis (sig. < 0.001) and red figures means the fold change of Cu contents between control and treatment.

from 0.5 to 0.9 μg/L. Cu concentration in seawater during 48 h larvae exposure decreased by about 20% in control and 40% in the 10 μg/L group (1 dpf) (Fig. S2). There was little difference in terms of Cu levels in seawater before and after addition of algal diet since the algae contained a low Cu level (only 11.5 μg/g normalized by dry weight).

The 48-hour survival test was first conducted to examine the short-term toxicity of Cu (Table S3). Survivorship of 1 dpf larvae declined slightly with increasing Cu levels, ranging from 97.2% to 89.3% at Cu concentration below 50 μg/L. *maximum* mortality (20%) was found in the 100 μg/L group. During chronic exposure to Cu (Fig. 3A–C), a smaller shell size was found, and all larvae were dead on 5 d and 13 d at 100 and 50 μg/L (Fig. 3A–B). Exposure to 20 μg/L led to more larval death during 21 d, but the size and growth rate were similar to those of the control treatment. Interestingly, exposure at 10 μg/L resulted in a promoting effect on larval growth and similar survival (Fig. 3A–C), about 15.74 ± 1.44 μm/d compared to 13.44 ± 2.01 μm/d in the control.

The proportion of settled larvae fluctuated around 30% within 24 h at the Cu concentrations of 2, 5, and 20 μg/L, which was comparable to the control. A higher Cu (50 μg/L Cu) subsequently reduced the settlement rate to 23%. At 10 μg/L, we also observed a higher settlement rate within 24 h (Fig. 3D), with 34.5% (p-value = 0.0519, independent t-test, indicating that the possibility to misjudge the difference between these two was only 5.19%). There were only about 27% larvae attached to the plastic plate in the control group. One-way ANOVA showed significant variation among different levels of Cu addition (p-value = 0.0266).

3.3. Gene prediction and annotation

A total of 203,932 genes were obtained via de novo assembly by Trinity, based on the 1,582,671,460 paired reads from 31 samples. After reducing the redundant sequences based on the coding proteins of *C. gigas*, 24,257 non-redundant genes were selected, with an average length of 1593 bp and the L50 value of 5618 bp (Table S4). In contrast, only 10,462 contigs with an average length of 723 bp were obtained in the previous study on the larval stages (Qin et al., 2012). Among these, 16,541 and 7051 genes were annotated in the GO and KEGG database, respectively. BUSCO results showed that 66.9% of 24,257 genes were targeted in the conserved Mollusca database (Table S4).

3.4. Differential response analysis under 10 μg/L Cu

In the present study, three larval stages exhibited different levels of response upon exposure to 10 μg/L Cu for 48 h. During the exposure, no difference between control and Cu exposure was observed in size and mortality. The Cu concentration doubled in 1 dpf larvae (39.6 μg/g) under 10 μg/L Cu exposure compared to the group without Cu addition (19.7 μg/g) (Fig. 2C). In 4 dpf larvae, there was about 4.5-times change between Cu exposure (104.2 μg/g) and control (23.2 μg/g). A much more dramatic increase was observed in 17 dpf larvae, with a 7.75-fold change (678.3 μg/g after 10 μg/L Cu exposure for 48 h compared to 87.5 μg/g in control, Fig. 2C).

These exposed larvae were collected for RNA sequencing. After Cu exposure for 48 h, there were a total of 1226 genes identified as DEGs, with 603 genes in 1 dpf larvae, 662 genes in 4 dpf larvae, and 188 genes in 17 dpf larvae (Fig. 4). Among them, only 17 genes were observed with differential expressions in all three stages, which accounted for only 1.4% of the total identified DEGs. The hypergeometric test was widely used in transcriptomic and proteomic research, checking whether some functions were altered under given conditions (Li et al., 2020; Young et al., 2010; Zhang et al., 2015). Among the top 10 enriched terms of GO molecular functions, only one (serine-type endopeptidase activity) was observed in all three stages (1 dpf, 4 dpf, and 17 dpf)

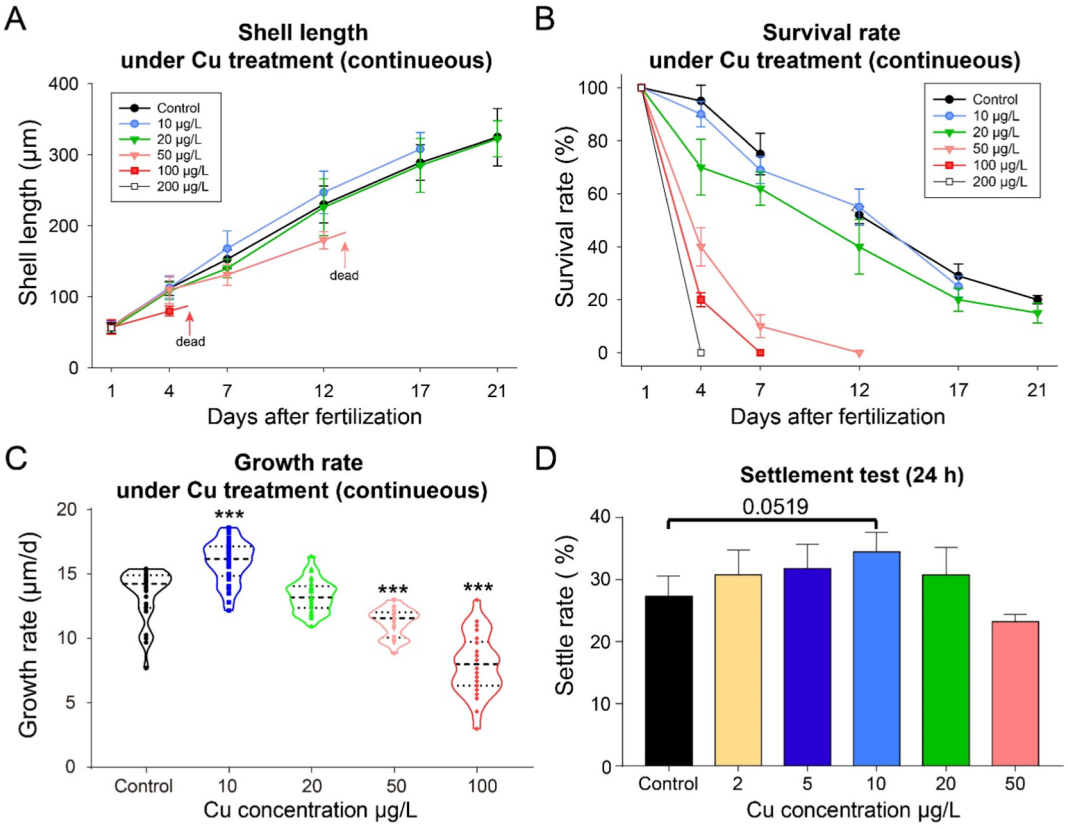

Fig. 3. The effects of Cu on oyster larvae. (A) The change of shell length of oyster larvae under different levels of Cu continuously (n = 30). Dead signifies all oyster larvae were dead. (B) The survival rate under different levels of Cu continuously (n = 3). (C) The calculated growth rate under varying levels of Cu continuously (n = 30). *** means a significant difference in independent student's t-test analysis (sig. < 0.001) compared to control. (D) The settle rate of oyster larvae under different levels of Cu for 24 h.

