BMC Genomics

RESEARCH Open Access

Comparative transcriptome analysis reveals differences in immune responses to copper ions in *Sepia esculenta* under high-temperature conditions

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Abstract

Sepia esculenta is one of the most abundant extant squid populations in Southeast Asia and is of interest due to its rapid reproductive rate and high commercial value. In recent years, with the rapid development of industrialization, issues such as global warming and heavy metal pollution in the oceans have emerged, posing a serious threat to the life activities of marine organisms. In this study, we used transcriptomic techniques to investigate the differences in Cu exposure immune responses in *S. esculenta* larvae under different temperature conditions. The enrichment of solute carrier family (SLC) genes and genes related to DNA replication and damage was significantly higher in the CuT group than in the Cu group. Functional enrichment analysis revealed that the FcγR-mediated phagocytosis and autophagy pathways were enriched in the CuT group. Based on the analysis of differentially expressed genes (DEGs) and functional enrichment results, we can preliminarily infer that the CuT group caused more severe disruption of intercellular ion transport and DNA replication and repair in larvae compared to the Cu group. This may have further interfered with the normal physiological activities of *S. esculenta* larvae. Overall, at high temperatures, Cu exposure induces a more intense inflammatory response. The results of this study provide a theoretical foundation for researchers to further understand the effects of environmental factors on the immunity of *S. esculenta* larvae, as well as preliminary insights into the enhanced toxic effects of metallic copper on aquatic organisms under high-temperature conditions.

Keywords Transcriptome, Cu co-exposure, Heavy metal, Immunity, *Sepia esculenta*

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Introduction

As industrialization progresses, heavy metal pollution in the sea is becoming more and more serious [1]. Recent studies have demonstrated that heavy metals can severely influence the growth and development of marine organisms, inducing tissue damage and immune dysfunction [2]. Harmful effects of heavy metals on aquatic organisms include tissue damage and impairment of the immune system [3]. For example, in fish, high concentrations of heavy metals significantly increase the rate of malformations and morbidity, and previous studies have shown that heavy metal enrichment has serious effects on molluscan physiological processes such as immunity and metabolism [4].

The rapid development of industrialization has not only led to the intensification of heavy metal pollutions but has also increased the rate of global warming [5]. Previous studies have shown that temperature induces changes in the toxicity of heavy metals to marine organisms [6]. Ren et al. found that high temperatures enhanced the intoxication of heavy metals to Ruditapes philippinarum, significantly inhibiting its growth and development [7]. Malhotra et al. showed that temperature would affect the toxicity induced by Cu exposure. Compared with low temperature (15 ± 1℃), the dissolution rate and aggregation of copper ions were enhanced at high temperature (26 ± 1℃) [8]. However, the effect of high temperatures on Cu exposure toxicity has not been thoroughly studied in mollusks. Therefore, understanding whether high temperatures have an effect on copper-induced toxicity is important for mollusk research, aquaculture, and the environmentally sound management of marine ecosystems.

S. esculenta is one of the most abundant squid populations surviving in Southeast Asia, with tasty meat, high protein content, and rapid reproduction [9], Therefore, it quickly attracted the attention of scholars. As a result of the degradation of the marine environment and the large-scale exploitation of marine resources by human beings, the population of *S. esculenta* has declined drastically, and in recent years, it has even become an endangered species [10]. Usually, *S. esculenta* is cultured in captivity in shallow coastal waters. High concentrations of heavy metals and high temperatures along the ocean coast have a profound effect on the growth and development of *S. esculenta* [11]. Therefore, it is essential to understand the consequences of high temperatures and heavy metals on *S. esculenta*.

RNA sequencing technology has developed rapidly due to its high throughput and accuracy, and it has been widely used in recent years to study the immune mechanisms of aquatic organisms. Examples include the use of RNA-Seq to study the molecular response of Cu to *Argopecten purpuratus* larvae, as well as the study of the molecular mechanisms of high temperature on *Octopus vulgaris* and the response of the transcriptome to Cu exposure in *Mytilus coruscus* [12–14]. Therefore, we can use RNA-Seq to preliminarily explore the toxicological mechanism of Cu exposure in *S. esculenta* larvae at high temperature.

In our study, we used high-throughput transcriptome sequencing to explore differences in Cu exposure immunity in *S. esculenta* larvae at different temperatures. By GO and KEGG functional enrichment analysis, it was found that high temperature significantly affected the Cu exposure immune response and induced a more intense inflammatory response in *S. esculenta* larvae. At the same time, high temperatures can induce more severe DNA damage and ion transport disorders. Our findings provide a basis for further exploration of differences in Cu exposure imaginal responses in *S. esculenta* larvae under different temperature conditions and provide more scientific breeding suggestions for golden squid and cephalopods.

Materials and methods

Collection of *S. esculenta* larvae and stress experiments

During the breeding season of *S. esculenta*, captures were made from the Yellow Sea area to obtain wild parents(Active and apparently healthy adult S. esculenta (manth length: 14.82 ± 0.21 cm; weight: 351.88 ± 11.75 g) were collected from the Yellow Sea near Qingdao, China.), and the captured wild parents were placed in workshop pools (Approximately 8 m long, 1 m wide and 1.2 m high, made of concrete) for transient rearing. After a week, the female parents began to lay eggs, with the collected eggs placed in moving seawater and temporarily raised until they hatched. At the same time, *S. esculenta* eggs hatched into larvae and were transferred to white buckets (100 L capacity). The larvae were divided into three groups including control (Group C), normal temperature Cu exposure (Cu Group), and high temperature Cu exposure (CuT Group), with 100 *S. esculenta* larvae in each group (mantle length (body size) = 6.2 ± 0.2 mm; weight = 62.8 ± 8.2 mg). The control group was grown in normal seawater (water temperature of 20.5 ± 1℃, dissolved oxygen of 5.6 ± 0.2 mg/L, pH of 8.3 ± 0.1, salinity of 30.1 ± 0.4, and continuous oxygenation) for 24 h. Anhydrous CuCl2 was added to seawater to achieve a Cu concentration of 50 μg/L in the Cu group and the CuT group(*S. esculenta* larvae were too small to measure Cu content in the body). The CuT group raised the temperature to 28 °C, as determined by preliminary experiments and references [15]. Finally, during the experiment, *S. esculenta* larvae were collected at 0 h (C_0h), 4 h (C_4h), and 24 h (C_24h) in the control group, at 4 h (Cu_4h) and 24 h (Cu_24h) in the Cu group, and at 4 h (CuT_4h)

and 24 h (CuT_24h) in the CuT group. All samples were stored in liquid nitrogen tanks until RNA extraction.

RNA extraction, library construction and sequencing

RNA extraction, library construction, and sequencing were performed by Beijing Novozymes Technology Co., Ltd., which used the TRIzol method for RNA extraction. The RNA samples were then subjected to stringent quality control by the Agilent 2100 bioanalyzer. The kit used for library construction was the NEBNext® Ultra™ RNA Library Prep Kit for Illumina®. Nine larvae were selected from each group, and three were randomly selected to form one replicate, for a total of three replicates. We sequenced *S. esculenta* larval RNA using the Illumina NovaSeq 6000 platform (Illumina, USA) [16].

Screening of differential genes and annotation of gene functions

First, we removed some raw reads, reads containing adapter sequences, and more than 10% of unidentified bases, as well as low-quality reads, and mapped the clean reads to the *S. esculenta* reference genome using HISAT2 software (unpublished). We used the DESeq2 method to identify DEGs and compared these genes with SwissProt, KEGG, GO, and other databases to find out their functions and used them for functional enrichment analysis. In our study, the *p*-value threshold was set to ≤ 0.05 [17, 18].

Enrichment analysis

We performed GO and KEGG analyses of genes using the DAVID bioinformatics resource (https://david.ncifcrf.gov/tools.jsp). Terms and pathways with *p*-values ≤ 0.05 were selected to be used in subsequent analyses.

Quantitative RT-PCR analysis

In this experiment, we screened 15 DEGs and designed primers using Primer Premier 5.0 (Table S1). Since the expression level of β-actin tends to be stable, we chose the β-actin gene as the reference gene for quantitative reverse transcription polymerase chain reaction. qRT-PCR was performed according to Liu et al. (2017) and Wang et al. (2023). Then, qRT-PCR was conducted in a 20 μL solution containing 10 ng of template cDNA and SYBR Premix Ex Taq II (TaKaRa) using a LightCycler 480 at 95 °C for 5 min pre-incubation, followed by 45 cycles of 95 °C for 15 s and 60 °C for 45 s. Finally, we analyzed the melting curve to detect single amplification. Fluorescent signal accumulation was recorded during the 60 °C for 45 s phase of each cycle using the LightCycler 480. The $2-\Delta\Delta Ct$ comparative Ct method was used to calculate the relative quantities of the target genes expressed as fold variation over β-actin. Quantitative RT-PCR was applied for the expression verification of key and hub genes in this investigation [19, 20].

Results

Transcriptome sequencing results

Sequencing of healthy, copper stress, and elevated temperature conditions copper stress *S. esculenta* larvae using RNA-seq methods; specific sequencing results are shown in Table S2. On average, 87.68% of clean reads were mapped to our reference genome. The structure and function of unigenes were annotated into several databases, including NR, SwissProt, KEGG, GO, and PFAM.

Analysis of DEGs

By means of a dynamic multi-group difference scatter plot (Fig. 1A), we found that a total of 787 DEGs were detected in the Cu_4h vs. C_4h comparison group. Of

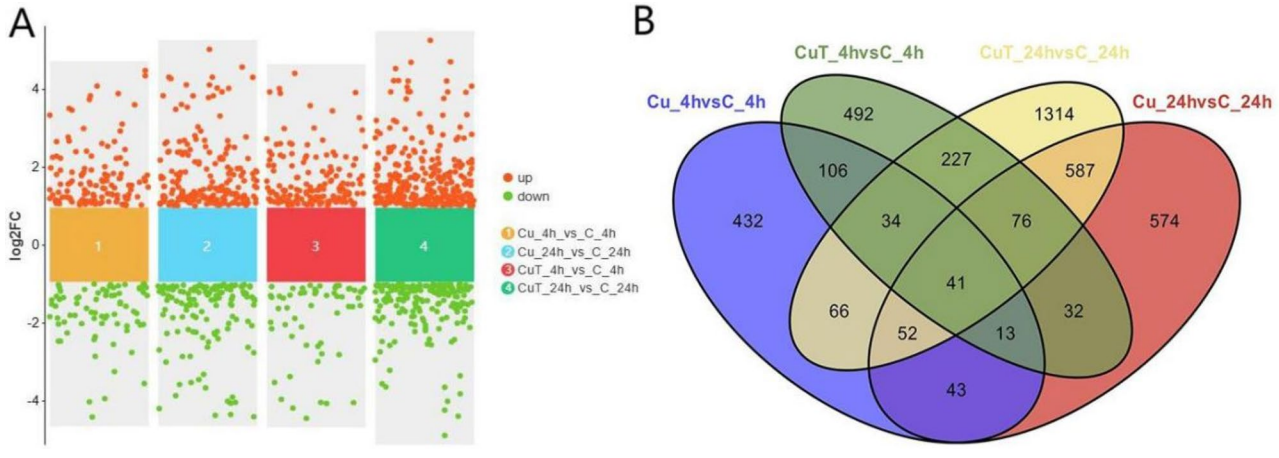

Fig. 1 **A**: Dynamic multi-group difference scatter plot of the distributional expression of DEGs, with numbers representing different groups, red dots representing up-regulation of DEGs, and green dots representing down-regulation of DEGs, and each dot representing a gene; **B**: Venn diagram of DEGs, with blue, green, yellow, and red representing DEGs in different groups, and other different colors representing the number of shared genes between different groups

these, 463 were up-regulated genes and 324 were down-regulated genes. A total of 1,418 DEGs were detected in the Cu_24h vs. C_24h comparison group, of which 680 were up-regulated genes and 738 were down-regulated genes. A total of 1,021 DEGs were detected in the CuT_4h vs. C_4h comparator group, with 566 up-regulated genes and 455 down-regulated genes. A total of 2,397 DEGs were found in the CuT_24h vs. C_24h comparison group, of which 1,071 were up-regulated and 1,326 were down-regulated.