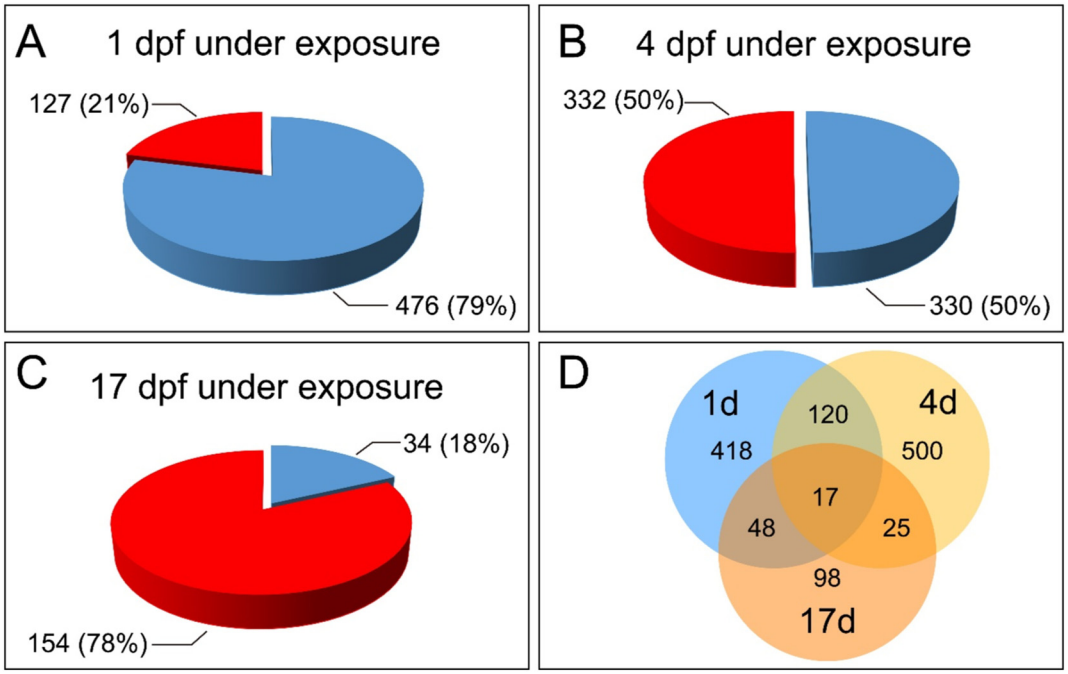

Fig. 4. The differentially expressed genes of three oyster larvae under 10 μg/L Cu exposure for 48 h. (A) 1 dpf oyster larvae, (B) 4 dpf oyster larvae, (C) 17 dpf oyster larvae, (D) the Venn diagram of DEGs in three stages of oyster larvae. In the pie chart, red color means upregulation, blue means downregulation. In pie chart, red and blue colors represent the proportions of upregulated genes and downregulated ones, respectively.

under 10 μg/L Cu for 48 h, while distinct effects were observed (Fig. 5 and Table S5).

3.5. Network analysis

A network was constructed by WGCNA to compare the relationship between the gene expressions and traits (i.e., Cu contents and the developmental stages in this study). Consequently, 16,030 genes were divided into 29 modules according to the similarity of gene expression, among which 15 modules were significantly correlated with traits (Cu content in soft tissue and the developmental stages), as shown in Fig. 6 (p-value < 0.05). 11 modules were observed in both Cu and the development stages, implying that Cu might participate in the development of oyster larvae during the swimming stage (Fig. 6). The functional enrichment was employed to screen out functions involved in larval development in which Cu might play essential roles. 129 terms (molecular function in Gene Ontology) were highlighted from 15 modules (Fig. 5A and Table S6), which were involved in various types of parts, including "transferase activity", "protein binding", "DNA binding", "RNA binding", "catalytic activity", "transcription factor activity", "chitin binding", "hydrolase activity", and "kinase activity".

A total of 440 transporters were filtered from the transcriptional profile of oyster larvae using KEGG pathway ko02000 ("Transporters"), as shown in Fig. 8A. Of these, there were 83 highlighted transporters since their expression was positively correlated with the Cu contents with the larval development (co-efficiency > 0.55). Most of them belonged to the solute carrier family (SLC), sharing about 65% (54 of 83 transporters, Fig. 8A). Based on the affinity to matters, 52 of 83 transporters were also classified as two major groups (Fig. 8B). 15 genes were responsible for inorganic molecules, including Zn^{2+}, Cu^{+}, Ca^{2+}, K^{+}, and Na^{+}. As for the organic transporters, 35 genes belonged to the SLC family, which were responsible for the transportation of organic matters, including amino acids (11 genes), monocarboxylate (4 genes), dicarboxylate (3 genes), tricarboxylate (1 gene), organic cation (7 genes), glucose (4 genes), myo-inositol (3 genes) and bile acid (2 genes).

4. Discussion

4.1. Newly constructed transcriptional profile

A new dataset containing 24,257 annotated genes was predicted from massive NGS reads, which owned a longer gene length (the average length of 1593 bp and the L50 value of 5618 bp). In contrast, only 10,462 contigs with an average length of 723 bp were obtained in a previous study about the larvae stages (Qin et al., 2012). Unlike the homogeneity of DNA, there were specific expressions of genes in different growing stages (larvae and adult) and different organs (i.e., gill, mantle, gonad, heart, etc.) (Hasegawa et al., 2015; Sonawane et al., 2017). Also, some of the genes are only stimulated when organisms were stressed by external factors (O'Rourke et al., 2020). In this regard, a high-quality database during the swimming stages of oyster *C. angulata* larvae at the RNA level was constructed, although only 66.9% completeness in BUSCO, which could provide the basic information to investigate further the dynamic change and the response to Cu at the molecular level.

4.2. Increased Cu demand with the larval development

In this study, high tolerance to Cu in oyster *C. angulata* was observed from the 48 h survival test on 1 dpf larvae, with only 20% mortality under 100 μg/L Cu. Compared to the other oyster species, *C. angulata* appeared to be more tolerant of the higher level of Cu in seawater. In oyster *C. sikamea*, for example, the exposure of 100 μg/L Cu for 48 h resulted in the death of larvae, from 50% to 90% (Weng and Wang, 2014). The median lethal concentration (LC_{50}) value in *C. gigas* larvae was approximately 10.66 μg/L Cu within 48 h (Hall et al., 2016).

In addition, Cu concentrations in the oyster larvae increased with development. Given the rapid growth (biomass and size per larva) during the larval stage, the total amount of Cu accumulated in the oyster larvae should increase from 1 dpf to 17 dpf, implying an increased demand for Cu (21 dpf > 17 dpf > 7 dpf > 4 dpf > 1 dpf). In addition, the effects of additional Cu on oyster larvae offered some supports for it. Upon Cu exposure at 10 μg/L, the later larval stage also responded more

Fig. 5. The GO enrichment of DEGs in three stages of oyster larvae under Cu exposure for 48 h. In the pie chart, red color means upregulation, and blue means downregulation.

significantly than the earlier stages, e.g., 7.5-fold in 17 dpf, 4.50-fold in 4 dpf, and 2.01-fold in 1 dpf. Surprisingly, there was a negative correlation between the number of downregulated genes and Cu demand (quantified by the fold change of Cu concentration) after Cu exposure (Fig. S3). Much fewer effects were found in the 17 dpf oyster larvae (higher Cu demand), with only 34 downregulated genes and up to a 7.5-fold change of Cu accumulation after exposure. In contrast, more critical effects were found in 1 dpf and 4 dpf larvae, with 476 and 332 downregulated genes, respectively.