The DEGs Venn diagram (Fig. 1B) clearly shows the unique and shared DEGs between each group. In both the Cu and CuT groups, we found the presence of genes from the SLC family (SLC6A9) and the ATP-binding cassette(ABC) subfamily (ABCC10, ABCC1, ABCB1). In the CuT group, we found that the SLC family (SLC16A4, SLC16A14, SLC22A21, SLC35B2, SLC6A9, SLC12A2) and the ABC subfamily (ABCF2) were present. Genes related to DNA replication and damage were found in genes unique to the CuT group, such as RFC4, RFC5, POLD3, RPA2, and RPA3.

The results of the clustering heat map showed that the expression pattern of the CuT group was obviously not the same as that of the Cu group (Fig. 2).

GO analysis

120, 208, 85, and 236 GO terms were enriched in the Cu_4h vs. C_4h, Cu_24h vs. C_24h, CuT_4h vs. C_4h, and CuT_24h vs. C_24h groups, respectively. The first 10 terms of the three clusters (biological process, cellular component, molecular function) included in each set of GO functional enrichment analyses are shown in the figure (Fig. 3A-D). Immunity-related overlapping terms between all four groups of the top 30 GO terms were identified through the GO Venn diagram (Fig. 3E) as including zinc ion binding (GO:0008270), oxidoreductase activity (GO:0016491), and alcohol dehydrogenase (NAD) activity (GO:0004022). Unique and immunologically relevant in the CuT group are apoptotic processes (GO:0006915) and DNA repair (GO:0006281).

We selected the GO terms with a large amount of DEGs enrichment and high significance to observe their copper stress and elevated temperature conditions copper stress changes in the amount of DEGs, as shown in the line chart (Fig. 3F). We found that during the Cu exposure process, the increase in temperature would lead to changes in the amount of DEGs enrichment in these terms, which showed an upward trend; the longer the high-temperature duration, the greater the number of enriched genes.

KEGG analysis

14, 18, 8, and 23 pathways were enriched in the Cu_4h vs. C_4h, Cu_24h vs. C_24h, CuT_4h vs. C_4h, and CuT_24h vs. C_24h groups, respectively, and the bubble plots show the six pathways (Fig. 4A-D). According to the KEGG Venn diagram (Fig. 4E), the immune-related pathways shared by the Cu group and CuT group included the DNA replication pathway, tight junction pathway, and cell cycle pathway, and the CuT group was enriched with more DEGs in the pathways compared to the Cu group. In addition, the nucleotide excision repair pathway, base excision repair pathway, mismatch repair pathway, nucleoside, FcγR-mediated phagocytosis pathway, and autophagy pathway were identified as unique to the CuT group and immunologically relevant.

Many DEGs are concentrated in the secondary KEGG signaling pathway associated with immunity (Fig. 5A-D), including the immune system and immune diseases. More DEGs were enriched in immune-related pathways in the CuT group than in the Cu group, which may imply that Cu exposure at high temperatures has a greater impact on the immune system of *S. esculenta* larvae.

qRT-PCR to verify DEGs

We verified the accuracy of 15 genes related to copper stress and elevated temperature conditions copper stress genes using qRT-PCR, and the results showed that the folding changes of DEGs detected by qPCR were consistent with the folding changes determined by RNA-Seq expression analyses, suggesting that the results of the RNA-Seq expression analyses were reliable (Fig. 6).

Discussion

Expression analysis of DEGs

The response of *S. esculenta* larvae to copper (Cu) exposure is a complex process involving various regulatory pathways and genes [21]. Our findings revealed that SLC family genes, as well as ABC family genes, were more highly enriched in the CuT group compared to the Cu group. Additionally, we identified DEGs associated with DNA damage, repair, and replication processes.

Analysis of SLC and ABC family-related DEGs

Sheng et al. demonstrated that the SLC family can regulate the function of macrophages and T cells. It plays an important role in various diseases involving inflammation and immune responses. These transporter cells can improve immune responses, such as T-cell activation, so SLCs play an important role in the immune process [22].

It has been shown that the ABC family is considered to play an essential role in the first line of cellular defense. When inflammation occurs in the body, genes in the ABC family reduce inflammation by positively regulating the NF-κB pathway and promoting the expression of antimicrobial peptides [23]. ABC transporter proteins are also expressed in a wide range of immune cells and are a key link between innate and adaptive immunity. In

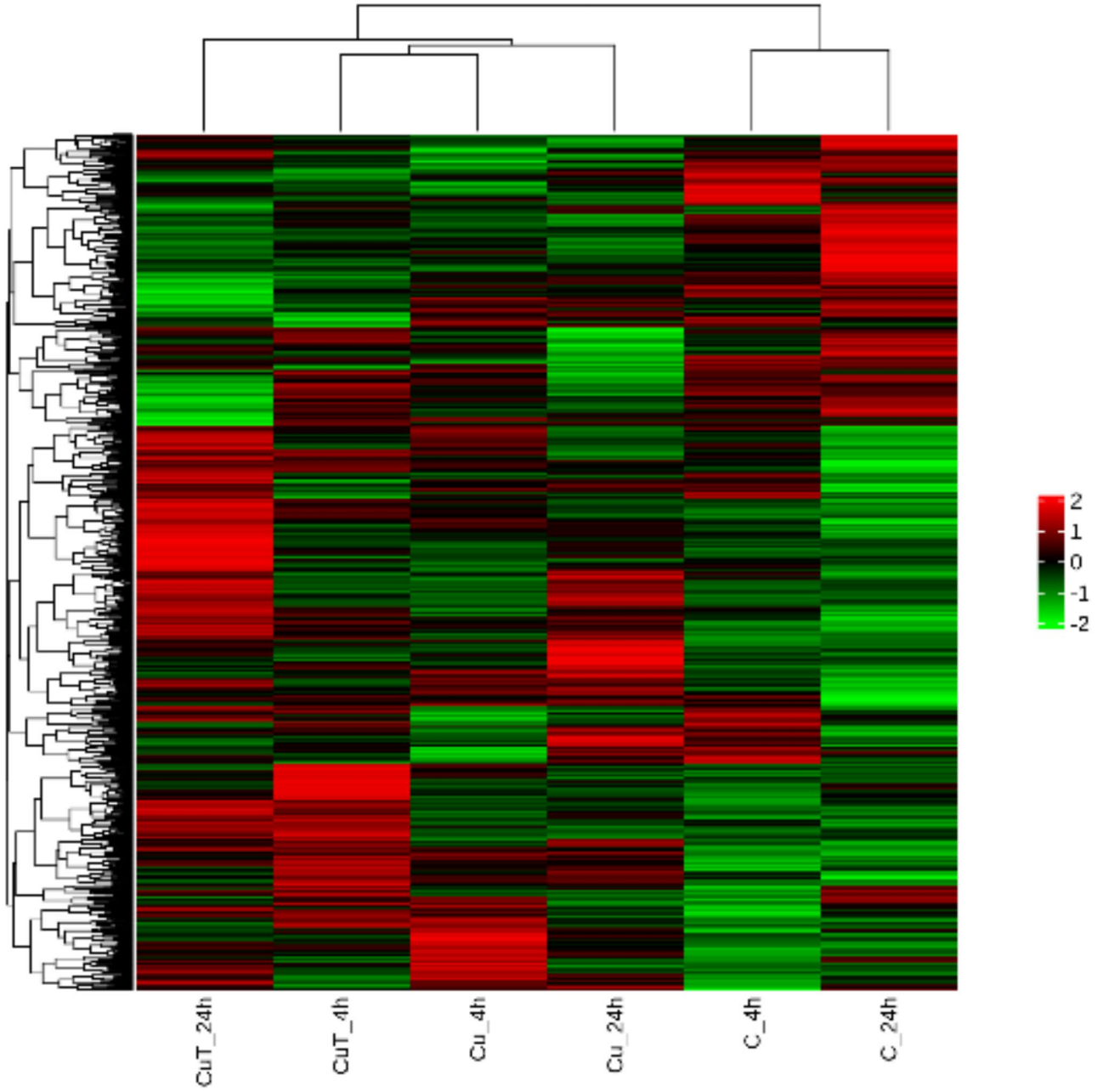

Fig. 2 DEGs heat map of hierarchical clustering. Each pillar represents a group, and each row represents a gene. Green to red indicates low to high expression levels

invertebrates, ABCB and ABCC transporter proteins regulate the entry of exogenous substances from the external environment and are the main mode of defense in invertebrates [24].

Based on the functions of the ABC and SLC families, we hypothesize that the accumulation or dissolution rate of copper ions in *S. esculenta* larvae may increase under elevated temperatures, leading to a stronger immune response. In this study, the number of DEGs in the SLC and ABC families varied before and after the temperature increase. We speculate that copper (Cu) exposure under high-temperature conditions may exacerbate the inflammatory response in *S. esculenta* larvae.

Analysis of DEGs related to DNA replication, damage, and repair

Furthermore, in the comparison of the Cu group with the CuT group, we newly identified other key DEGs enriched in multiple pathways in the CuT group; for example, RFC4, RFC5, POLD3, RPA2, and RPA3, and their expressions were down-regulated. These genes play a key role in DNA damage, replication, and repair [25–27]. The