A putative developmental route (Fig. S4) was constructed from their gene expression patterns (based on the principal component analysis). Generally, the position of samples was largely decided by the time post-fertilization, and the addition of 10 μg/L Cu did not change the fundamental pattern of transcription. Compared to the control, the position of 1 dpf larvae under Cu exposure was closer to the starting point, but those of 4 dpf and 17 dpf were closer to the endpoint, implying some promoting effects of Cu on 4 dpf and 17 dpf larvae. The increased Cu demand with the larval development could explain the different effects of Cu on those three stages and indicate the critical role of Cu on oyster larvae.

4.3. Cu effect on larval growth

Cu is one of the essential elements in living organisms, including oysters. To date, most studies are based on model organisms, for example, humans, mice, zebrafish, and plants. Therefore, the role of Cu in oysters remains obscure, although oysters could hyper-accumulate Cu from the external environment (up to 4000 μg/g dw in gill and mantle) (Shi et al., 2015). We monitored the morphological change of oyster larvae at different levels of Cu continuously within the swimming stage, detecting a faster growth rate at 10 μg/L Cu than the control and other higher levels of Cu (Fig. 3). Such effects can be considered as the

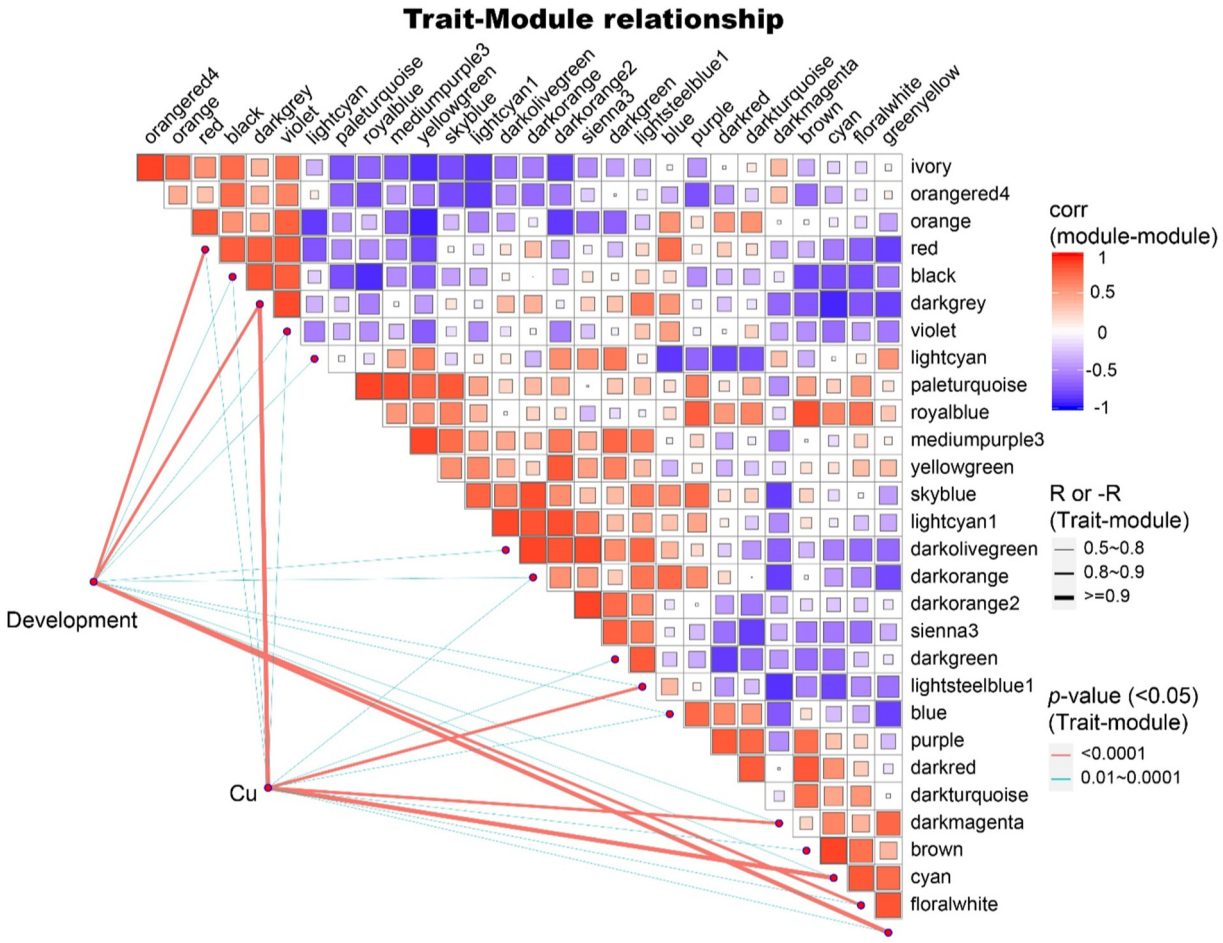

Fig. 6. The relationship between modules and trait (development and Cu content). corr: the coefficient value among 29 identified modules. R or -R: the absolute value of the coefficient value between modules and traits. *p*-Value: the significance of trait-module relationship.

hormesis of Cu on oyster larval growth. There should be some corresponding responses at the molecular level to support the promoting growth under 10 μg/L Cu, which was uncovered by the integrated results between the network analysis (WGCNA) of five developmental stages and differential expression of genes under Cu exposure.

Cu might pose threats to the early larvae, especially for the 1 dpf with newly developing calcified shell. Among the Top 10 highlighted GO terms from 1 dpf larvae under Cu exposure, only "GTPase activity" owned more upregulated genes than downregulated ones. In the putative developmental route, the position of 1 dpf larvae under Cu exposure was closer to the starting point, but those of 4 dpf were closer to the endpoint (Fig. S4). In contrast, much more positive effects were observed on 4 dpf larvae treated by the same Cu level. Even though there were 5 GO terms enriched in both Cu-treated 1 dpf and 4 dpf larvae, they showed distinct differentiation (Fig. 5). The following analysis mainly focuses on the positive effects in 4 dpf larvae at the mRNA level to explore the roles of Cu in the oyster.

"Structural constituent of ribosome" (GO:0003735) was highlighted by the "green-yellow" module from network analysis, indicating the essential role in the development. Moreover, it was one of the most significant GO terms in 4 dpf larvae responding to Cu addition, with all 28 DEGs expressing higher than control. The ribosome is the indispensable organelle in all living organisms, which is responsible for the translation of genetic information. The biogenesis of ribosome depends on the abundance of those ribosomal protein-encoding mRNAs (RP-mRNAs) and the following translation (de la Cruz et al., 2015; Dermit et al., 2020). Protein is necessary for the generation of organs and the intense cellular processes with larval development. Therefore, higher expression of RP-mRNAs indicated more generated ribosome, thus promoted the molecular change.

Among the 39 DEGs belonging to "calcium ion binding" (GO:0005509) in 4 dpf larvae, 25 genes exhibited higher mRNA abundance than the control. Some of the calcium-binding proteins are part of the shell matrix in the oyster as the interactor with minerals (Hincke et al., 2010; Huang et al., 2007). In terms of organisms with calcified shells, including oysters, the formation of the calcified shell is the most key process. The upregulation of calcium-binding proteins might help oyster *C. virginica* to fight against ocean acidification, without toxicity and other damage under the stress of elevated CO_2 levels (Richards et al., 2018). Against the database of shell proteins identified in Pacific oyster *C. gigas* by proteomics (Zhang et al., 2012), 297 mRNA sequences were matched in the constructed profile. In the present study, Cu stimulated the mRNA abundance of 10 genes in the oyster larvae, 8 genes in the 4 dpf, and 2 genes in the 17 dpf. The upregulation of calcium binding proteins and shell proteins would lead to faster deposition in the shell, thus facilitating the shell formation and growth of larvae.

4.4. Cu effect on larval settlement

The larval settlement, also named bioadhesion, is one of the most critical processes during the life history of marine mollusks. As mentioned before, more pediveliger attached to the substrates when there were higher levels of Cu in the seawater (Nelson and Mantoura, 1984; Prytherch, 1934). However, the Cu concentrations reported in these much earlier studies were much higher than the Cu levels in estuaries nowadays (from 0.04 to 31.0 μg/L in seawater) (Lu et al., 2017).

Foulon et al. (2018) observed the existence of Cu in the adhesive footprint of *C. gigas* settled larvae by Energy Dispersive X-ray spectroscopy (EDS) spectra. Therefore, it is necessary to investigate whether Cu could promote the settlement of oyster larvae at the environmental level.

Traditionally, the significant difference between two independent groups was set as p-value < 0.05. Exposure at 10 μg/L Cu led to a further 7.5% increase of larvae attachment to substrate compared to the control (27%) within 24 h (Fig. 3D). Although the p-value (0.0519) between control and 10 μg/L treatments was slightly higher the threshold, it was still regarded that 10 μg/L Cu promoted the larval settlement. Since the attached larvae were hard to collect from the substrate, 17 dpf larvae were instead selected to simulate the molecular effects under Cu addition during the settlement. Obviously, Cu displayed differential effects on 17 dpf larvae from the previous two stages (Figs. 4 and 5), which was ascribed to the increased demand for Cu. Except for the downregulation of "double-stranded RNA bind" and "metal ion transmembrane transporter activity", the other 8 terms exhibited higher activities among the top 10 most enriched GO terms (Fig. 5). Among them, four upregulated functions were also observed from the key modules in the WGCNA (Fig. 7), i.e., methyltransferase activity (GO:0008168), chitin binding (GO:0008061), catalytic activity (GO:0003824), and hydrolase activity (GO:0004553 and GO:0016798).

With the transition from the pelagic stage to benthic stages, a proteinaceous secretion produced by the pseudopod is the critical step for bioadhesion in oyster *C. gigas* (Foulon et al., 2018). Both chitin and chitin binding proteins have been identified in the mollusk shell (Schönitzer and Weiss, 2007; Suzuki et al., 2007; Suzuki et al., 2009; Zhang et al., 2012; Zhao et al., 2018), which are synthesized and secreted by the mantle. They are interspersed in the layers of shell, playing roles in the mineral formation and making differences in the mechanical properties of the shell (Chan et al., 2018; Currey, 1999). In barnacles, which own a similar life cycle as oyster, chitin is the indispensable factor of the successful adhesion of barnacle larvae (Aldred et al., 2020).

The result of differential analysis under Cu exposure showed that 5 genes with chitin-binding domain expressed higher in the 17 dpf larvae than control. Chondroitin proteoglycan 2 (2.95-fold) existed in the extracellular region and involved cell adhesion and migration (Avram et al., 2014). Peritrophin-44 (2.46-fold), the other known chitin binding proteins, could work as barriers in the chitinous matrix to prevent microorganisms' invasion (Elvin et al., 1996). Both genes were expressed actively during the whole developmental stages, and their upregulation would promote the settlement of oyster larvae. Specifically, apart from cell migration, the generation of the proteinous matrix (between the pseudopod and substrate) occurred with the settlement. The higher expression of peritrophin-44 indicated the enhanced antimicrobial activity, which contributed to the successful bio-adhesion. The other three upregulated genes were identified with chitin binding activity, but their functions were not yet well-studied. Therefore, the stimulation of Cu on larval settlement might be associated with the elevated expression of genes encoding chitin binding proteins, further promoting the process of bio-adhesive layer and the shell formation.

4.5. The putative mechanisms of Cu accumulation

Apart from the vital role of Cu on larval development, it is also important to investigate the Cu accumulation and transport across cells at the molecular level (i.e., transporters). After 48 h exposure at 10 μg/L Cu, there was up to 7.75-times change in 17 dpf larvae but only 2.0-times in 1 dpf and 4.5-times in 4 dpf larvae, which indicated that the late stage of larvae displayed a higher ability to accumulate Cu. There were a few transporters (ko02000) expressed differentially in the Cu-treated larvae (2 in 1 dpf, 8 in 4 dpf, and 3 in 17 dpf), implying that the increased Cu content resulted from the elevated external Cu concentration instead of the upregulated the transporters (Fig. S5). Nonetheless, higher Cu content in soft tissue was observed in 21 dpf larvae compared to the earlier stages whereas the Cu concentration in seawater ranged from 0.5 to 0.9 μg/L during the development for 21 d (Fig. 2). The possible reason might be the higher expression of transporters in the transition stage, thus the bigger capacity to accumulate Cu actively. Therefore, network analysis was employed to compare the dynamic gene expression of 5 developmental stages and further investigate the putative transporters participating in the Cu accumulation with larval development.

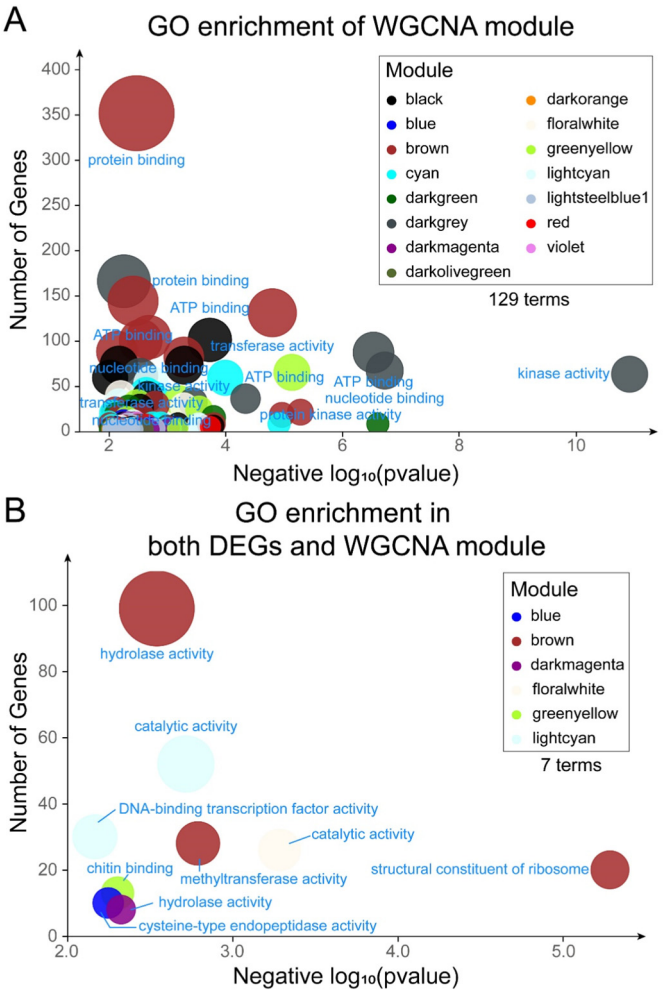

Fig. 7. (A) The GO enrichment of genes in 15 modules obtained from WGCNA (p-value < 0.01). (B) The shared GO terms were found in both DEGs under Cu exposure and genes from modules in WGCNA.