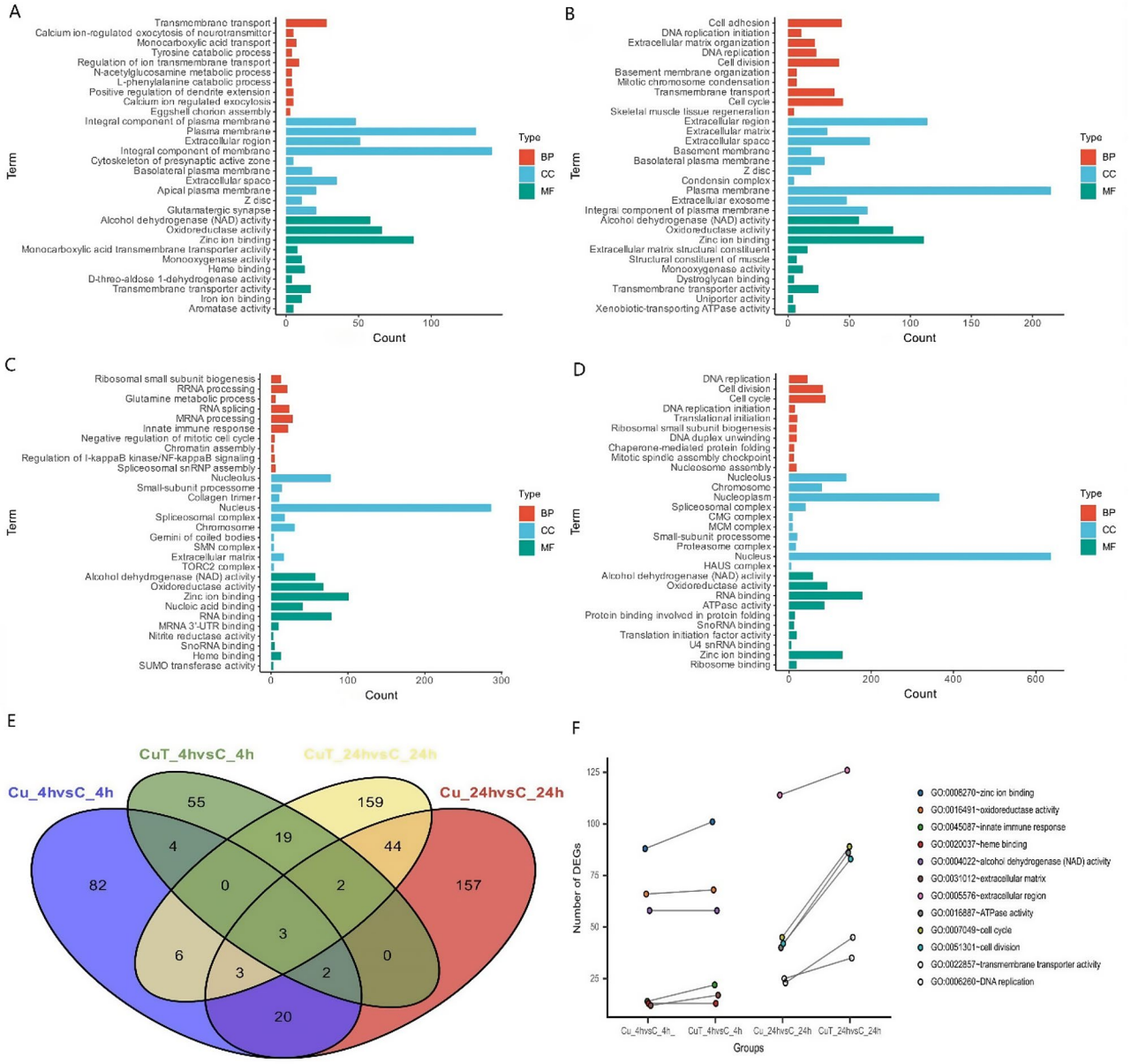

Fig. 3 **A**: Enriched GO terms for the Cu_4h vs. C_4h group; **B**: Enriched GO terms for the Cu_24h vs. C_24h group; **C**: Enriched GO terms for the CuT_4h vs. C_4h group; **D**: Enriched GO terms for the CuT_24h vs. C_24h group. The horizontal coordinate indicates the number of DEGs enriched to the term; the vertical coordinates denote specific GO-based terms, and the three colors represent the three ontologies. **E**: Venn diagram of GO, with red, green, yellow, and blue representing the different groups and the other colors representing GO terms shared between the different groups; **F**: Line plots of the four groups of GO terms in the Cu_4h vs. C_4h and CuT_4h vs. C_4h, Cu_24h vs. C_24h, and CuT_24h vs. C_24h DEGs, with the vertical coordinates representing the number of DEGs, the horizontal coordinates representing the groups, and the dots in different colors representing the different GO terms

downregulation of the expression of these genes, which we speculate is due to increased inflammation, leads to dysfunctional DNA replication and repair in *S. esculenta* larvae.

In summary, we speculate that Cu exposure effects on ion transport, as well as DNA replication and repair, are stronger in *S. esculenta* larvae at high temperatures, and that high temperatures may be able to lead to higher rates of aggregation or solubilization of Cu ions in *S. esculenta* larvae.

GO functional enrichment analysis

Analysis of common GO terms between Cu and cut groups

In the GO functional enrichment analysis, we found three GO terms the Cu group shared with the CuT group, namely zinc ion binding (GO:0008270), oxidoreductase

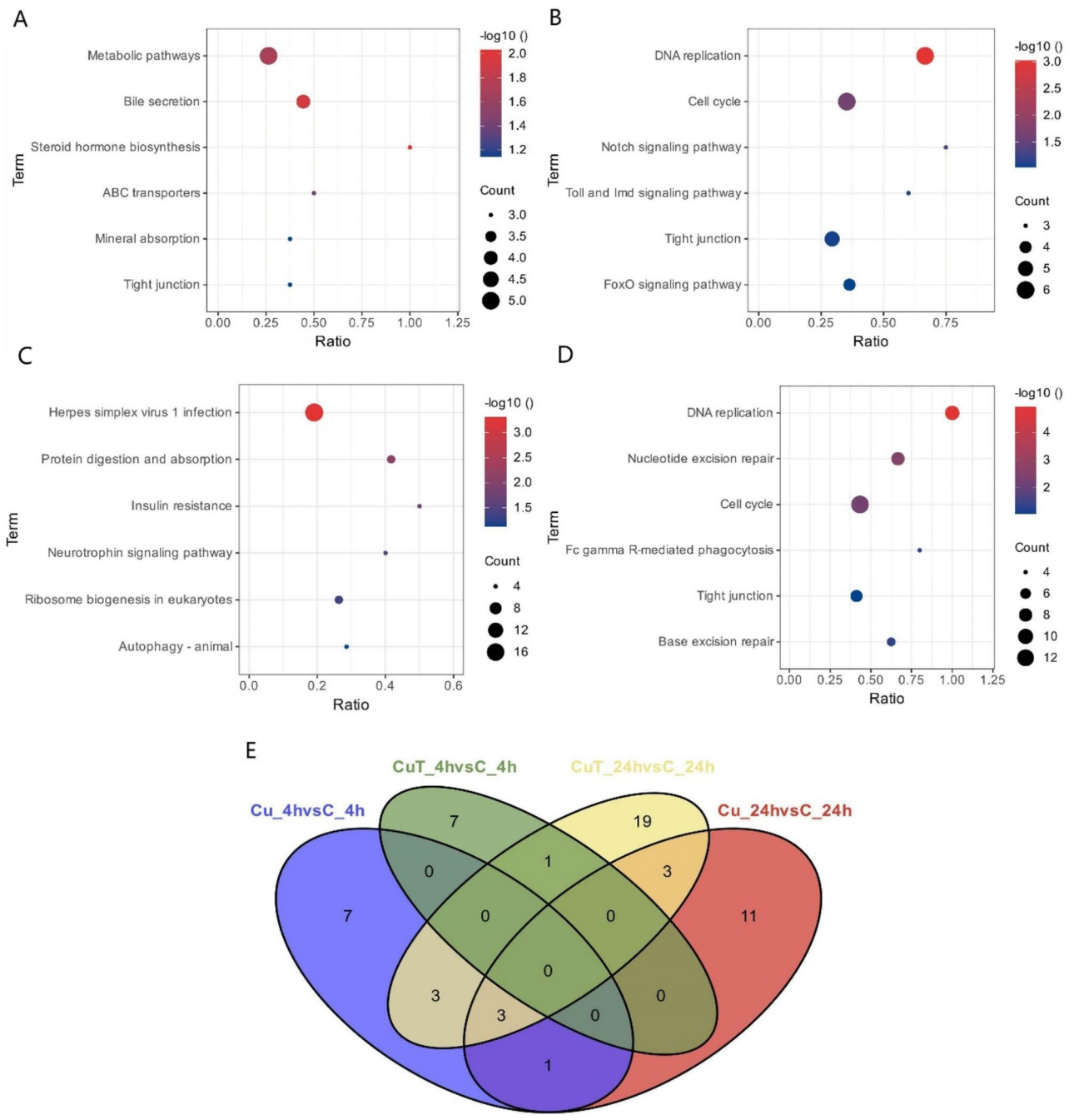

Fig. 4 A Enriched KEGG pathways for the Cu_4h vs. C_4h group; **B**: Enriched KEGG pathways for the Cu_24h vs. C_24h group; **C**: Enriched KEGG pathways for the CuT_4h vs. C_4h group; **D**: Enriched KEGG pathways for the CuT_24h vs. C_24h group. KEGG bubble plot, with the size of the dots representing the number of DEGs and the colors representing significance; the vertical coordinate is the name of the pathway, and the horizontal coordinate is the ratio of DEGs to the total number of DEGs in the pathway. **E**: KEGG Venn diagram, with red, green, yellow, and blue representing the different groups and the other colors representing pathways shared between the different groups

activity (GO:0016491), and alcohol dehydrogenase (NAD) activity (GO:0004022).

Previous studies have shown that zinc ions play an important role in immune processes and signal regulation, and that a deficiency of zinc ions leads to dysfunction in many organs and the immune system [28]. Oxidoreductase is an important regulator of immune pathways. An increase in oxidoreductase activity promotes the generation of reactive oxygen species, and these by-products may modulate pro-inflammatory pathways [29]. Alcohol dehydrogenase activity is closely related to autoimmune inflammation, and alcohol

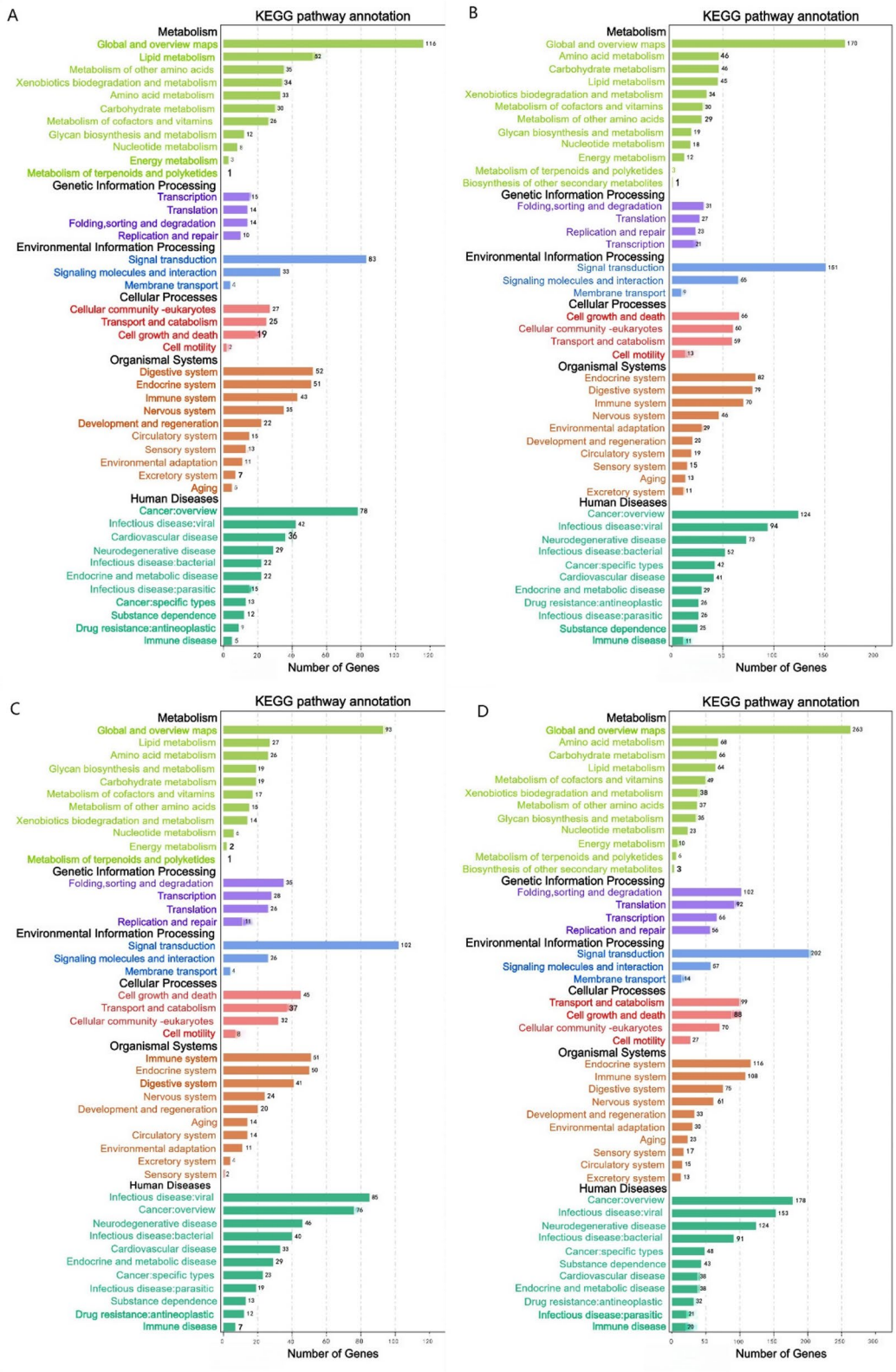

Fig. 5 Class 2 KEGG signaling pathway annotations; vertical coordinates indicate the type 2 KEGG category, and horizontal coordinates indicate the number of corresponding DEGs. **A**: Enriched Class 2 KEGG signaling pathways for the Cu_4h vs. C_4h group; **B**: Enriched Class 2 KEGG signaling pathways for the Cu_24h vs. C_24h group; **C**: Enriched Class 2 KEGG signaling pathways for the CuT_4h vs. C_4h group; **D**: Enriched Class 2 KEGG signaling pathways for the CuT_24h vs. C_24h group