In natural surface seawater, part of Cu exists as free ions, including Cu^{2+} and Cu^{+}. Among these, Cu^{+} ion is easily oxidized to Cu^{2+} ion (Nelson and Mantoura, 1984; Sharma and Millero, 1988). Some zinc transporters (ZIPs) are involved in copper transportation in both *Arabidopsis thaliana* (Wintz et al., 2003) and humans (Antala and Dempski, 2012). The expression of two ZnTs (ZnT2 and ZnT9) and two ZIPs (ZIP1 and ZIP14) increased dramatically in 21 dpf larvae, coupling with the increasing Cu content (Fig. 8B). Surprisingly, the change of Zn contents in oyster larvae under Cu exposure would support the hypothesis that Cu could be imported via zinc transporters, with the decreased Zn concentration at 1 dpf and 4 dpf but not at 17 dpf larvae (Fig. S6). There were limited expressions of zinc transporters in the early stages of larvae (1 dpf and 4 dpf), thus the elevated Cu level might occupy the channels. In contrast, the rising expressions of transporters in the later stages could allow more Cu and Zn to be transported. The present study also showed the potential of these transporters to co-transport Cu into oysters, including Na^{+}, NH_4^{+}, K^{+}, Ca^{2+}, and SO_4^{2-}. However, there were limited studies about the transportation of Cu

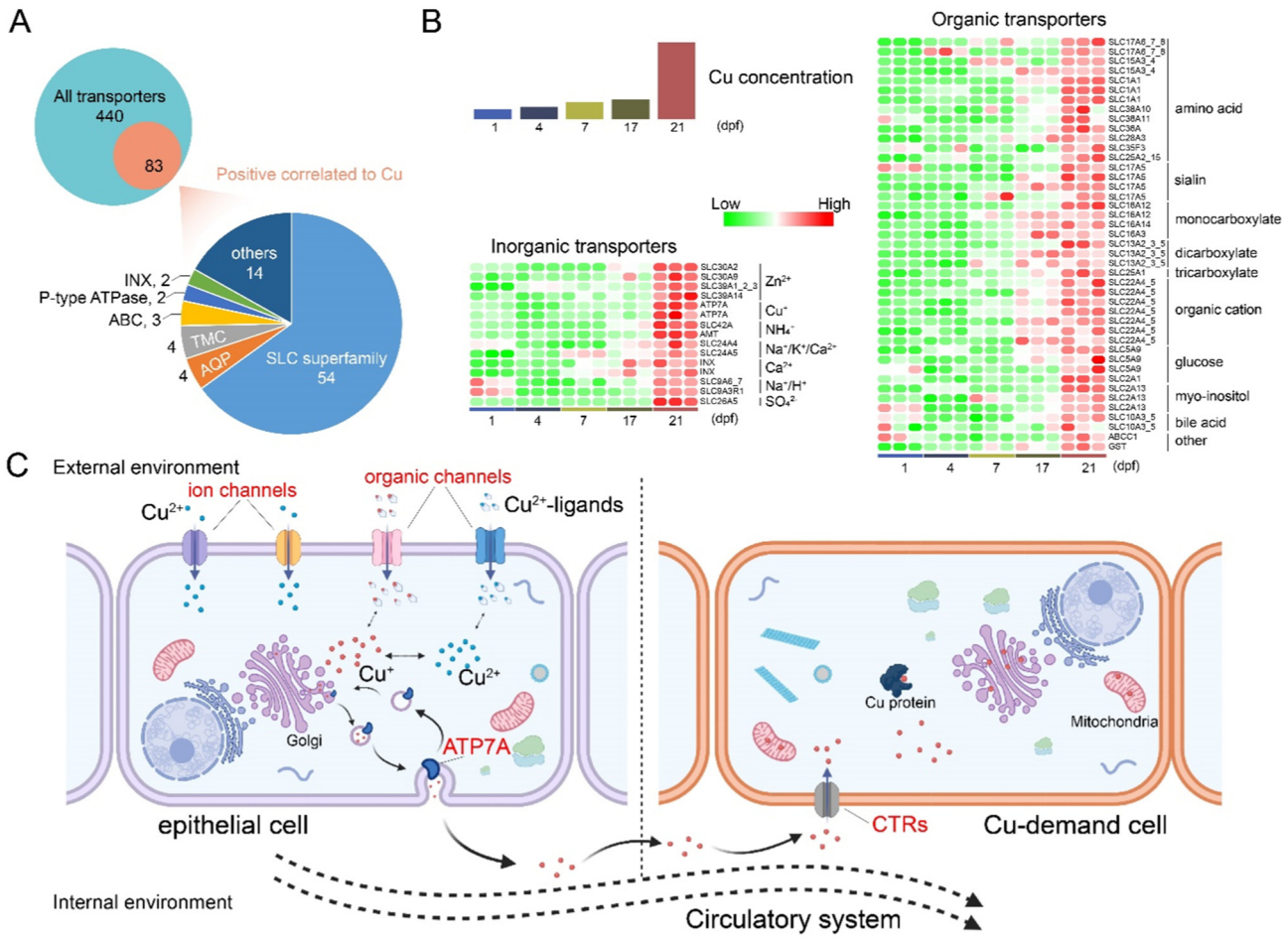

Fig. 8. Identification of putative Cu transporters. (A) Proportions of transcripts identified as transporters in the transcriptional profile and WGCNA. (B) The expression level of transcripts from two major groups, responsible for organic and inorganic molecules. The bar chart represents the Cu levels of five oyster larvae. (C) The proposed route of Cu accumulation in the late stages of oyster larvae. Items with red color mean high expression. Created with BioRender.com.

ions. In lobster (*Homarus americanus*), calcium transporters contributed to the copper transport in hepatopancreatic mitochondria (Chavez-Crooker et al., 2002). The overexpression of potassium transporter could significantly increase the content of a broad range of cations in the plant, including Cu^{2+} (Lan et al., 2010).

On the other hand, Cu-ligand complexes are a relatively stable form in seawater than free ions (Muller and Batchelli, 2013; Whitby et al., 2017). The ligands include amino acids (Wu et al., 2010), H_2O (Yabutani et al., 2018), humic substances and thiol types (Whitby et al., 2018), carboxylate (Bunting and Thong, 1970), bile acid (Lewis, 1973), and other substances from organisms or human activities (Wiramanaden et al., 2008). Considering the large Cu-ligand complexes structure, they might not be transported across the lipid bilayer by ion channels, whereas organic channels might work. Cu amino acid complexes which owned a faster absorption than Cu^{2+} could be imported faster in the Caco-2 cell, and the absorption channel was not the traditional Cu transporters (Gao et al., 2014). In our study, a set of organic channels (37 genes) was identified to be positively against the increasing Cu content in oyster larvae (Fig. 8B). These Cu-ligand complexes could be assimilated by the organic channels, and then Cu^{+} could be released from the complex within the epithelial cell. Our study therefore showed the potential role of these transporters in the Cu accumulation of oyster *C. angulata*, which might need more in-depth investigations to verify.

Both ionic and organic channels might contribute to the Cu accumulation in the epithelial cell, resulting in higher Cu content than the other cells that were not contacted to the external environment directly but might need more Cu. Therefore, the transportation of Cu across cells would help balance the gap among cells. Unlike the external environment, imported Cu^{2+} was reduced into Cu^{+} under redox oxidation, and the cell is likely to utilize Cu^{+} (Fig. 8C). Copper-transporting ATPase 1 (ATP7A), located on the Golgi, is responsible for Cu homeostasis via pumping Cu^{+} into the Golgi and then forming transport vesicles to exclude Cu^{+} from the cell efficiently (Lutsenko, 2020; Yi and Kaler, 2014). Two genes were annotated as ATP7A in this study, which expressed in the late stages of oyster larvae (17 dpf and 21 dpf) actively. After that, internalized Cu^{+} in the cytosol could be exported from the epithelial cell by the ATP7A and the transport vesicles, then spreading in the whole body via the open circulatory system (Fig. 8C).

Before utilization as the cofactor of Cu-proteins, Cu^{+} needs to be imported into the Cu-demand cell with the help of Cu transporters (CTRs) (Fig. 8C). CTRs are in the basolateral border (i.e., blood side) in mammalian cells, which are responsible for the Cu distribution among cells (Kaplan and Lutsenko, 2009). Consistently, our result showed that three CTRs expressed with slight fluctuation during the larval development of oyster *C. angulata,* and their expression was not affected by Cu addition in three stages (Fig. S7).

5. Conclusion

This study monitored the morphological effects of Cu (from 0.5 to 200 μg/L Cu) on oyster *C. angulata* larvae during the pelagic larval stage. Not only was high tolerance to Cu observed in oyster larvae (1 dpf), but some promoting hormesis effects of Cu on growth and

settlement (10 μg/L). Besides, the Cu accumulation in the soft tissue of larvae increased with development, which implied the increasing Cu demand and the significant role of Cu on oyster larvae. With the constructed transcriptional profile, the dynamic change with the larval development and the molecular effects of Cu on oyster larvae were studied for the first time. A putative route for Cu accumulation and transportation in the oyster was proposed by WGCNA. Both ion channels and organic transporters participated in the internalization of Cu from seawater (free Cu ions and Cu-ligands). After secreted by ATP7A and entering the open circulatory system, the accumulated Cu could be further distributed across cells via the CTRs-based absorption. Higher expressions of genes in the ribosome and calcium binding process were observed in oyster larvae after exposure to 10 μg/L Cu, which might be the reason for the stimulated growth from Cu. The supplementation of Cu would promote the chitin binding process, thus facilitating the settlement of pediveliger larvae. Our findings shed insight into the molecular mechanisms of Cu accumulation and the possible role of Cu on the larval oyster *C. angulata*. Further in-depth and molecular experiments are needed to investigate the optimal Cu level and the corresponding function in oysters.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147159.

CRediT authorship contribution statement

Yunlong: Conceptualization, Investigation, Writing
Karl Tsim: Writing
Wen-Xiong Wang: Conceptualization, Writing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We thank the anonymous reviewers for their comments on this work, which was supported by the Natural Science Foundation of China (21777134), the General Research Fund of Hong Kong Research Grants Council (CityU 16102918), and the TUYF fund (19SC01).

References

Aldred, N., Chan, V.B.S., Emami, K., Okano, K., Clare, A.S., Mount, A.S., 2020. Chitin is a functional component of the larval adhesive of barnacles. Commun. Biol. 3, 31.
Alyagor, I., Berkun, V., Keren-Shaul, H., Marmor-Kollet, N., David, E., Mayseless, O., Issman-Zecharya, N., Amit, I., Schuldiner, O., 2018. Combining developmental and perturbation-seq uncovers transcriptional modules orchestrating neuronal remodeling. Dev. Cell 47, 38–52 e36.
Antala, S., Dempski, R.E., 2012. The Human ZIP4 Transporter has two distinct binding affinities and mediates transport of multiple transition metals. Biochemistry 51, 963–973.
Avram, S., Shaposhnikov, S., Buiu, C., Mernea, M., 2014. Chondroitin sulfate proteoglycans: structure-function relationship with implication in neural development and brain disorders. Biomed. Res. Int. 2014, 642798.
Basantani, M.K., Gupta, D., Mehrotra, R., Mehrotra, S., Vaish, S., Singh, A., 2017. An update on bioinformatics resources for plant genomics research. Curr. Opin. Plant Biol. 11-12, 33–40.
Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.
Bunting, J.W., Thong, K.M., 1970. Stability constants for some 1:1 metal–carboxylate complexes. Can. J. Chem. 48, 1654–1656.
Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., Madden, T.L., 2009. BLAST+: architecture and applications. BMC Bioinforma. 10, 421.
Chan, V.B.S., Johnstone, M.B., Wheeler, A.P., Mount, A.S., 2018. Chitin facilitated mineralization in the eastern oyster. Front. Mar. Sci. 5, 347.
Chavez-Crooker, P., Garrido, N., Ahearn, G.A., 2002. Copper transport by lobster (*Homarus americanus*) hepatopancreatic mitochondria. J. Exp. Biol. 205, 405.
Chen, C., Chen, H., Zhang, Y., Thomas, H.R., Frank, M.H., He, Y., Xia, R., 2020. TBtools: an integrative toolkit developed for interactive analyses of big biological data. Mol. Plant 13, 1194–1202.
Costa, V., Aprile, M., Esposito, R., Ciccodicola, A., 2013. RNA-Seq and human complex diseases: recent accomplishments and future perspectives. Eur. J. Hum. Genet. 21, 134–142.
de la Cruz, J., Karbstein, K., Woolford Jr., J.L., 2015. Functions of ribosomal proteins in assembly of eukaryotic ribosomes in vivo. Annu. Rev. Biochem. 84, 93–129.
Currey, J.D., 1999. The design of mineralised hard tissues for their mechanical functions. J. Exp. Biol. 202, 3285.
De Wit, P., Durland, E., Ventura, A., Langdon, C.J., 2018. Gene expression correlated with delay in shell formation in larval Pacific oysters (*Crassostrea gigas*) exposed to experimental ocean acidification provides insights into shell formation mechanisms. BMC Genomics 19, 160.
Dermit, M., Dodel, M., Lee, F.C.Y., Azman, M.S., Schwenzer, H., Jones, J.L., Blagden, S.P., Ule, J., Mardakheh, F.K., 2020. Subcellular mRNA localization regulates ribosome biogenesis in migrating cells. Dev. Cell 55, 298–313 e210.
van Dijk, E.L., Auger, H., Jaszczyszyn, Y., Thermes, C., 2014. Ten years of next-generation sequencing technology. Trends Genet. 30, 418–426.
Duncan, P.F., 2003. SHELLFISH | commercially important molluscs. In: Caballero, B. (Ed.), Encyclopedia of Food Sciences and Nutrition, Second edition Academic Press, Oxford, pp. 5222–5228.
Eierman, L.E., Hare, M.P., 2015. Reef-specific patterns of gene expression plasticity in eastern oysters (*Crassostrea virginica*). J. Hered. 107, 90–100.
Elvin, C.M., Vuocolo, T., Pearson, R.D., East, I.J., Riding, G.A., Eisemann, C.H., Tellam, R.L., 1996. Characterization of a major peritrophic membrane protein, peritrophin-44, from the larvae of lucilia cuprina: cDNA and deduced amino acid sequences. J. Biol. Chem. 271, 8925–8935.
FAO, 2018. FAO Online Query Panels. http://www.fao.org/fishery/topic/16140/en.
Fisher, W., 2004. Antimicrobial activity of copper and zinc accumulated in eastern oyster amebocytes. J. Shellfish Res. 23, 321–351.
Foulon, V., Artigaud, S., Buscaglia, M., Bernay, B., Fabioux, C., Petton, B., Elies, P., Boukerma, K., Hellio, C., Guérard, F., Boudry, P., 2018. Proteinaceous secretion of bioadhesive produced during crawling and settlement of *Crassostrea gigas* larvae. Sci. Rep. 8, 15298.
Foulon, V., Boudry, P., Artigaud, S., Guérard, F., Hellio, C., 2019. In silico analysis of pacific oyster (*Crassostrea gigas*) transcriptome over developmental stages reveals candidate genes for larval settlement. Int. J. Mol. Sci. 20, 197.
Gao, S., Yin, T., Xu, B., Ma, Y., Hu, M., 2014. Amino acid facilitates absorption of copper in the Caco-2 cell culture model. Life Sci. 109, 50–56.
Ginger, K.W.K., Vera, C.B.S., D., R., Dennis, C.K.S., Adela, L.J., Yu, Z., Thiyagarajan, V., 2013. Larval and post-larval stages of Pacific oyster (*Crassostrea gigas*) are resistant to elevated CO_2. PLoS ONE 8, e64147.
Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644.
Hall, M., Cheung, E., Abbasi, M.D., Churches, N., 2016. A new behavioral phenotyping strategy for pacific oyster (crassostrea gigas) larvae reveals cohort-level effects on copper toxicity swimming response. Ann. Aquac. Res. v, 3.
Hart, P.J., Balbirnie, M.M., Ogihara, N.L., Nersissian, A.M., Weiss, M.S., Valentine, J.S., Eisenberg, D., 1999. A structure-based mechanism for copper-zinc superoxide dismutase. Biochemistry 38, 2167–2178.
Hasegawa, Y., Taylor, D., Ovchinnikov, D.A., Wolvetang, E.J., de Torrenté, L., Mar, J.C., 2015. Variability of gene expression identifies transcriptional regulators of early human embryonic development. PLoS Genet. 11, e1005428.
Hincke, M.T., Nys, Y., Gautron, J., 2010. The role of matrix proteins in eggshell formation. J. Poult. Sci. 47, 208–219.
Huang, J., Zhang, C., Ma, Z., Xie, L., Zhang, R., 2007. A novel extracellular EF-hand protein involved in the shell formation of pearl oyster. Biochim. Biophys. Acta Gen. Subj. 1770, 1037–1044.
Hunt, H., Scheibling, R., 1997. Role of early post-settlement mortality in recruitment of benthic marine invertebrates. Mar. Ecol. Prog. Ser. 155, 269–301.
Hwang, J.E.C., de Bruyne, M., Warr, C.G., Burke, R., 2014. Copper overload and deficiency both adversely affect the central nervous system of *Drosophila*. Metallomics 6, 2223–2229.
Kaplan, J.H., Lutsenko, S., 2009. Copper transport in mammalian cells: special care for a metal with special needs. J. Biol. Chem. 284, 25461–25465.
Lan, W.-Z., Wang, W., Wang, S.-M., Li, L.-G., Buchanan, B.B., Lin, H.-X., Gao, J.-P., Luan, S., 2010. A rice high-affinity potassium transporter (HKT) conceals a calcium-permeable cation channel. Proc. Natl. Acad. Sci. U. S. A. 107, 7089–7094.
Langfelder, P., Horvath, S., 2008. WGCNA: an R package for weighted correlation network analysis. BMC Bioinforma. 9, 559.
Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357.
Lee, J., Prohaska, J.R., Thiele, D.J., 2001. Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. Proc. Natl. Acad. Sci. U. S. A. 98, 6842.
Lewis, K.O., 1973. The nature of the copper complexes in bile and their relationship to the absorption and excretion of copper in normal subjects and in Wilson's disease. Gut 14, 221–232.
Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinforma. 12, 323.
Li, Y., Wang, W.-X., 2021. Protein molecular responses of field-collected oysters *Crassostrea hongkongensis* with greatly varying Cu and Zn body burdens. Aquat. Toxicol. 232, 105749.
Li, Y., Zhang, X., Meng, J., Chen, J., You, X., Shi, Q., Wang, W.-X., 2020. Molecular responses of an estuarine oyster to multiple metal contamination in Southern China revealed by RNA-seq. Sci. Total Environ. 701, 134648.