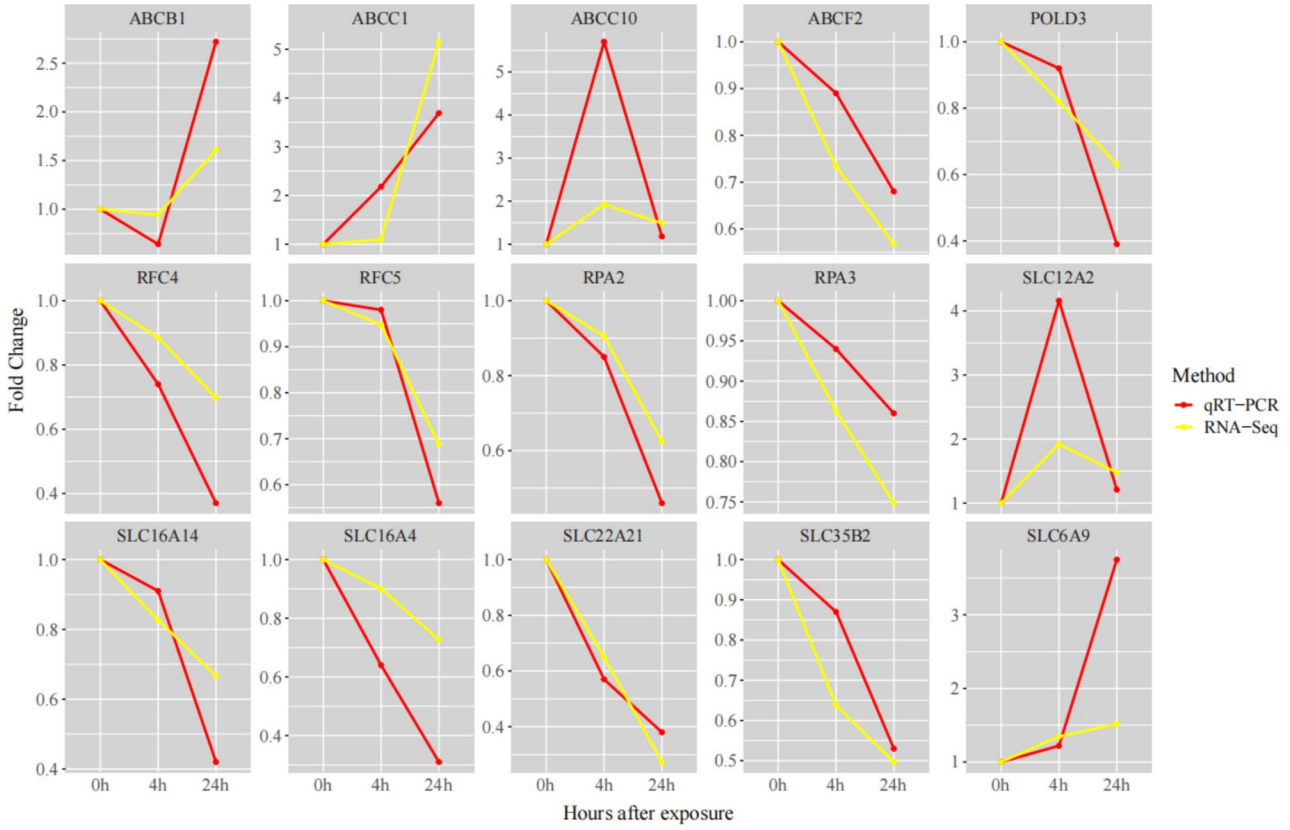

Fig. 6 qRT-PCR and RNA-Seq results of key DEGs. The vertical coordinates represent the fold change, and the horizontal coordinate represents the duration of exposure

dehydrogenase is essential for the metabolism of organisms and the regulation of macrophage function. It is mainly used to remove apoptotic cells from the body [30].

The enrichment of these terms may indicate that Cu exposure may lead to a decrease in ion binding ability and reductase activity in *S. esculenta* larvae, which may result in an imbalance of the immune system of *S. esculenta* larvae or promote inflammation. We also found that the number of DEGs in the terms zinc ion binding (GO:0008270) and oxidoreductase activity (GO:0016491) differed between the Cu group and CuT group, with an increase in DEGs in the CuT group. This trend may indicate that high temperature can enhance the harmful effects of copper exposure on *S. esculenta*.

Analysis of unique GO terms in the CuT group

Unique GO terms enriched in the CuT group compared to the Cu group that are relevant to immunity include apoptosis (GO:0006915) and DNA repair (GO:0006281).

Apoptosis is important in the maintenance of tissue homeostasis and the reduction of inflammation, and organisms can gradually eliminate or reduce inflammation by renewing cells through apoptosis in vivo [31]. DNA damage repair and innate immunity are two conserved mechanisms that play a role in both cellular stress responses, and it has been found that DNA repair is closely linked to inflammation and that organisms can effectively control inflammation through their own DNA repair [32].

Through GO enrichment analysis, we were able to speculate that Cu exposure inflammation in *S. esculenta* larvae is more severe under high-temperature conditions and that *S. esculenta* larvae may reduce inflammation in vivo by accelerating apoptosis of damaged cells themselves. Moreover, Cu exposure at high temperatures may lead to DNA repair dysfunction in *S. esculenta* larvae, thereby affecting their immune function and potentially resulting in more severe inflammation.

KEGG functional enrichment analysis

Analysis of common KEGG pathways between Cu and CuT groups

The pathways found to be common to the Cu and CuT groups and immunologically relevant through KEGG functional enrichment analysis were the DNA replication pathway, the tight junction pathway, and the cell cycle pathway.

DNA replication is associated with innate immunity in animals, and some innate immune errors are caused by mutations in DNA replication factors. The DNA

replication pathway is a complex signaling pathway that plays an important role in regulating the repair of DNA damage and inducing cell apoptosis [33]. In our research, the significant enrichment of the DNA replication pathway in *S. esculenta* larvae is likely due to Cu exposure leading to errors in DNA replication in *S. esculenta* larvae, which results in innate immune disorders in *S. esculenta* larvae. Research has found that inflammation can increase damage to the nervous system by degrading tight junction proteins. There is an interaction between tight junction proteins and inflammation, and protecting these proteins can also alleviate inflammatory responses, thereby reducing the potential for impaired neurological function [34]. Genes in the tight junction pathway, such as TUBA1C and MICALL2, showed down-regulated expression, and we speculate that *S. esculenta* larvae undergo in vivo inflammation following Cu exposure and degradation of tight junction proteins. Cell cycle activity is related to immune function, and dysregulation of the cell cycle often indicates the presence of abnormal immune cells. Some cell cycle regulators can modulate the cell cycle to exert immunomodulatory effects, and cell cycle programs may be linked to immune behavior through mechanisms that can influence immunity [35, 36]. We found down-regulation of the CDK1 gene, a cell cycle protein-dependent kinase, in our transcriptome results. CDK1 influences its catalytic activity through unique interactions with various cell cycle protein complexes, ensuring that cell cycle progression is not impaired. Additionally, CDK1 plays a significant regulatory role in modulating various cellular immune responses during inflammation [37, 38]. Therefore, the downregulation of the CDK1 gene after copper induction in *S. esculenta* larvae likely indicates an abnormality in the cell cycle.