Lillis, A., Eggleston, D.B., Bohnenstiehl, D.R., 2013. Oyster larvae settle in response to habitat-associated underwater sounds. PLoS ONE 8, e79337.
Liu, Z., Zhang, Y., Zhou, Z., Zong, Y., Zheng, Y., Liu, C., Kong, N., Gao, Q., Wang, L., Song, L., 2020. Metabolomic and transcriptomic profiling reveals the alteration of energy metabolism in oyster larvae during initial shell formation and under experimental ocean acidification. Sci. Rep. 10, 6111.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.
Lu, G., Zhu, A., Fang, H., Dong, Y., Wang, W.-X., 2019. Establishing baseline trace metals in marine bivalves in China and worldwide: meta-analysis and modeling approach. Sci. Total Environ. 669, 746–753.
Lu, G.-Y., Ke, C.-H., Zhu, A., Wang, W.-X., 2017. Oyster-based national mapping of trace metals pollution in the Chinese coastal waters. Environ. Pollut. 224, 658–669.
Lutsenko, S., 2020. Sending copper where it is needed most. Science 368, 584.
Meng, J., Wang, W.-X., Li, L., Zhang, G., 2018. Tissue-specific molecular and cellular toxicity of Pb in the oyster (*Crassostrea gigas*): mRNA expression and physiological studies. Aquat. Toxicol. 198, 257–268.
Moreira, A., Figueira, E., Libralato, G., Soares, A.M.V.M., Guida, M., Freitas, R., 2018. Comparative sensitivity of *Crassostrea angulata* and *Crassostrea gigas* embryo-larval development to As under varying salinity and temperature. Mar. Environ. Res. 140, 135–144.
Muller, F.L.L., Batchelli, S., 2013. Copper binding by terrestrial versus marine organic ligands in the coastal plume of River Thurso, North Scotland. Estuar. Coast. Shelf Sci. 133, 137–146.
Nell, J.A., Holliday, J.E., 1986. Effects of potassium and copper on the settling rate of Sydney rock oyster (*Saccostrea commercialis*) larvae. Aquaculture 58, 263–267.
Nelson, A., Mantoura, R.F.C., 1984. Voltammetry of copper species in estuarine waters: part I. Electrochemistry of copper species in chloride media. J. Electroanal. Chem. Interfacial Electrochem. 164, 237–252.
Ono, H., Ishii, K., Kozaki, T., Ogiwara, I., Kanekatsu, M., Yamada, T., 2015. Removal of redundant contigs from de novo RNA-Seq assemblies via homology search improves accurate detection of differentially expressed genes. BMC Genomics 16, 1031.
O'Rourke, J.A., McCabe, C.E., Graham, M.A., 2020. Dynamic gene expression changes in response to micronutrient, macronutrient, and multiple stress exposures in soybean. Funct. Integr. Genomics 20, 321–341.
Prytherch, H.F., 1934. The role of copper in the setting, metamorphosis, and distribution of the American oyster, *Ostrea virginica*. Ecol. Monogr. 4, 47–107.
Qin, J., Huang, Z., Chen, J., Zou, Q., You, W., Ke, C., 2012. Sequencing and de novo analysis of *Crassostrea angulata* (Fujian oyster) from 8 different developing phases using 454 GSFlx. PLoS ONE 7, e43653.
Revelle, W.R., 2020. psych: Procedures for Psychological, Psychometric, and Personality Research. Northwestern University, Evanston, Illinois R package version 2.0.9. https://CRAN.R-project.org/package=psych.
Richards, M., Xu, W., Mallozzi, A., Errera, R.M., Supan, J., 2018. Production of calcium-binding proteins in crassostrea virginica in response to increased environmental CO2 concentration. Front. Mar. Sci. 5.
Schönitzer, V., Weiss, I.M., 2007. The structure of mollusc larval shells formed in the presence of the chitin synthase inhibitor Nikkomycin Z. BMC Struct. Biol. 7, 71.
Seppey, M., Manni, M., Zdobnov, E.M., 2019. BUSCO: assessing genome assembly and annotation completeness. In: Kollmar, M. (Ed.), Gene Prediction: Methods and Protocols. Springer, New York, New York, NY, pp. 227–245.
Sharma, V.K., Millero, F.J., 1988. Effect of ionic interactions on the rates of oxidation of Cu (I) with O2 in natural waters. Mar. Chem. 25, 141–161.
Shi, B., Huang, Z., Xiang, X., Huang, M., Wang, W.-X., Ke, C., 2015. Transcriptome analysis of the key role of GAT2 gene in the hyper-accumulation of copper in the oyster *Crassostrea angulata*. Sci. Rep. 5, 17751.
Shi, B., Wang, T., Zeng, Z., Zhou, L., You, W., Ke, C., 2019. The role of copper and zinc accumulation in defense against bacterial pathogen in the Fujian oyster (*Crassostrea angulata*). Fish Shellfish Immunol. 92, 72–82.
Sonawane, A.R., Platig, J., Fagny, M., Chen, C.-Y., Paulson, J.N., Lopes-Ramos, C.M., DeMeo, D.L., Quackenbush, J., Glass, K., Kuijjer, M.L., 2017. Understanding tissue-specific gene regulation. Cell Rep. 21, 1077–1088.
Suzuki, M., Sakuda, S., Nagasawa, H., 2007. Identification of chitin in the prismatic layer of the shell and a chitin synthase gene from the Japanese pearl oyster, *Pinctada fucata*. Biosci. Biotechnol. Biochem. advpub, 0706060434-0706060434.