In conclusion, the activation of the DNA replication pathway, tight junction pathway, and cell cycle pathway following Cu exposure may be linked to the presence of inflammation in *S. esculenta* larvae. We also observed a differential distribution of DEGs across the three shared pathways between the Cu and CuT groups, with an increased number of DEGs in the CuT group. This trend suggests that copper exposure under elevated temperature conditions may exacerbate the adverse effects on *S. esculenta*.

Analysis of unique KEGG pathways in the CuT group

Comparison of the Cu group with the CuT group revealed that the pathways unique to the CuT group related to immunity were the nucleotide excision repair pathway, base excision repair pathway, FcγR-mediated phagocytosis pathway, and autophagy pathway.

Nucleotide excision repair is unique in that it recognizes and removes truncated damage in DNA and is involved in the repair of DNA, which is closely linked to autoimmunity and inflammation present in the self [39–41]. Base excision repair (BER) is the primary pathway for eukaryotic cells to resolve single-base damage, enabling rapid and efficient DNA damage repair [42]. Therefore, we deduced that the enrichment of DEGs in the nucleotide excision repair pathway and base excision repair pathway was most likely caused by high-temperature Cu exposure, resulting in further disruption of the replication of *S. esculenta* larval DNA. Phagocytosis is a biological process; phagocytosis is usually accompanied by inflammation; phagocytosis is fundamental to the immune response and ensures that the body removes pathogens and apoptotic cells. Activation of phagocytosis by FcγR stimulates phospholipase D (PLD) activity and stimulates the macrophage plasma membrane to produce phosphatidic acid (PA), thereby increasing phagocytic efficiency [43–45]. Activation of the phagocytosis pathway in *S. esculenta* larvae may further exacerbate inflammation in vivo due to Cu exposure at elevated temperatures, and phagocytosis is required to remove damaged cells in vivo and thereby inhibit inflammation. Autophagy is associated with many essential biological processes, which include immunity, cell development, and the differentiation of cells through the regulation of inflammation. Through autophagic cells, damaged or harmful components can be eliminated [46, 47]. We found that the Prkcd and Sqstm1 genes were enriched and up-regulated in this pathway. Therefore, we postulate that high-temperature Cu exposure and inflammation in *S. esculenta* larvae result in an increase in the number of damaged cells in the body and an increase in autophagy, which accelerates the clearance of damaged cells from the body and thus reduces the inflammatory response.

In conclusion, high temperatures enhanced the toxicity of metallic copper ions, which further led to DNA damage in *S. esculenta* larvae and interfered with the ability of the larvae to replicate and repair DNA. Cu exposure under high-temperature conditions was able to cause more severe inflammation in *S. esculenta* larvae, which attenuated the inflammatory response by enhancing autophagy and phagocytosis in vivo.

Conclusion

We employed transcriptome sequencing to investigate the impact of copper (Cu) exposure on the immune responses of *S. esculenta* larvae under high-temperature conditions. Through the analysis of (DEGs and functional enrichment pathways, we preliminarily observed that the CuT group exhibited a more pronounced disruption of intercellular ion transport, immune function, and DNA replication and repair compared to the Cu-only group. This disruption likely interfered with the normal physiological processes of *S. esculenta* larvae, potentially leading

to enhanced inflammation. Inflammatory responses were more intense in the CuT group, as indicated by the increased DNA damage and stronger immune response observed in the larvae. This study contributes to bridging the knowledge gap regarding the combined effects of temperature and heavy metals on *S. esculenta* larvae, offering new insights into how their interactions exacerbate the health risks to these organisms. Furthermore, our findings provide a valuable reference for understanding the survival and reproductive challenges faced by other cephalopods in similar environmental conditions. In the context of rising global temperatures and increasing heavy metal pollution, the present study is an important practical guide for the breeding of *S. esculenta* and other cephalopods. The reference data and theories we provide will help aquaculture enterprises or individuals to better understand and cope with these challenges, thus promoting the sustainable development of related industries.

Abbreviations

RNA-seq	RNA sequencing
RT-qPCR	Quantitative reverse-transcription PCR
DEGs	Differentially expressed genes
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11418-y.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
Not applicable.

Author contributions
Zhao. Y. wrote the original manuscript; Chang. D., Zheng. Y., and Zhang. Y. analyzed the data; Wang. Y., Bao. X., and Sun. G. provided help with the software; Feng. Y. and Liu. X. organized the graphs and tables; Li. Z., Liu. X., and Yang. J. provided research ideas and revised the manuscript; all authors have reviewed and approved the manuscript.

Funding
This research was supported by the earmarked fund for the Modern Agro-industry Technology Research System (CARS-49), the Natural Science Foundation of Shandong Province (No. ZR2019BC052).

Data availability
The datasets generated and analysed during the current study are available in the NCBI repository, accession number SRR19578100, SRR19578102, SRR19578103, SRR19578104, SRR19578105, SRR19578106, SRR19578107, SRR19578108, SRR19578109, SRR19578110, SRR19578111, SRR19578112, SRR19578113, SRR19578114, SRR31864754, SRR31864755, SRR31864756, SRR31864757, SRR31864758, SRR31864759 at the following link: https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA844162%26;o=library_name_s%3Aa.

Declarations

Ethics approval and consent to participate
The experimental protocols were reviewed and approved by the Animal Care and Ethics Committee of the Ludong University (protocol number: LDU-IRB20210308NXY). The present study was reported according to the recommendation of ARRIVE guidelines for animal research. The method and process were carried out in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 17 December 2024 / Accepted: 28 February 2025
Published online: 17 March 2025

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