Suzuki, M., Saruwatari, K., Kogure, T., Yamamoto, Y., Nishimura, T., Kato, T., Nagasawa, H., 2009. An acidic matrix protein, Pif, is a key macromolecule for nacre formation. Science 325, 1388.
Tan, Q.-G., Wang, Y., Wang, W.-X., 2015. Speciation of Cu and Zn in two colored oyster species determined by X-ray absorption spectroscopy. Environ. Sci. Technol. 49, 6919–6925.
Wang, H., Qian, L., Liu, X., Zhang, G., Guo, X., 2010. Classification of a common cupped oyster from Southern China. J. Shellfish Res. 29, 857–866 810.
Wang, W.-X., Yang, Y., Guo, X., He, M., Guo, F., Ke, C., 2011. Copper and zinc contamination in oysters: subcellular distribution and detoxification. Environ. Toxicol. Chem. 30, 1767–1774.
Wang, W.-X., Meng, J., Weng, N., 2018. Trace metals in oysters: molecular and cellular mechanisms and ecotoxicological impacts. Environ Sci Process Impacts 20, 892–912.
Weng, N., Wang, W.-X., 2014. Improved tolerance of metals in contaminated oyster larvae. Aquat. Toxicol. 146, 61–69.
Weng, N., Jiang, H., Wang, W.-X., 2019. Novel insights into the role of copper in critical life stages of oysters revealed by high-resolution NanoSIMS imaging. Environ. Sci. Technol. 53 (24), 14724–14733.
Whitby, H., Hollibaugh, J.T., van den Berg, C.M.G., 2017. Chemical speciation of copper in a salt marsh estuary and bioavailability to thaumarchaeota. Front. Mar. Sci. 4, 178.
Whitby, H., Posacka, A.M., Maldonado, M.T., van den Berg, C.M.G., 2018. Copper-binding ligands in the NE Pacific. Mar. Chem. 204, 36–48.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer.
Wintz, H., Fox, T., Wu, Y.-Y., Feng, V., Chen, W., Chang, H.-S., Zhu, T., Vulpe, C., 2003. Expression profiles of arabidopsis thaliana in mineral deficiencies reveal novel transporters involved in metal homeostasis. J. Biol. Chem. 278, 47644–47653.
Wiramanaden, C.I.E., Cullen, J.T., Ross, A.R.S., Orians, K.J., 2008. Cyanobacterial copper-binding ligands isolated from artificial seawater cultures. Mar. Chem. 110, 28–41.
Wu, Z., Fernandez-Lima, F.A., Russell, D.H., 2010. Amino acid influence on copper binding to peptides: cysteine versus arginine. J. Am. Soc. Mass Spectrom. 21, 522–533.
Xu, F., Zhang, G., 2020. Transcriptomic and proteomic dynamics during metamorphosis of Pacific oyster *Crassostrea gigas*. bioRxiv 2020.2003.2025.004614.
Yabutani, T., Kishibe, S., Kamimura, M., Nozoe, K., Yamada, Y., Takayanagi, T., 2018. Copper speciation for natural water by on-site sample treatment/solid-phase extraction/inductively coupled plasma mass spectrometry. Anal. Sci. 34, 725–728.
Yan, L., Su, J., Wang, Z., Yan, X., Yu, R., Ma, P., Li, Y., Du, J., 2017. Transcriptomic analysis of *Crassostrea sikamea* × *Crassostrea angulata* hybrids in response to low salinity stress. PLoS ONE 12, e0171483.
Yi, L., Kaler, S., 2014. ATP7A trafficking and mechanisms underlying the distal motor neuropathy induced by mutations in ATP7A. Ann. N. Y. Acad. Sci. 1314, 49–54.
Young, M.D., Wakefield, M.J., Smyth, G.K., Oshlack, A., 2010. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol. 11, R14.
Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Yang, P., Zhang, L., Wang, X., Qi, H., Xiong, Z., Que, H., Xie, Y., Holland, P.W.H., Paps, J., Zhu, Y., Wu, F., Chen, Y., Wang, J., Peng, C., Meng, J., Yang, L., Liu, J., Wen, B., Zhang, N., Huang, Z., Zhu, Q., Feng, Y., Mount, A., Hedgecock, D., Xu, Z., Liu, Y., Domazet-Lošo, T., Du, Y., Sun, X., Zhang, S., Liu, B., Cheng, P., Jiang, X., Li, J., Fan, D., Wang, W., Fu, W., Wang, T., Wang, B., Zhang, J., Peng, Z., Li, Y., Li, N., Wang, J., Chen, M., He, Y., Tan, F., Song, X., Zheng, Q., Huang, R., Yang, H., Du, X., Chen, L., Yang, M., Gaffney, P.M., Wang, S., Luo, L., She, Z., Ming, Y., Huang, W., Zhang, S., Huang, B., Zhang, Y., Qu, T., Ni, P., Miao, G., Wang, J., Wang, Q., Steinberg, C.E.W., Wang, H., Li, N., Qian, L., Zhang, G., Li, Y., Yang, H., Liu, X., Wang, J., Yin, Y., Wang, J., 2012. The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490, 49.
Zhang, Y., Sun, J., Mu, H., Li, J., Zhang, Y., Xu, F., Xiang, Z., Qian, P.-Y., Qiu, J.-W., Yu, Z., 2015. Proteomic basis of stress responses in the gills of the Pacific oyster *Crassostrea gigas*. J. Proteome Res. 14, 304–317.
Zhao, R., Takeuchi, T., Luo, Y.-J., Ishikawa, A., Kobayashi, T., Koyanagi, R., Villar-Briones, A., Yamada, L., Sawada, H., Iwanaga, S., Nagai, K., Satoh, N., Endo, K., 2018. Dual gene repertoires for larval and adult shells reveal molecules essential for molluscan shell formation. Mol. Biol. Evol. 35, 2751–2761.